Peptide Conjugated Phosphorodiamidate Morpholino Oligomers Increase Survival of Mice Challenged with Ames *Bacillus anthracis*

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Targeting bacterial essential genes using antisense phosphorodiamidate morpholino oligomers (PMOs) represents an important strategy in the development of novel antibacterial therapeutics. PMOs are neutral DNA analogues that inhibit gene expression in a sequence-specific manner. In this study, several cationic, membrane-penetrating peptides were conjugated to PMOs (PPMOs) that target 2 bacterial essential genes: acyl carrier protein (*acpP*) and gyrase A (*gyrA*). These were tested for their ability to inhibit growth of *Bacillus anthracis*, a gram-positive spore-forming bacterium and causative agent of anthrax. PPMOs targeted upstream of both target gene start codons and conjugated with the bacterium-permeating peptide (RFF)₃R were found to be most effective in inhibiting bacterial growth *in vitro*. Both of the gene-targeted PPMOs protected macrophages from *B. anthracis* induced cell death. Subsequent, *in vivo* testing of the PPMOs resulted in increased survival of mice challenged with the virulent Ames strain of *B. anthracis*. Together, these studies suggest that PPMOs targeting essential genes have the potential of being used as antisense antibiotics to treat *B. anthracis* infections.

Introduction

BACILLUS ANTHRACIS is a gram-positive, spore-forming bacterium and causative agent of anthrax disease. The most effective drugs to treat human anthrax are ciprofloxacin, a broad-spectrum fluoroquinolone class of antibiotic and doxycycline, a tetracycline antibiotic (Brook, 2002). However, the rising incidences of resistance to current antibiotics (Brook et al., 2001; Choe et al., 2000; Price et al., 2003) and the possibility of developing engineered antibiotic resistant bacteria pose a potential threat to human health and national security. Identifying unique chemical scaffolds and novel targets and approaches will be important for development of broad-spectrum countermeasures that have therapeutic potential against the emerging resistant and multi-resistant bacteria.

Antisense phosphorodiamidate morpholino oligomers (PMOs) and their derivatives downregulate target gene expression in a sequence-dependent manner by interfering with the binding of ribosome to mRNA and thereby inhibiting protein translation (Iversen, 2001). The PMO backbone is made of morpholino rings with phosphorodiamidate linkage,

which protects them from nuclease degradation while still maintaining the complementary base pairing. Covalent conjugation of the PMO with membrane-penetrating peptides has enhanced their cellular uptake by mammalian cells (Moulton et al., 2004) as well as bacteria (Tilley et al., 2006).

The potential application of PMO-based antisense technology targeting bacterial pathogens is being explored for the development of a new class of antibacterial drugs. Phosphorodiamidate morpholino oligomers (Geller et al., 2003; Greenberg et al., 2010) have been shown to silence expression of bacterial genes. Gene-silencing oligomers decrease expression of reporter genes such as luciferase, activate endogenous genes such as β -galactosidase, and inhibit growth by targeting essential genes (Geller et al., 2005). Some of the attractive targets for antisense antibacterial therapy include essential genes such as acyl carrier protein (*acpP*) encoding the fatty acid biosynthesis protein (Geller et al., 2005; Greenberg et al., 2010), bacterial RNA polymerases (Bai et al., 2011), *fabI*-encoding enoyl-acyl carrier protein reductase and 16S rRNA (Nielsen, 2010). Prior studies have shown that PMO targeting the *acpP* gene was able to inhibit *E. coli* viability *in vitro* and

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in vivo in mice (Geller et al., 2005). To further improve the uptake of the PMOs by the bacteria, PMOs conjugated with various peptides (PPMOs) and targeting the *acpP* gene were shown to enhance bacterial growth inhibition and viability of *E. coli* and *Salmonella enterica* Serovar Typhimurium (Tilley et al., 2006). More recently PPMOs targeting the *acpP* gene inhibited *Burkholderia cepacia* complex (Bcc) *in vitro* and increased survival of mice challenged with a clinical isolate of Bcc (Greenberg et al., 2010).

