AD_____
(Leave blank)

Award Number: W81XWH-08-2-0042

TITLE: Catalytic Bioscavengers Human Butyrylcholinesterase and Paraoxonase Sequestered to the center for CNS

PRINCIPAL INVESTIGATOR: Ruben J. Boado, Ph.D.

CONTRACTING ORGANIZATION: ArmaGen Technologies, Inc.
Santa Monica, CA 90401

REPORT DATE: April 2010

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

■ Approved for public release; distribution unlimited

□ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2010	Final	01 APR 2008 - 31 MAR 2010

4. TITLE AND SUBTITLE
Catalytic Bioscavengers Human Butyrylcholinesterase and Paraoxonase Sequestered to the center for CNS

5a. CONTRACT NUMBER
W81XWH-08-2-0042

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Ruben J. Boado, PhD

Email: rboado@armagen.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

ArmaGen Technologies, Inc.
914 Colorado Ave.
Santa Monica, CA 90401

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
USAMRMC
504 Scott St.
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this research was 2-fold. The first specific aim was to genetically engineer, express, and validate 2 novel biopharmaceutical fusion proteins, designated AGT-185 and AGT-186. Both proteins are fusion proteins of human paraoxonase (PON)-1 variants and a genetically engineered monoclonal antibody (MAb) against the human insulin receptor (HIR). The 2 proteins are intended for development as new treatments of the brain in organophosphate chemical nerve gas attacks. The second specific aim is the performance of a plasma pharmacokinetics (PK) and brain uptake study of AGT-185 in the adult Rhesus monkey. Both specific aims were accomplished. Both AGT-185 and AGT-186 were engineered, expressed, and shown to retain high binding to the HIR and high PON1 enzyme activity, and the proteins were shipped to the Institute for Chemical Defense for testing against nerve gas agents. The PK study in the primate was the first PK study performed for a variant of human PON1 and showed the IgG-PON1 fusion protein is rapidly cleared from blood following intravenous injection, and is rapidly taken up by all organs including brain. The blood-brain barrier permeation constant for AGT-185 was high compared to a blood volume marker and comparable to the permeation constant for the HIRMAb alone without the fused PON1. AGT-185 is the first stable, field-deployable formulation of human PON1. AGT-185 is the first brain penetrating form of human PON1.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	unlimited	11	Dr. Ruben J. Boado
unclassified	unclassified	unclassified			**19b. TELEPHONE NUMBER** *(include area code)* 310-917-1275

Table of Contents

Page

Introduction

The purpose of this research is 2-fold. The first specific aim is to genetically engineer, express, and validate 2 novel biopharmaceutical fusion proteins, designated AGT-185 and AGT-186. Both proteins are fusion proteins of human paraoxonase (PON)-1 variants and a genetically engineered monoclonal antibody (MAb) against the human insulin receptor (HIR). The 2 proteins are intended for development as new treatments of the brain in organophosphate chemical nerve gas attacks. The second specific aim is the performance of a plasma pharmacokinetics (PK) and brain uptake study of AGT-185 in the adult Rhesus monkey.

Chemical nerve gas agents are organophosphates (OP), and rapidly degraded by enzymes with organophosphatase activity. Human PON-1 is the most potent human protein with organophosphatase activity, and is being considered as a new treatment of OP toxicity. However, OP agents kill via a mechanism of action within the central nervous system (CNS). Therefore, it is important that new agents against OP toxicity be developed that are active within the brain. PON-1, like other large molecule biopharmaceuticals, does not enter the brain, because brain penetration is blocked by the blood-brain barrier (BBB).

Recombinant proteins such as PON1 can be re-engineered for transport across the human BBB with molecular Trojan horse technology. A molecular Trojan horse is a peptide, or peptidomimetic MAb, that undergoes receptor-mediated transport across the BBB via an endogenous BBB receptor. The most potent molecular Trojan horse known is a HIRMAb, which enters brain via the endogenous BBB insulin receptor. The HIRMAb is biologically active in humans and Old World primates, such as the Rhesus monkey, but is not active in New World primates, or in rodents, or other lower species.

