A biotechnological product and its potential as a new immunomodulator for treatment of animal phlebovirus infection: Punta Toro virus 

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ABSTRACT

Intracellular pathogens with widespread drug-resistance contribute substantially to the increasing rates in morbidity and mortality due to emerging and reemerging diseases. Thus, the development of new drugs, including those that can enhance the immune response, is urgently needed. The immunomodulator, P-MAPA, a proteinaceous aggregate of ammonium and magnesium phospholinolate-palmitoleate anhydride derived from Aspergillus oryzae, have been shown to induce antitumor activities. The ability of this compound to elicit protective immunity against viral infections has not been fully explored. Here, we report findings on the use of P-MAPA as an antiviral agent in a mouse model of acute phleboviral (Punta Toro virus) disease. A dose administered i.p. 24 h post-infectious challenge (100 mg/kg dose of P-MAPA) was remarkably effective at preventing death due to Punta Toro virus infection. This dose also reduced systemic viral burden and liver discoloration assayed on day 3 of infection. Taken together, our findings indicate that non-specific immunotherapy with P-MAPA appears to be an effective treatment for blocking Punta Toro virus-induced disease and suggest that further exploration with other viral disease models is warranted.

Punta Toro virus, has been adapted to induce a Rift Valley fever virus-like disease in C57BL/6 mice. Since this virus is recommended biosafety level 2 (BMBL), does not require high-level biologic concern, this virus has been used as a model to study the potential role of immunomodulating substances in therapies (Sidwell et al., 1992).

A spectrum of immunomodulators has been studied for Punta Toro virus such as Ampligen (Ampligen, 2008), 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinonone (bropirimine) (Vroegop et al., 1999), poly[ICLC] (NCI, 2007), AM-3 (Immunoferon) (Santos et al., 2004), MVE-1 (Sidwell et al., 1992), 5-amino-3-β-p-ribofuranosylthiazolo[4,5-d]pyrimidine-2,7(3H,6H)-dione(7-thia-8-oxoguanosine, immunosine) (Colic et al., 2000; Smeee et al., 1991a,b), rIL-2 (Mead et al., 1991) and type-1 IFN-inducer in the form of dsRNA poly[1:C] and poly[I:C12U] (Sidwell et al., 1992, 1994; Gowen et al., 2007a,b), cationic liposome–DNA complexes (CLDC-JuvImmuneTM; from Juvaris) (Dow et al., 1999; Sellins et al., 2005; Gowen et al., 2006a,b, 2008a; Romani et al., 1997; Samuel, 2001), and IFN alfacon-1 (trade name Infugen®) (Gowen et al., 2008b).

Besides these immunomodulators above mentioned, many antiviral compounds against PTV were also studied (Sidwell et al., 1988a,b; Smeee et al., 1990, 1996; Neyts et al., 1996; Gabrielsen et al., 1992; Gowen et al., 2007a,b).

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1. Introduction

Phleboviruses of the Bunyaviridae family are considered as one of the species causing serious disease in animals and humans producing significant hepatic disease that resembles severe disease caused by the related human and livestock pathogen, Rift Valley fever virus (RVFV) (Anderson et al., 1990; Fisher et al., 2003; Pifat and Smith, 1987). Among these are the Chagres, Alenquer, Candidura and Punta Toro (PTV) viruses. The latter is endemic to Central and South America, and appears to be infectious for man. Intensive antiviral studies using Punta Toro virus in a murine system have indicated that immunomodulators also appear to be viable alternatives for therapy of phlebovirus infections (Sidwell et al., 1992).

* All animal procedures were carried out in accordance with all ethical guidelines established by the institutions involved.

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An immunologic parameter common to all these substances is their ability to induce Type I interferon. The active immunomodulators were significantly effective when therapy was initiated as late as 48 h after virus inoculation, at a time when clinical signs of the Punta Toro virus disease were manifested in the animal (Sidwell et al., 1994).