In the present study, several PPMOs targeting 2 bacterial essential genes *acpP* and gyrase A (*gyrA*) (Christen et al. 2011; Gil et al., 2004) were tested for their ability to *in vitro* inhibit growth of *B. anthracis*, a gram-positive spore-forming bacterium. Our studies revealed that their ability to inhibit bacterial growth was dependent on the base position of the PMO relative to the start codon of the targeted mRNA and the nature of the peptide sequence. In a cell-based assay, select PPMOs increased viability of macrophages infected with *B. anthracis*. *In vivo*, PPMOs improved the survival of mice challenged with the highly virulent Ames strain of *B. anthracis*.

Materials and Methods

Antisense peptide tagged phosphorodiamidate morpholino oligomers (PPMOs)

The peptide-tagged neutral or charged PMOs were synthesized by AVI Biopharma Inc. (Corvallis, OR) as described previously (Summerton and Weller, 1993; Tilley et al., 2006; Weller and Hassinger, 2008; Fox M.J. et al., 2009). PMOs targeting the 2 essential genes acyl carrier protein (*acpP*; gene id. No. 2849363) and gyrase A (*gyrA*; gene id. No. 2851434) with sequence complementarities at various base positions relative to the start codon of the targeted mRNA were designed and are listed in Table 1. Neutral PMOs were tagged through an XB linker with peptide RFFRFFRFFR [(RFF)₃R] or RXRRX RXRRXR [(RXR)₄] where R=arginine; F=phenylalanine;

X=6-aminohexanoic acid; and B=β-alanine. The peptides were attached at either the 5' or 3' end of the PMOs.

Bacterial strains

The Sterne strain of *Bacillus anthracis* was used to test all the PPMOs in the bacterial growth inhibition studies. Select PPMOs were then tested *in vitro* and *in vivo* in mice using the virulent Ames strain of *B. anthracis*.

Minimum inhibitory concentration studies

In vitro inhibition of bacterial growth and subsequent minimum inhibitory concentrations (MICs) were determined by the broth microdilution methods (NCCLS, 2003). Briefly, *B. anthracis* cultures (5×10^5 CFU/mL) in log-phase growth were resuspended in Mueller-Hinton broth. Absorbance at 600 nm was measured and concentration calculated. Bacterial cultures (100 μL, 5×10^5 CFU/mL) were grown aerobically in 96-well plates with various concentrations of PPMOs and scrambled control PPMOs ranging from 0 to 80 μM. Plates were incubated at 37°C for 16–20 hours and cell growth was determined by measuring the absorbance at 600 nm. All experiments were done in duplicate and repeated 3 times.

Cell based infection assays

J774A.1 macrophages (6×10^5) were infected with Sterne strain of *B. anthracis* at a multiplicity of infection of 5 in the presence of indicated concentrations of test or scrambled control PMOs. After 4-hour incubation at 37°C, bacterial growth was inhibited by adding antibiotics penicillin (100 IU) and streptomycin (100 μg/mL). Cell viability was determined by adding Sytox green, a dye impermeant to live cells. After 15 minutes at 37°C, cells were washed twice with phosphatase buffered saline and analyzed by flow cytometry. All experiments were repeated 3 times.

TABLE 1. MINIMUM INHIBITORY CONCENTRATIONS OF PEPTIDE-CONJUGATED PHOSPHORODIAMIDATE MORPHOLINO OLIGOMERS TARGETING BACTERIAL ESSENTIAL GENES FOR ACYL CARRIER PROTEIN (*acpP*), GYRASE A (*gyrA*), AND FtsZ (*ftsZ*) AGAINST *B. ANTHRACIS*