The engineering of a fusion protein of the HIRMAb and PON-1 represents a re-engineering of the PON1 protein to enable brain penetration and transport across the BBB. In the first generation fusion protein, AGT-185, the full PON-1 amino acid sequence, from Met-1 to Leu-355, was fused to the carboxyl terminus of the heavy chain of the HIRMAb. This sequence includes the PON1 signal peptide, from Met-1 to Ala-15, since the PON1 signal peptide is not cleaved in vivo. In the second generation fusion protein, AGT-186, a PON1 variant is fused to the HIRMAb, whereby the 15 amino acid signal peptide is deleted, and the His-115 is mutated to Trp-115, the H115W mutation.

Body

Original Statement of Work (SOW):

<u>01 year (months 1-12):</u>

(1) AGT-185

- (a) Dual transfection of COS cells with pCD-HC185 and pCD-LC1 with Lipofectamine 2000 in COS cells under SFM conditions (10xT500 flasks)
- (b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-185 in the presence of 1 mM calcium
- (c) Western blotting of purified AGT-185 with primary antibodies to human IgG and human PON-1
- (d) HIR binding assay using purified HIR extracellular domain as the receptor, and comparison of affinity of AGT-1 and AGT-185 (lot to be shipped to USAMRICD)

(e) Shipment of at least 25 ug of purified AGT-185 to USAMRICD for determination of activity against chemical nerve gas agents, and comparison with recombinant BCE or PON mutants (BCE or PON mutants provided by USAMRICD)

(2) AGT-186
(a) receipt from USAMRICD of a single plasmid DNA encoding mutated BCE or PON
(b) PCR of BCE or PON mutant cDNA and subcloning into pCD-UTV1 to produce pCD-HC186
(c) Bi-directional DNA sequencing of pCD-HC186 expression cassette, which includes CMV promoter, AGT-186 heavy chain fusion gene, and BGH transcription termination sequence
(d) Dual transfection of COS cells with pCD-HC186 and pCD-LC1
(e) Protein A affinity purification of AGT-186 from COS cell conditioned SFM
(f) Western blotting with primary antibodies to human IgG and human BCE/PON (BCE/PON antibody provided by USAMRICD if commercial antibody not satisfactory)
(g) HIR binding assay for comparison of binding of AGT-1, AGT-185, and AGT-186

02 year (months 13-24):

(1) AGT-185
(a) Dual transfection of COS cells with pCD-HC185 and pCD-LC1
(b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-185 in the presence of 1 mM calcium
(c) Radio-labeling of AGT-185 and purification
(d) Intravenous injection of radiolabeled-AGT-185 in anesthetized adult Rhesus monkey, and measurement of plasma pharmacokinetics, brain uptake, peripheral organ uptake, and capillary depletion analysis.

(2) AGT-186
(a) Dual transfection of COS cells with pCD-HC186 and pCD-LC1 with Lipofectamine 2000 in COS cells under SFM conditions (10xT500 flasks)
(b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-186
(c) Western blotting of purified AGT-186 with primary antibodies to human IgG and either human PON-1 or human BCE
(d) HIR binding assay using purified HIR extracellular domain as the receptor; comparison of affinity of AGT-1, AGT-185, and AGT-186
(e) Shipment of at least 25 ug of purified AGT-186 to USAMRICD for determination of activity against chemical nerve gas agents, and comparison with recombinant BCE or PON mutants (BCE or PON mutants provided by USAMRICD)

Key Research Accomplishments

- With respect to AGT-185, COS cells were dual transfected with the heavy chain (HC) and the light chain (LC) expression plasmids encoding AGT-185. Conditioned serum free medium (>2L) was collected, and AGT-185 was purified by protein A affinity chromatography in the presence of 1 mM CaCl2. The bi-functionality of the purified AGT-185 was verified by an HIR binding ELISA, a

fluorometric enzymatic assay of PON-1 organophosphatase activity, and by human IgG and PON1 Western blotting.