The immunomodulator developed by Farmabrasilis (Nunes, 2008), is a proteinaceous aggregate of magnesium and ammonium phospholipolate-palmitoleate anhydride (P-MAPA) derived from A. oryzae (Nunes et al., 2004). P-MAPA has previously demonstrated anti-tumor activity in several mouse models (Justo et al., 2003). Extensive toxicology studies suggest that the compound is a safe drug, since in acute, subchronic and chronic toxicity trials were significantly effective when therapy was initiated as late as 48 h after virus inoculation, at a time when clinical signs of the disease were manifested in the animal (Sidwell et al., 1988a,b). Briefly, a specific volume of liver homogenate or serum was serially diluted and added to triplicate wells of LLC-MK2 cell monolayers in 96-well microplates. The viral cytopathic effect (CPE) was determined 6–7 days post-virus exposure and the 50% endpoints were calculated as described (Reed and Muench, 1938). The detection limit for the virus titer assays was 2.8 log_{10} cell culture 50% infectious doses (CCID_{50})/0.1 g of liver tissue or mL of serum. In samples presenting no detectable liver or serum virus, a value of <2.8 log_{10} was assigned. Therefore, a mean virus titer value proceeded by "<" indicates that at least one of the samples had undetectable levels of virus and is likely an overestimate of the actual mean viral load. For statistical analysis, a value of 1.8 log_{10} was assigned for samples with undetectable levels of virus.

2. Materials and methods

2.1. Prophylactic and therapeutic treatment of Punta Toro virus infection in mice with P-MAPA

2.1.1. Animals
Female 7-week C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and held for 5 days prior to experimentation. They were fed standard mouse chow and tap water ad libitum.

2.1.2. Virus
Adames strain PTV was provided by Dr. Dominique Pfat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The virus used was from a stock prepared after four passages of the original virus stock through LLC-MK2 rhesus monkey kidney cells and one passage in hamsters. The virus (0.2 mL) was inoculated via the subcutaneous (s.c.) route.

2.1.3. Compounds
P-MAPA was provided by Dr. Iseu Nunes (Farmabrasilis, Campinas, SP, Brazil). Ribavirin was supplied by ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Both drugs were prepared in sterile saline for intraperitoneal (i.p.) administration.

2.1.4. Liver and serum virus titers
Virus titers were assayed using an infectious cell culture assay as previously described (Sidwell et al., 1988a,b). Briefly, a specific volume of liver homogenate or serum was serially diluted and added to triplicate wells of LLC-MK2 cell monolayers in 96-well microplates. The viral cytopathic effect (CPE) was determined 6–7 days post-virus exposure and the 50% endpoints were calculated as described (Reed and Muench, 1938). The detection limit for the virus titer assays was 2.8 log_{10} cell culture 50% infectious doses (CCID_{50})/0.1 g of liver tissue or mL of serum. In samples presenting no detectable liver or serum virus, a value of <2.8 log_{10} was assigned. Therefore, a mean virus titer value proceeded by "<" indicates that at least one of the samples had undetectable levels of virus and is likely an overestimate of the actual mean viral load. For statistical analysis, a value of 1.8 log_{10} was assigned for samples with undetectable levels of virus.

2.1.5. Serum alanine aminotransferase (ALT) determinations
Serum ALT levels were measured using the ALT (SGPT) Reagent Set purchased from Pointe Scientific, Inc. (Lincoln Park, MI) following the manufacturer’s recommendations. Reagent volumes were adjusted for analysis with 96-well microplates.

2.1.6. Prophylactic and therapeutic treatment with P-MAPA
15–25 mice from each group were treated with several doses of P-MAPA, ribavirin or saline, as indicated in Table 1. Animals were challenged with 4.1 × 10^{3} CCID_{50} of PTV. P-MAPA was administered once per day for 6 days starting either 24 h prior to or after virus challenge. Ribavirin was given twice a day for 5 days beginning 4 h pre-virus challenge. Five mice from each group were sacrificed on day 4 of infection and their livers were removed, weighed and scored on a scale of 0–4 for hepatic icterus, 0 being normal and 4 being maximal yellow coloration. Serum was collected for ALT determinations and infectious virus titers were determined for both liver homogenates and serum samples. The remaining animals in each group were observed for death within 21 days. For comparison, three sham-infected animals were included as normal controls in order to establish baselines for all parameters tested. The amounts of P-MAPA tested for antiviral activity were also evaluated for signs of overt toxicity in uninfected mice.

2.1.7. Single dose therapeutic treatment with P-MAPA
15–25 mice from each group were treated with several doses of P-MAPA, ribavirin or saline, as indicated. Animals were challenged with 1.3 × 10^{4} CCID_{50} of PTV. P-MAPA was administered as a single dose 24 h post-infections challenge. Ribavirin was given twice a day for 5 days beginning 4 h pre-virus challenge. Five mice from each group were sacrificed on day 3 of infection, and their livers were removed and examined as described above.