AVI NG-	Gene target	Gene ID No.	Base position*	Sequence 5'→3'	Peptide	MIC (μM)
05-0544	acpP	2849363	4 to 14	AAA ACA TCT GC	(RFF) ₃ R	80
06-0759	acpP	2849363	−9 to +2	ATT CCA TTC AC	(RFF) ₃ R	80
06-0760	acpP	2849363	−17 to −7	CAC CTC CCC TC	(RFF) ₃ R	1.25
08-0971	acpP	2849363	−17 to −7	CAC CTC CCC TC	3'-(RFF) ₃ R	1.25
07-1031	acpP	2849363	−9 to +2	ATT CCA TTC AC	(RXR) ₄	80
07-1032	acpP	2849363	−17 to −7	CAC CTC CCC TC	(RXR) ₄	80
07-1088	acpP	2849363	−17 to −7	CAC CTC CCC TC	3'-(RXR) ₄	80
05-0546	gyrA	2851434	4 to 14	TGA TTG TCT GA	(RFF) ₃ R	80
07-1087	gyrA	2851434	−16 to −6	AGC ACC TCC TC	(RFF) ₃ R	5
07-1161	ftsZ	2848148	−23 to −13	CTA GAA CCT CA	(RFF) ₃ R	2.5
Controls						
05-0655	Scrambled			TCT CAG ATG GT	(RFF) ₃ R	160
06-0078	Scrambled			TCT CAG ATG GT	(RXR) ₄	40
06-0093	Scrambled	Cat10		TCT TTC TTC T	(RFF) ₃ R	>160
	ciprofloxacin					0.1
	vancomycin					0.8
	Polymyxin B					29
	nisin					6

*Numbering from first base of start codon.

MIC, minimum inhibitory concentration.

PPMO resistance

Strains of spontaneous resistant mutants were selected in liquid broth cultures. Each day for 10 days, an overnight culture of *B. anthracis* Sterne 34F2 was diluted 1:50 in Mueller-Hinton 2 broth supplemented with 20 μ M (RFF)₃R-AcpP (06-0760) and grown aerobically at 37°C for 15–18 hours. After 10 days, the culture was diluted and spread on lysogeny broth (LB) agar plates. Ten colonies were selected at random and characterized for their resistance by measuring the MIC of (RFF)₃R-AcpP. The MIC of (RFF)₃R-AcpP PPMO in one of the isolates (named BAPR1) was 4 times higher than in the parent strain.

Ciprofloxacin-resistant mutant

One hundred microliters of a 15-hour LB broth culture of *B. anthracis* Sterne 34F2 was spread and grown for 15 hours at 37°C on an LB agar plate that included 0.05 μ g/mL ciprofloxacin. Colonies were picked and grown for 15 hours at 37°C in liquid LB broth with 0.1 μ g/mL ciprofloxacin. This cycle of alternate growth on agar and liquid broth was repeated twice with increasing concentrations of ciprofloxacin up to 4 μ g/mL. One colony was isolated on 2 μ g/mL ciprofloxacin and further characterized.

B. anthracis mice challenge studies

Mice (8–10 weeks old C57BL/6 strain, 10 mice/group) were treated via the intraperitoneal (i.p.) route with the PPMOs (100 μ g/mouse/day or 5 mg/kg). After 1 hour, mice were infected via the i.p. route with ~500 CFU of Ames *B. anthracis* spores. Subsequent treatment with the PPMOs was continued at times +1 hour, +6 hours and +24 hours post infection. Mice survival was observed for 10 days. The experiments were repeated 2 independent times. To evaluate the statistical significance, the samples were subjected to Kaplan Meier survival analysis and log rank tests to compare survival curves among groups. The procedures involving the manipulation of infectious material were conducted within biological safety cabinets by personnel wearing appropriate protective clothing and equipment in the BSL3 containment suite.

All research was conducted under an approved protocol and in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals, and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The facility at which this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Results

In vitro bacterial growth inhibition

Peptide conjugated phosphorodiamidate morpholino oligomers (PPMOs) targeting the 2 bacterial essential genes *acpP* and *gyrA* were tested for their ability to *in vitro* inhibit growth of *B. anthracis*. As shown in Table 1, inhibition of growth was dependent on the base position of the PMO relative to the start codon of the targeted mRNA. PPMO base sequences targeting the nontranslated upstream regions of

both genes, which often include the ribosome binding site, inhibited growth. (RFF)₃R-AcpP (06-0760), which is complementary to the region upstream of the start codon (–17 to –7) had an MIC = 1.25 μ M. This was more effective than either of the other AcpP PPMOs (05-0544 and 06-0759) that target regions of *acpP* farther downstream (MIC = 80 μ M each). Similarly, (RFF)₃R-GyrA (07-1087), which targets the region upstream of the start codon, was more effective than (RFF)₃R-GyrA (05-0546), which targets a downstream region. (RFF)₃R-AcpP (06-0760) was more effective in reducing bacterial growth than was (RFF)₃R-GyrA (07-1087).