- The COS-derived AGT-185 was shipped to the USAMRICD, and shown to have activity against chemical nerve gas agents that was comparable to the PON1 control.
- With respect to AGT-186, this fusion protein is identical to AGT-185 with the following exceptions: (a) the 15-amino acid PON1 signal peptide is deleted, and (b) the PON1 sequence contains the H115W mutation. The PON1 cDNA, minus the sequence encoding for the 15 amino acid signal peptide was produced by PCR, and spliced at the 3'-end of the coding region of the heavy chain (HC) of the chimeric monoclonal antibody (MAb) against the HIR. The engineering of this intermediate plasmid, designated pCD-HC185SP, was validated by DNA sequencing and expression of the fusion protein, designated AGT-185SP, in COS cells. AGT-185SP is identical to AGT-185, except the HC does not contain the 15 amino acid PON1 signal peptide. Using site-directed mutagenesis (SDM) and custom oliogodexoynucleotides (ODN), we mutated the DNA sequence of pCD-HC185SP encoding position 115 from histidine (H) to tryptophan (W), and this SDM was confirmed by DNA sequencing. This SDM of pCD-HC185SP resulted in the engineering of the new HC expression plasmid, pCD-HC186, which is the HC expression plasmid encoding AGT-186. COS cells were dual transfected with pCD-HC-186 and pCD-LC-1, where the latter is the AGT-186 light chain (LC) expression plasmid DNA. COS cells were initially transfected in 6-well dishes, and then in 10xT500 flasks in serum free medium, followed by purification of AGT-186 with protein A affinity chromatography. The identity and potency of AGT-186 was confirmed by SDS-PAGE, human IgG and PON1 Western blotting, by human insulin receptor (HIR) binding, and by paraoxonase (PON)-1 enzyme activity. The AGT-186 was shipped to the USA ICD for testing against nerve gas agents.
- We performed additional work on a new fusion protein, AGT-184. AGT-186 is a variant of AGT-185, which contains the H115W mutation, and also lacks the 15 amino acid (AA) PON-1 signal peptide. Subsequently, we engineered AGT-184, which is identical to AGT-185, except the 15 AA PON-1 signal peptide is removed. Both AGT-184 and AGT-186 lack the 15 AA PON-1 signal peptide, but AGT-186 includes the additional modification of the H115W mutation. Both AGT-184 and AGT-186 were expressed in COS cells, and affinity purified with protein A chromatography. For testing of AGT-184 and AGT-186 activity against chemical nerve gas agents, we shipped to Steve Kirby on 8-4-9 183 ug of AGT-184 and 235 ug of AGT-186.
- With respect to the plasma PK and brain uptake of AGT-185 in the Rhesus monkey, we originally planned to co-inject the primate with [3H]-AGT-185 and [125I]-PON-1. However, we purchased recombinant paraoxonase (PON)-1 from 2 different vendors, and both batches of PON1 were not pure on reducing SDS-PAGE and Coomasie blue staining. We felt the results would not be interpretable given the impurity of the PON1. Therefore, we removed the PON1 from the study, since the co-injection of PON1 was not part of the original aims of the project. We have performed pilot tritiations of fusion protein with [3H]-N-