2.1.8. Statistical analysis
The log-rank test for survival analysis was performed using JMP statistical software (SAS, Cary, NC). The Fisher’s exact test (two-tailed) was used for evaluating increases in total survivors. The Mann–Whitney test (two-tailed) was performed to analyze the differences in mean day of death (MDD), virus titers, serum ALT levels and liver score comparisons.

3. Results and discussion
The survival curve of mice treated either 24 h prior to or after inoculation with PTV is shown in Fig. 1. Treatment was most effective when initiated post-infection and at the highest dose of 5 mg/kg. This treatment schedule protected 70% of mice from death. All other doses and schedules failed to significantly protect challenged animals as determined by log-rank analysis. Notably, the
Effect of i.p. P-MAPA treatment on PTV infection in mice.

Table 1

<table>
<thead>
<tr>
<th>Treatment/start</th>
<th>Dosage/day</th>
<th>Surv/total</th>
<th>MDD ± S.D.</th>
<th>Disease parameterb</th>
<th>Liver</th>
<th>Serum</th>
<th>Score ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean virus titerc ± S.D.</td>
<td>ALTd ± S.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-MAPA/-24 h</td>
<td>5 mg/kg</td>
<td>5/10</td>
<td>4.4 ± 0.5</td>
<td>&lt;2.8 (0)</td>
<td>&lt;4.8 ± 1.9 (80)</td>
<td>2784 ± 2384</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/kg</td>
<td>1/10</td>
<td>5.2 ± 0.4</td>
<td>&lt;3.0 ± 0.6 (40)</td>
<td>5.3 ± 0.8 (100)</td>
<td>3306 ± 2986</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>P-MAPA/24 h</td>
<td>5 mg/kg</td>
<td>7/10</td>
<td>5.7 ± 0.6</td>
<td>&lt;2.8 ± 0.1 (20)</td>
<td>&lt;3.7 ± 1.3 (40)</td>
<td>791 ± 1428</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/kg</td>
<td>3/10</td>
<td>6.6 ± 1.4</td>
<td>&lt;3.8 ± 1.4 (40)</td>
<td>&lt;5.4 ± 1.5 (80)</td>
<td>2979 ± 2457</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>50 mg/kg</td>
<td>10/10c</td>
<td>&gt;21</td>
<td>&lt;2.8 (0)</td>
<td>&lt;2.8 (0)</td>
<td>14 ± 11c</td>
<td>0.5 ± 0.5c</td>
</tr>
<tr>
<td>Saline</td>
<td>–</td>
<td>4/20</td>
<td>5.1 ± 0.5</td>
<td>&lt;2.8 (0)</td>
<td>6.1 ± 1.1 (100)</td>
<td>3674 ± 2771</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Sham-infected</td>
<td>–</td>
<td>3/3</td>
<td>&gt;21</td>
<td>&lt;2.8 (0)</td>
<td>&lt;2.8 (0)</td>
<td>9 ± 11</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Animals: female 8-week-old C57Bl/6 mice; Virus: Punta Toro, Adames strain; Drug diluent: saline; Treatment schedule: qd × 6 beg – 24 or 24 h (ribavirin; bid × 5 beg – 4 h); Treatment route: i.p.; Expt. duration: 21 days.

a Mean day of death of mice dying prior to day 21.

b Determined on day 4 of infection; 5 mice per treatment group.

c Log10 cell culture 50% infectious dose (CCID50) of liver or mL of serum. Percentage of animals presenting with detectable virus levels are indicated in parenthesis.

d Alanine aminotransferase; measured in international units per liter.
e Score of 0 (normal liver) to 4 (maximal discoloration).

*P<0.05 compared to saline placebo-treated mice.
**P<0.01 compared to saline placebo-treated mice.
***P<0.001 compared to saline placebo-treated mice.

low-dose P-MAPA treatment initiated 24 h after infection extended the time of death of infected animals compared to the placebo (Fig. 1 and Table 1). The MDD was also greater in the therapeutic 5 mg/kg P-MAPA treatment, in contrast to groups where treatment started prior to infection. The positive control, ribavirin, afforded complete protection to the 80% lethal challenge dose.