A comparison of the 2 peptides that were conjugated to the AcpP PPMOs showed that (RFF)₃R-AcpP (06-0760) was more effective than (RXR)₄-AcpP (07-1032). Furthermore, changing the (RFF)₃R peptide from the 5' to 3' end of the AcpP PPMO had no effect on the MIC (compare 06-0760 with 08-0971).

Thus, overall these studies suggest that PPMOs that are targeted upstream of the start codon near the ribosome binding site and tagged with (RFF)₃R peptide inhibit growth of *B. anthracis* *in vitro*.

Cell-based infection assays

B. anthracis produces lethal toxin that *in vitro* causes the lysis and death of susceptible macrophages (Friedlander et al., 1993). To determine if the PPMOs could prevent *B. anthracis*-induced cell death, macrophages were treated with PPMOs and infected with *B. anthracis*. Cell viability was determined by measuring the uptake of the membrane impermeant Sytox green dye using flow cytometry. As shown in Fig. 1, macrophages infected with *B. anthracis* in the presence of the (RFF)₃R-AcpP (06-0760) or (RFF)₃R-GyrA (07-1087) showed increased cell viability compared with the scrambled control or infection alone. At a concentration of as little as 1 μ M (RFF)₃R-AcpP was efficient in protecting infected macrophages. In contrast, a higher concentration (10–20 μ M) of the (RFF)₃R-GyrA was required to achieve similar protection of infected macrophages. Thus, these studies show that PPMOs protected macrophages from the lethal effects of infection with *B. anthracis*.

Resistance to PPMOs

A spontaneous PPMO-resistant strain (BAPR1) was isolated by growing *B. anthracis* Sterne in broth supplemented with 20 μ M (RFF)₃R-AcpP. BAPR1 was further characterized for its susceptibility to ciprofloxacin and PPMOs. As depicted in Table 2, the MIC results show that BAPR1 was fully susceptible to ciprofloxacin but was mildly resistant (4-fold) to the PPMO (06-0760) used for selection. Furthermore, BAPR1 was 8-fold more resistant to (RFF)₃R-FtsZ (07-1161) compared with the parental Sterne strain. (RFF)₃R-FtsZ is targeted to a noncoding region upstream of the start codon of *ftsZ*, which is an essential gene required for bacterial cell division. These results suggest that the mechanism of resistance to PPMOs in BAPR1 is not specific for *acpP*. In contrast, BAPR1 was fully susceptible to (RFF)₃R-GyrA (NG-07-1087). Apparently the mechanism of resistance is not linked to the peptide, but appears to be linked to the sequence of the PMO.

A ciprofloxacin-resistant strain of *B. anthracis* was characterized for its susceptibility to (RFF)₃R-AcpP. Results indicate that the susceptibility of ciprofloxacin-resistant *B. anthracis* to (RFF)₃R-AcpP (NG-06-0760) was similar to the ciprofloxacin-

FIG. 1. Cell viability of *Bacillus anthracis*-infected macrophages. Cell viability of macrophages treated with indicated concentrations of targeted peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) and infected with *B. anthracis* was measured by uptake of Sytox green dye using flow cytometry. The percentage of live and dead cells are indicated.

sensitive parental strain of *B. anthracis* (Table 2). Thus, together these studies suggest that that resistance to PPMOs does not cause a change in the resistance to antibiotics and resistance to antibiotics does not result in resistance to PPMOs.

In vivo efficacy studies

The *in vivo* efficacy of the PPMOs targeting *acpP* (NG-06-0760) or the *gyrA* (NG-07-1087) was tested in the virulent Ames *B. anthracis* infection model. Mice were pretreated with the PPMOs (5 mg/kg) and after 1 hr infected with the virulent

Ames strain of *B. anthracis*. Additional treatments were given at +1 hour, +6 hours and +24 hours post infection. As shown in Fig. 2, mice treated with the gene-targeted PPMOs showed a statistically significant increase in the survival rate ($P=0.0003$) compared with scramble or phosphate buffered saline controls that succumbed to the pathogen as early as 48 hours post challenge. A statically significant increase in survival was observed in mice treated with *acpP* ($P=0.0008$) or *gyrA* ($P<0.0001$) PPMOs compared to scrambled treated controls. Thus these studies confirm that PPMOs targeting bacterial essential genes are capable of protecting mice in a lethal challenge model of *B. anthracis*.