succinimidyl propionate (NSP), which we obtain freshly prepared from American Radiolabeled Chemicals, Inc., and this has proven to be an excellent method of radiolabeling fusion proteins without the denaturing effects coincident with oxidative radio-iodination associated with the chloramine T method. We then tritiated the AGT-185 to a specific activity of 2.6 uCi/ug, and a trichloroacetic acid (TCA) precipitation of 99%, and this product was injected intravenously into an adult female Rhesus monkey of 5.1 kg at the UCLA Dept. of Lab Animal Medicine under subcontract with that institution. The UCLA facility was site visited by the US Army in early 2010. The animal was injected intravenously (IV) with 1436 uCi of [^{3}H]-AGT-185 fusion protein in 0.6 mL of Tris buffered saline(pH=8.0), formulated with 0.02% Terigitol NP-10 and 1 mM CaCl2, by bolus injection over 30 seconds in the left femoral vein. The dose of fusion protein was 0.13 mg/kg. The animal was initially anesthetized with intramuscular ketamine, and anesthesia was maintained by 1% isoflurane by inhalation. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Following intravenous drug administration, femoral venous plasma was obtained at 1, 2.5, 5, 15, 30, 60, and 120 min for determination of ^{3}H radioactivity. The animal was euthanized, and samples of major organs (heart, liver, spleen, lung, skeletal muscle, and omental fat) were removed, weighed, and processed for determination of radioactivity. The cranium was opened and the brain was removed. Samples of frontal cortical gray matter, frontal cortical white matter, cerebellar gray matter, and cerebellar white matter were removed for radioactivity determination. Samples (~2 gram) of frontal cortex were removed for capillary depletion analysis. The brain was homogenized in 8 mL cold PBS in a tissue grinder. The homogenate was supplemented with 9.4 mL cold 40% dextran (70 kDa), and an aliquot of the homogenate was taken for radioactivity measurement. The homogenate was centrifuged at 3200 g at 4C for 10 min in a fixed angle rotor. The brain microvasculature quantitatively sediments as the pellet, and the post-vascular supernatant is a measure of capillary depleted brain parenchyma. The vascular pellet and supernatant were counted for ^{3}H radioactivity in parallel with the homogenate. The volume of distribution (VD) was determined for each of the 3 fractions from the ratio of total ^{3}H radioactivity in the fraction divided by the total ^{3}H radioactivity in the 120 min terminal plasma. Plasma and tissue samples were analyzed for ^{3}H radioactivity with a liquid scintillation counter (Tricarb 2100TR, Perkin Elmer). All samples for ^{3}H counting were solubilized in Soluene-350 and counted in the LSC in Opti-Fluor O (Perkin Elmer).

- The ^{3}H radioactivity in plasma, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a bi-exponential equation,

  $$\%ID/mL = A1e^{-k1t} + A2e^{-k2t}$$

  The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the median residence time (MRT), the central volume of distribution (Vc), the steady state volume of distribution (Vss), the area under the plasma concentration curve (AUC), and the systemic clearance (CL). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd, Cork,

Ireland). Data were weighted by $1/(\%ID/mL)^2$. The organ clearance (μL/min/g), also called the permeability-surface area (PS) product, is computed from the terminal organ uptake (%ID/g) and the 120 min plasma AUC (%IDmin/mL) as follows:

$$\text{organ PS product} = [(\%ID/g)/AUC]*1000$$

Reportable Outcomes

(1) The plasma concentration decay curve for AGT-185 is shown in Figure 1. The % of injected dose (ID)/mL decreases rapidly in plasma following IV injection. This plasma decay curve was fit to the bi-exponential equation described above to yield the PK parameters shown in Table 1. The data show a high systemic volume of distribution (Vss) relative to the central volume (Vc), which is indicative of rapid egress of AGT-185 from the vascular compartment. This is also illustrated by the very high rate of systemic clearance (CL) of AGT-185 (Table 1).

(2) AGT-185 penetrated brain and peripheral tissues, as reflected by the high %ID/100 grams tissue (Table 2). The brain uptake data were normalized by the 2 hour plasma area under the concentration curve (AUC) to yield the BBB permeability-surface area (PS) product. Figure 2 shows the BBB PS product for AGT-185 is high compared to that of a plasma volume marker, human IgG1, which indicates that AGT-185 is transported across the BBB. The BBB PS produce shown in Figure 2 for AGT-185 is comparable to the previously reported value for the chimeric HIRMAb, without the fused PON1. These findings indicate that fusion of PON1 to the heavy chain of the HIRMAb has no effect on the penetration of the BBB by the antibody.