Due to the day 4 time of sacrifice chosen in this study in order to maximize liver disease parameters, liver viral burden was only barely detectable in a few of the treated groups, with no detectable virus present in the placebo-treated animals (Table 1). This is likely due to the fact that peak liver burden actually occurs on day 2 of infection and wanes considerably by day 4 (Gowen et al., 2006b). The later sacrifice time, as opposed to day 3, was selected for viral and liver disease parameter analysis since ALT levels and hepatic icterus are more pronounced on day 4. Only the 5 mg/kg/day dose of P-MAPA was successful at reducing serum viral loads with only 2 of the 5 mice presenting detectable virus, compared to 100% for the placebo-treated animals. Moreover, liver disease was also diminished as reflected by the much reduced mean ALT levels and liver scores (Table 1). No other treatment schedule had an appreciable impact on the tested parameters. Ribavirin, as expected, was highly efficacious in limiting viral burden and liver disease.

These findings suggest that frequent administration (once per day for 6 days) may not be the best treatment strategy. Perhaps a higher single dose may provide greater protection since a repeated daily immune stimulation may induce tolerance. Further, handling stress is minimized by a single injection, which improves the fitness of the animals to combat the infection. Therefore, to verify the potential immunomodulatory activity of P-MAPA against PTV infection, a simple dose regime given 24 h post-infection was tested.

As seen in Fig. 2, only the 100 mg/kg dose of P-MAPA was able to elicit adequate host defense in mice infected with Punta Toro virus 24 h prior and provided complete protection from an infectious dose that killed 65% of the mice in the saline placebo-treated group.
group. Ribavirin treatment, included as a positive control, protected 100% of challenged mice (Fig. 2). Treatment with lower doses of P-MAPA did not have a significant impact on survival outcome. Moreover, of the animals that died prior to day 14, there was no appreciable difference in the MDD of the P-MAPA-treated animals that failed to survive the infection compared to the placebo-treated mice (Table 2). As expected (Farmabrasilis, 2009b) for uninfected mice, there was no apparent overt toxicity at the tested doses of P-MAPA as the animals appeared healthy and maintained group weights comparable to the saline-treated animals (Table 3). These findings agree with previous toxicology data of the compound (Farmabrasilis, 2009b).

We also evaluated several disease parameters in groups of mice infected and treated in parallel. In this experiment, very little liver virus could be detected in liver homogenates with the exception of mice in the 10 and 1 mg/kg P-MAPA treatment groups (Table 2). Although there may be some indication that such doses of P-MAPA may increase liver virus levels, the data are only suggestive, as statistically significant differences were not evident. On the other hand, systemic viral loads were dose dependent since the 100 mg/kg treatment reduced viral burden by $\geq 1$ log$_{10}$, whereas serum samples from mice in the 10 and 1 mg/kg treatment groups presented virus titers similar to those seen for the placebo group (Table 1). Virus levels were undetectable in the ribavirin-treated animals; however, these mice began treatment 4 h prior to infection with Punta Toro virus and the drug was given twice daily for 5 days. Consequently, ribavirin also significantly limited liver disease measured by serum ALT levels and hepatic icterus scores (Table 2).

Although the mean levels of ALT found in the P-MAPA-treated mice were all lower than those observed for the placebo group, the differences were not significant. Slight, but appreciable decreased in the mean liver scores were detected in the 100 and 10 mg/kg P-MAPA groups. The ALT and liver score data suggest that the higher doses of P-MAPA are reducing liver disease severity, albeit slightly.

When P-MAPA in a single dose of 100 mg/kg/day after 24 h infection and with a mean liver virus titer and mean serum virus titer (log$_{10}$) of $<2.8$ and $<4.9$, respectively, is compared with some immunomodulators highly active against Punta Toro virus, such as Ampligen (0.005–5 mg/kg/day) (Sidwell et al., 1992), with CLDC immunomodulators highly active against Punta Toro virus, such as Ampligen (0.005–5 mg/kg/day) (Sidwell et al., 1992), with CLDC (0.020 mg/kg/single dose after 24 h) (Gowen et al., 2006a,b) and with ribavirin (50 mg/kg × 5 dose/day-4 h before infection) (this paper) which have mean liver virus titer and mean serum virus titer (log$_{10}$) at the same levels, it is possible to conclude that P-MAPA appears as a promising new, non-toxic immunomodulator to treat Punta Toro virus-induced disease.