TABLE 2. MINIMUM INHIBITORY CONCENTRATIONS OF PEPTIDE-CONJUGATED PHOSPHORODIAMIDATE MORPHOLINO OLIGOMERS AGAINST WILD TYPE, PHOSPHORODIAMIDATE MORPHOLINO OLIGOMER-RESISTANT AND CIPROFLOXACIN-RESISTANT *BACILLUS ANTHRACIS*

Antibacterial	Gene target	Peptide	MIC (μ M)		
			Sterne (parent, susceptible)	PMO-Resistant BAPR1	Ciprofloxacin resistant <i>B. anthracis</i> (CR1.4)
NG-06-0760	<i>acpP</i>	5'-(RFF) ₃ R	1.25	5	1.25
NG-07-1087	<i>gyrA</i>	5'-(RFF) ₃ R	5	5	N.D.
NG-07-1161	<i>ftsZ</i>	5'-(RFF) ₃ R	2.5	20	N.D.
Controls					
NG-05-0655	scrambled	(RFF) ₃ R	160	160	N.D.
ciprofloxacin			0.1	0.1	1.0

MIC, minimum inhibitory concentration; N.D., not determined; PMO, phosphorodiamidate morpholino oligomer.

FIG. 2. *In vivo* testing of PPMOs in the virulent Ames *B. anthracis* infection model. C57BL/6 mice ($n=20$) pretreated for 1 hr with targeted PPMOs (5 mg/kg) were infected with the virulent Ames strain of *B. anthracis*. Subsequent treatment was continued at +1 hour, +6 hours and +24 hours. The percentage of mice that survived the challenge was plotted. Statistical analysis revealed a significant increase in survival rates in *B. anthracis* infected mice that were treated with *acpP* ($P=0.0008$) or *gyrA* ($P<0.0001$) PPMOs compared with scrambled treated controls.

Discussion

In this study, antisense technology was tested for efficacy against *B. anthracis*, a proven agent of bioterrorism. Two bacterial essential genes *acpP* and *gyrA* were targeted, and variations in the target position were investigated. Previous studies in *E. coli* have revealed that an optimal targeting strategy for PMOs is to position the oligomers immediately upstream of the start codon (Deere et al., 2005). In *B. anthracis*, our results showed a similar effect. Targeting positions immediately upstream of the start codons of either *acpP* or *gyrA* was more effective than targeting positions downstream. A likely explanation for this effect is an overlap with the apparent ribosome binding site, which is separated 7–9 bases upstream from the start codon (Vellanoweth and Rabinowitz, 1992). Although these results show a correlation between MIC and position of the PPMO targeting upstream of the start codon, the sample size is too small to make a generalized statement. There is a possibility that the high G/C rich content of these PPMO sequences may have contributed to the lower MIC values. However, analysis of a large number of PPMO sequences in *E. coli* has shown no correlation between G/C content and MIC (Geller et al., unpublished results).

PMOs by themselves are not efficiently taken up by bacteria. However, the sequence-specific effects of the PMO on bacterial gene expression have been significantly improved by attaching a membrane-penetrating peptide to the end of the PMO (Geller et al., 2003; Tilley et al., 2006; Mellbye et al., 2009; Tilley et al., 2007). The peptides themselves are known to traverse through the lipid bilayer, often without causing lysis of the bacterial cell, or without killing the bacteria (Vaara and Porro, 1996). Presumably, the movement of the peptide through the bilayer delivers the PMO to the intracellular compartment. Importantly, mixtures of PMOs and membrane-penetrating peptides are ineffective, which indicates that the peptide must be covalently attached to the PMO for entry into the cell (Geller and Iversen, unpublished data).

