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Doxycycline as an inhibitor of p-glycoprotein in the alpaca for the purpose of maintaining avermectins in the CNS during treatment for parelaphostrongylosis

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ABSTRACT

Meningeal worms (*Parelaphostrongylus tenuis*) are a common malady of alpacas, often refractory to conventional treatments. Ivermectin is a very effective anthelmintic used against a variety of parasites but this drug is not consistently effective against alpaca meningeal worms once the parasite has gained access to the CNS, even if used in a protracted treatment protocol. Ivermectin is not effective against clinical cases of *P. tenuis*, raising the possibility that the drug is not sustained at therapeutic concentrations in the central nervous system (CNS). A specific protein (designated as p-glycoprotein (PGP)) effluxes ivermectin from the brain at the blood–brain barrier, thus hampering the maintenance of therapeutic concentrations of the drug in the CNS. Minocycline is a synthetic tetracycline antibiotic with an excellent safety profile in all animals tested to date. Minocycline has three unique characteristics that could be useful for treating meningeal worms in conjunction with ivermectin. First, minocycline is an inhibitor of PGP at the blood–brain barrier and this inhibition could maintain effective concentrations of ivermectin in the brain and meninges. Second, minocycline protects neurons *in vivo* through a number of different mechanisms and this neuroprotection could alleviate the potential untoward neurologic effects of meningeal worms. Third, minocycline is a highly lipid-soluble drug, thus facilitating efficient brain penetration. We thus hypothesized that minocycline will maintain ivermectin, or a related avermectin approved in ruminants (abamectin, doramectin, or eprinomectin), in the alpaca CNS. To test this hypothesis, we cloned the gene encoding the alpaca PGP, expressed the alpaca PGP in a heterologous expression system involving MDCK cells, and measured the ability of minocycline to inhibit the efflux of avermectins from the MDCK cells; doxycycline was used as a putative negative control (based on studies in other species). Our *in vitro* studies surprisingly revealed that doxycycline was effective at inhibiting the efflux of ivermectin and doramectin (minocycline had no effect). These two avermectins, in combination with doxycycline, should be considered when treating meningeal worms in alpacas.

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1. Introduction

Meningeal worms (*Parelaphostrongylus tenuis*) are nematodes that infect alpacas (and other species), often resulting in death once neurologic signs are manifested (Johnson et al., 2006). Treatment once involved a protracted high dose protocol of ivermectin (now supplanted by fenbendazole (Whitehead and Bedenice 2009)), but this drug failed to be consistently effective. Since ivermectin is not reliably effective against clinical cases of *P. tenuis*, it is possible that the drug is not sustained at therapeutic concentrations in the central nervous system (CNS) despite the elevated dose and exaggerated duration of treatment.

Meningeal worm treatment failures associated with ivermectin are potentially due to efflux of ivermectin from the brain. P-Glycoprotein (PGP) is a non-specific efflux pump that removes foreign substances, including ivermectin, from the brain at the blood–brain-barrier (Darby et al., 2011). The purpose of PGP is to prevent noxious accumulation of xenobiotics in the brain, as evidenced by the ivermectin-associated neuropathies in a subset of Collies (a breed of canines) that do not express a functional PGP (Mealey et al., 2001). That is, the homeostatic activity of PGP leads to an egress of ivermectin from the brain and this egress minimizes the therapeutic potential of ivermectin in the CNS.

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Minocycline has three unique characteristics that could be useful as an ivermectin potentiator for treating meningeal worms. First, minocycline is an established inhibitor of PGP at the blood–brain barrier (Milane et al., 2007) and this inhibition could maintain ivermectin in the brain and meninges. Second, minocycline protects neurons *in vivo* through a number of different mechanisms including anti-inflammation and anti-apoptosis (Stirling et al., 2005) and this neuroprotection could alleviate the potential untoward neurologic effects of meningeal worms and ivermectin. Third, minocycline is a highly lipid-soluble drug, thus facilitating efficient blood–brain-barrier penetration. We hypothesized that minocycline will inhibit the alpaca PGP and thus maintain ivermectin, or a related avermectin approved in ruminants (abamectin, doramectin, or eprinomectin), in tissue culture cells acting as a surrogate for the alpaca CNS. To test this hypothesis, we cloned the gene encoding the alpaca PGP, expressed the alpaca PGP in a heterologous expression system involving Madin–Darby Canine Kidney (MDCK) cells, and measured the ability of minocycline to inhibit the efflux of avermectins from the MDCK cells. The goal of this study was to identify an avermectin whose efflux is hindered by minocycline. This identification will provide the basis for a minocycline–avermectin co-treatment for parelaphostrongylosis in alpacas. Our results identified an alternative tetracycline, one that had not been previously associated with PGP inhibition, that apparently blocks the alpaca PGP and potentiates avermectins in a model of the CNS.