(3) Transport of AGT-185 across the BBB, as opposed to simple binding to the brain vasculature, was confirmed with the capillary depletion method (Table 3). The high brain VD in the post-vascular supernatant is evidence that the AGT-185 has penetrated the BBB and entered into brain parenchyma.

Conclusions

- AGT-185 is a genetically engineered IgG fusion protein, wherein human paraoxonase (PON)-1 is fused to the carboxyl terminus of the heavy chain of the chimeric monoclonal antibody (MAb) against the human insulin receptor (HIR). The HIRMAb part of AGT-185 acts as a molecular Trojan horse to ferry the fused PON1 across the blood-brain barrier (BBB) via transport on the endogenous BBB insulin receptor. The HIRMAb cross-reacts with the insulin receptor in Old World primates, such as the Rhesus monkey, but not lower species.
- The expressed AGT-185 was purified with protein A affinity chromatography and anion exchange chromatography. The purified protein passed specifications with a dozen analytic tests of purity, potency, and identity. AGT-185 bound the HIR with high affinity, KD=0.58±0.10 nM, and had high PON1 enzyme activity, 641± 26 nmol/hr/mg, based on a fluorometric enzymatic assay using a model organophosphate substrate.
- AGT-185 was radiolabeled with [3H]-N-succinimidyl propionate to a specific activity of 2.6 uCi/ug and a trichloroacetic acid precipitability of >99%. The [3H]-AGT-185 was injected intravenously (IV) into a 5.1 kg female Rhesus monkey for

a pharmacokinetics (PK) study at a systemic dose of 0.13 mg/kg. Blood was sampled at 2.5, 5, 15, 30, 60, 90, and 120 min after injection, and the animal was euthanized at 120 min after injection for sampling of brain.

- AGT-185 was rapidly removed from primate blood with a systemic clearance of 7.6±0.3 mL/min/kg and a systemic volume of distribution of 230±29 mL/kg. The systemic clearance of AGT-185 is several-fold faster than the clearance of the chimeric HIRMAb alone, which suggests the high clearance in vivo is due to the PON1 moiety.
- AGT-185 was taken up by brain and the 2 hour brain uptake was 0.47±0.01 % of injected dose per brain. The brain volume of distribution (VD) of AGT-185 was 362±27 uL/gram, which is high compared to the brain VD of a brain plasma volume marker, such as human IgG1, 14±1 uL/gram (Table 3). The capillary depletion method was used to verify that the AGT-185 actually penetrated brain parenchyma, and was not simply absorbed to the brain microvasculature. The BBB permeability-surface area (PS) product of AGT-185 was 2.0±0.1 uL/min/gram (Figure 2), which is high and comparable to the BBB PS product of the HIRMAb alone. These data indicate that AGT-185 penetrates the BBB, and that fusion of the PON1 does not inhibit transport of the HIRMAb across the BBB in vivo.

OVERALL CONCLUSIONS:

- AGT-185 is the first stable, field-deployable formulation of human PON1.
- This is the first PK study of a human PON1 formulation, and shows that a IgG-PON1 fusion protein has a high systemic clearance and systemic volume of distribution.
- AGT-185 is the first brain penetrating form of human PON1.

References

None.

Appendices

Figure 1. Rate of removal of AGT-185 from the plasma compartment following IV injection in the adult Rhesus monkey.