Peptides with various patterns of alternating cationic and hydrophobic amino acids have been shown to penetrate bacterial membranes without bactericidal effect (Vaara and Porro, 1996). The strategy of using membrane-penetrating peptides to carry synthetic antisense oligomers into bacterial cells was first shown by Good et al. (Good et al., 2001). In the present study, 2 peptides (RFF)₃R and (RXR)₄ (R = arginine; F = phenylalanine; X = 6-aminohexanoic acid) covalently conjugated to PMOs targeting *acpP* or *gyrA* gene were tested for their ability to *in vitro* inhibit growth of *B. anthracis*. PMOs conjugated with the (RFF)₃R peptide were most effective in inhibiting bacterial growth compared to those tagged with the (RXR)₄ peptide. The antibacterial effect was not modulated by moving the peptide from the 5' to the 3' end.

Our resistance results suggest that PPMO-resistance in one particular isolate, BAPR1, was not linked to the peptide or a specific target, but rather appeared to be dependent on the PMO sequence. Although the mechanism of resistance to the PPMOs is unknown, our observed results do not necessarily suggest a nonspecific effect. Cell growth may be more tolerant to changes in concentration of GyrA than AcpP or FtsZA. It has been shown that a small decrease in the concentration of some proteins (such as AcpP) may cause growth defects, whereas similar decreases in other proteins have little or no observable effects (Goh et al., 2009). It is also possible that a mutation in BAPR1 may somehow reduce, but not entirely eliminate the uptake of PPMOs. If PPMO resistance were linked to uptake of PPMOs, perhaps changes in PPMO uptake could have little effect on the threshold concentration of GyrA but a higher effect on AcpP or FtsZ that is required to maintain growth. Furthermore, a sequence-biased degradation of AcpP or FtsZ PPMO more than GyrA PPMO could conceivably contribute to the observed results. However, to test these hypotheses, assays will need to be developed to quantify PPMOs in biological samples.

An important challenge in antibacterial discovery is to successfully tackle antibacterial resistance. Our results show that a ciprofloxacin-resistant *B. anthracis* was susceptible to the (RFF)₃R-AcpP to the same extent as the parental strain. This suggests that PPMOs may be effective against antibiotic-resistant strains of *B. anthracis* because they have a completely different structure and mechanism of action than standard antibiotics.

The antibacterial activity of the antisense PPMOs was demonstrated in cell-based infection assays. Macrophages treated with the *acpP* PPMO and infected with *B. anthracis* showed much-reduced cell death compared to scrambled or infected control alone. A higher concentration of the (RFF)₃R-GyrA compared with (RFF)₃R-AcpP was required for the same effect.

The *in vivo* efficacy studies revealed that *AcpP* and *GyrA* PPMOs protected mice after lethal challenge with the highly virulent Ames strain of *B. anthracis*. Although the (RFF)₃R-AcpP was more effective than the (RFF)₃R-GyrA in the MIC assays, the results of the lethal challenge showed the opposite trend. The inverted trend is not surprising because growth conditions are quite different *in vivo* compared to *in vitro*. Bacterial growth rate, gene expression, mRNA abundance, and PPMO pharmacokinetics and -dynamics may all play roles in determining the efficacy of PPMOs *in vivo*.

The 2 scrambled PPMOs with the same base sequence but different peptides (06-0078 and 05-0655) had different MIC

values (see Table 1). This suggests that the efficacy of PPMOs may not be entirely gene specific. Off target or nonspecific effects of PPMOs have also been observed *in vitro* with *E. coli* (Wesolowski et al., 2011) and *Acinetobacter lwoffii* (Geller, unpublished data), and *in vivo* with *Burkholderia multivorans* (Greenberg et al., 2010). Some of this nonspecific effect may be due to partial complementarity of scrambled sequence controls to other genes (Wesolowski et al., 2011). Our data suggests that the nonspecific effect is dependent on the amino acid composition of the peptide. To further improve the antibacterial efficacy of the PPMOs while at the same time reducing the non-specific effects of the peptide, it would be useful to test the new generation of the cationic PMOs (Mellbye et al., 2010), and vary the composition of the peptide.

In summary, these studies show that PPMOs reduced growth of *B. anthracis* *in vitro* and *in vivo*. Furthermore, PPMOs were effective against ciprofloxacin-resistant *B. anthracis*. This suggests that antisense technology is a feasible approach for developing novel antibacterials for *B. anthracis*.

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Author Disclosure Statement

Drs. Bruce Geller and Patrick Iversen were employed at AVI Biopharma and Oregon State University during these studies.

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