### Table 2

<table>
<thead>
<tr>
<th>Treatment/start</th>
<th>Dosage</th>
<th>Surv/total</th>
<th>MDD ± S.D.</th>
<th>Disease parameterb</th>
<th>Mean virus titerc ± S.D.</th>
<th>ALT d ± S.D.</th>
<th>Liver</th>
<th>Scoree ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-MAPA</td>
<td>100 mg/kg</td>
<td>10/10&lt;sup&gt;a&lt;/sup&gt; &gt;14</td>
<td>&lt;2.8 (0)</td>
<td>Liver Serum Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>4/10</td>
<td>5.3 ± 1.2</td>
<td>&lt;3.3 ± 0.8 (60)</td>
<td>5.7 ± 1.0 (100)</td>
<td>2069 ± 2024</td>
<td>2.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>5/10</td>
<td>5.2 ± 0.4</td>
<td>&lt;3.3 ± 0.0 (20)</td>
<td>6.3 ± 0.8 (100)</td>
<td>2892 ± 2170</td>
<td>3.3 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribavirin</td>
<td>50 mg/kg/day</td>
<td>10/10&lt;sup&gt;c&lt;/sup&gt; &gt;14</td>
<td>&lt;2.8 (0)</td>
<td>Liver Serum Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>–</td>
<td>5.6 ± 0.9</td>
<td>&lt;2.8 (0)</td>
<td>Liver Serum Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-infected</td>
<td>–</td>
<td>3/3 &gt;14</td>
<td>&lt;2.8 (0)</td>
<td>Liver Serum Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Animals: female 8-week-old C57BL/6 mice; Drug diluent: saline; Treatment schedule: single dose, 24 h (ribavirin; bid × 5 beg – 4 h); Treatment route: i.p.; Expt. duration: 14 days.

<sup>a</sup> Mean day of death of mice dying prior to day 14.
<sup>b</sup> Determined on day 3 of infection; 5 mice per treatment group (4 mice for the ribavirin group).
<sup>c</sup> Log$_{10}$ cell culture 50% infectious dose (CCID<sub>50</sub>/0.1 g of liver or mL of serum. Percentage of animals presenting with detectable virus levels are indicated in parenthesis.
<sup>d</sup> Alanine aminotransferase; measured in international units per liter.
<sup>e</sup> Score of 0 (normal liver) to 4 (maximal discoloration).

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage</th>
<th>Survivors/total</th>
<th>Mean host weight change&lt;sup&gt;a&lt;/sup&gt; (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-MAPA</td>
<td>100 mg/kg</td>
<td>3/3</td>
<td>0.0</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>3/3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>3/3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Ribavirin</td>
<td>50 mg/kg/day</td>
<td>3/3</td>
<td>0.1</td>
</tr>
<tr>
<td>Saline</td>
<td>–</td>
<td>3/3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Difference between initial weight and weight on day 6.

Animals: female 8-week-old C57BL/6 mice; Drug diluent: saline; Treatment schedule: single dose, 24 h (ribavirin; bid × 5 beg – 4 h); Treatment route: i.p.; Expt. duration: 14 days.

4. Conclusions

Only the 5 mg/kg dose of P-MAPA, given i.p. for 6 days, was effective in protecting mice from death due to PTV infection when the initiation of treatment was delayed until 24 h after infectious challenge. Systemic virus titer reduction and limitation of liver disease were also apparent. The study examining the antiviral activity of a single dose of P-MAPA revealed that the 100 mg/kg dose, administered i.p. 24 h post-infection, was remarkably effective at preventing death due to PTV infection. This dose also reduced systemic viral burden and liver discoloration assayed on day 3 of infection. Overall, the 10 and 1 mg/kg doses did not have a significant impact on the outcome of infection. Compared to the 6-day course of treatment, the single dose strategy appears to be more efficacious.

Taken together, our findings indicate that non-specific immunotherapy with P-MAPA appears to be an effective treatment for blocking Punta Toro virus-induced disease and indicates that further exploration in other viral disease models may be warranted. By comparing P-MAPA with other immunomodulatory compounds with high efficacy against on Punta Toro virus, it is possible to conclude that P-MAPA also appears as a promising new immunomodulator. Further work is needed in the development of compound for use in treating viral infections. Use of P-MAPA as adjuvant immunotherapy with antivirals is a possibility.
Acknowledgements

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