2. Materials and methods

2.1. Cloning of the alpaca gene encoding PGP

The gene encoding the alpaca PGP was cloned from mRNA obtained from alpaca blood. Total RNA was isolated using the RNeasy blood kit (Qiagen) and cDNA was created using polyT and random primers. Segments of the alpaca PGP cDNA were PCR-amplified using degenerate primers designed based on published sequences of the ovine gene encoding PGP (accession number NP_001009790.1). The deduced amino acid sequence is presented in Fig. 1A and B.

2.2. Heterologous expression of the alpaca gene encoding PGP

The gene encoding the full-length alpaca PGP cDNA was synthesized (GenScript) and cloned into the eukaryotic expression vector pcDNA3.3 (Invitrogen). A control plasmid (the full-length alpaca PGP cDNA in the prokaryotic expression vector pUC57) was also synthesized.

MDCK cells, which are the standard for heterologous expression of PGP (Lam and Rajaraman, 2012), were maintained in 24-well tissue culture dishes in EMEM (ATCC) with 10% fetal bovine serum (ATCC) at 37 °C with 5% CO_2. For transfections, MDCK cells were seeded at 2×10^5 cells/well and then transfected 24 h later using lipofectamine (Invitrogen) and 1 μg vector DNA as per the manufacturer's protocol. Functional analysis ensued 48–72 h after transfection.

2.3. Assessment of avermectin efflux in MDCK cells heterologously expressing the alpaca PGP

At 48–72 h post-transfection, MDCK cells were incubated with either minocycline or doxycycline (both obtained from Sigma–Aldrich) or saline (vehicle). Both drugs were added to the cells at a final concentration of 5 μg/mL, which is the maximum plasma concentration (C_{max}) for standard parenteral doses in sheep (Castro Robles et al., 2012; Wilson and Green, 1986).

After one hour of incubation with either minocycline or doxycycline, avermectins (abamectin, doramectin, eprinomectin, and ivermectin) were separately added at 5 ng/mL which is the C_{max} for these drugs in sheep administered standard parenteral doses (Hennessy et al., 2000; Hodoscek et al., 2008; Lloberas et al., 2012). Avermectins were allowed to incubate with the cells for six hours after which the media was washed away and cells were lifted off the plate using trypsin.

Cells were then lysed using bead-beating as per Hansen et al. (2014). Lysates were submitted to the Pharmacology Analytical Support Team laboratory at Iowa State University for intracellular avermectin quantitation using LC–MS. Non-transfected cells were used as the background matrix.

2.4. Statistical analyses

Statistical comparisons were made using an analysis of variance with Tukey's *post hoc* test for multiple comparisons. $p < 0.05$ was deemed to indicate statistical significance.

3. Results

In order to determine if minocycline can inhibit the PGP in the alpaca, the alpaca PGP was heterologously expressed in MDCK cells and minocycline-mediated increases in intracellular avermectin concentrations were measured. Ivermectin, doramectin, abamectin, and eprinomectin were chosen as the avermectins since all four are available for use in ruminants.

As shown in Fig. 2, minocycline had no effect on the alpaca PGP-dependent efflux of any avermectin. The intracellular concentrations of all four avermectins were markedly lower when the PGP was expressed, including when minocycline was present, when compared to cells lacking PGP. However, doxycycline prevented the PGP-mediated efflux of ivermectin and doramectin in the heterologous expression system Fig. 3, In PGP-expressing cells, doxycycline elevated the concentrations of ivermectin and doramectin to that observed for cells lacking PGP. The concentrations of ivermectin and doramectin were significantly lower in PGP-expressing cells in the absence of doxycycline, indicating that the PGP was effluxing the avermectins.

4. Discussion

Meningeal worms cause fatalities in alpacas and treatment failures can be associated with an inability to deliver effective anthelmintic drug concentrations to the brain. Avermectins are effective at inhibiting meningeal worms if an adequate concentration reaches the worms, or if the drug can prophylactically kill the migrating larvae (Nagy, 2004). Avermectins are effluxed from the brain by the PGP present in the blood–brain-barrier, thus diminishing CNS concentrations of these drugs.

Minocycline is a synthetic tetracycline antibiotic that has activities unrelated to its antibacterial activity. Minocycline is an inhibitor of PGP (Milane et al., 2007) and it also has neuroprotective effects (Stirling et al., 2005). Therefore this drug appeared to be a good candidate for increasing CNS concentrations of avermectins while also protecting the brain from the insults presented by the meningeal worms and the potential neurologic side effects of elevated avermectin concentrations in the brain.

Interestingly, our results indicate that minocycline is not an inhibitor of the alpaca PGP but doxycycline, a second-generation tetracycline related to minocycline, is an inhibitor of this efflux protein. While doxycycline has not previously been associated with inhibition of the human PGP (Michel et al., 1984), the human and alpaca PGPs are only 83% identical (Fig. 1A). The alpaca and ovine PGPs are 95% identical (Fig. 1B), suggesting that doxycycline may also be a PGP inhibitor in other ruminants.

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