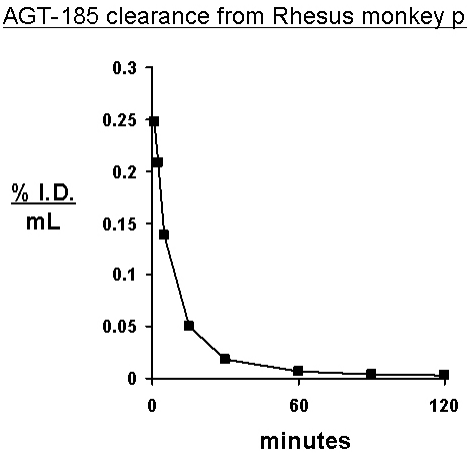

Figure 2. Blood-brain barrier (BBB) permeability-surface area (PS) product of AGT-185 (HIRMAb-PON1) and an isotype control antibody, human IgG1 (hIgG1), measured at 2 hours after IV injection in the adult Rhesus monkey. The PS product of hIgG1 reflects sequestration in the brain plasma volume, as the hIgG1 does not cross the BBB. The PS product for AGT-185 is high and comparable to the PS product for the HIRMAb alone without the fused PON1. These data show that AGT-185 traverses the BBB, and that fusion of the PON1 has no inhibitory effect on HIRMAb transport into brain.

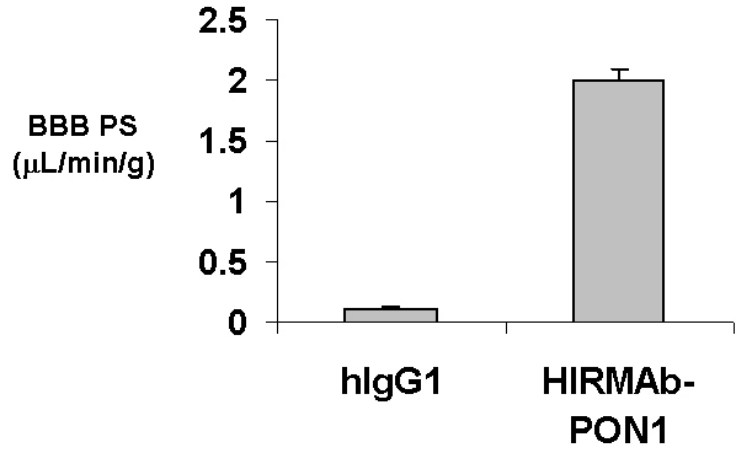

Table 1. Pharmacokinetic parameters of AGT-185 in the Rhesus monkey.

parameter	units	Value
A1	%ID/mL	0.245 ± 0.020
A2	%ID/mL	0.014 ± 0.004
k1	min-1	0.118 ± 0.011
k2	min-1	0.014 ± 0.003
MRT	min	30 ± 4
Vc	mL/kg	92 ± 7
Vss	mL/kg	230 ± 29
$AUC\Big\vert^{120}$	%IDmin/mL	2.92 ± 0.13
AUCss	%IDmin/mL	3.12 ± 0.14
CL	mL/min/kg	7.6 ± 0.3

Table 2. Organ uptake of [^{3}H]-AGT-185 in the Rhesus monkey

organ	**Organ uptake (% I.D./100grams)**
Frontal gray	0.47 ± 0.01
Frontal white	0.37 ± 0.01
Cerebellar gray	0.54 ± 0.07
Cerebellar white	0.50 ± 0.02
heart	0.55 ± 0.16
liver	38.8 ± 0.3
spleen	21.3 ± 1.1
lung	3.8 ± 0.2
Skeletal muscle	0.22 ± 0.02
fat	0.76 ± 0.03
kidney	13.8 ± 1.7

Table 3. Capillary depletion analysis of human IgG1 and AGT-185 distribution in Rhesus monkey brain

Parameter	**hIgG1 VD**	**AGT-185 VD**
Homogenate VD	14 ± 1	362 ± 27
Post-vascular supernatant VD	-	287 ± 17
Brain capillary pellet VD	-	32 ± 6

VD=volume of distribution (uL/g)