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Plasma protein binding limits the blood brain barrier permeation of the pyrethroid insecticide, deltamethrin

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HIGHLIGHTS

- Deltamethrin (DLM) is not a P-glycoprotein substrate or inhibitor.
- The extent of DLM permeation into the brain is dependent on protein binding.
- Transport of DLM is linear/passive at physiological albumin concentrations.

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ABSTRACT

Previous pharmacokinetic studies of deltamethrin (DLM) have revealed that brain levels of this highly lipophilic pyrethroid insecticide are only 15–20% of plasma levels. Experiments were performed to assess determinants limiting CNS access including plasma protein binding and the efflux transporter, P-gp. A human brain microvascular endothelial cell line, hCMEC/D3, was utilized as a model *in vitro* system to evaluate blood-brain barrier (BBB) permeation. Incubation of DLM with a series of human serum albumin (HSA) concentrations showed that unbound (f_u) DLM ranged from 80% with 0.01% HSA to ~20% at the physiologically-relevant 4% HSA. A positive correlation (R = 0.987) was seen between f_u and cellular uptake. Concentration-dependent uptake of DLM in 0.01% HSA was non-linear and was reduced at 4 °C and by the P-gp inhibitor cyclosporine (CSA), indicative of a specific transport process. Cellular accumulation of [^{3}H]-paclitaxel, a P-glycoprotein (P-gp) substrate, was increased by CSA but not by DLM, suggesting that DLM is neither a substrate nor an inhibitor of P-gp. The concentration-dependent uptake of DLM from 4% HSA was linear and not significantly impacted by temperature or CSA. In situ brain perfusion studies monitoring brain association of DLM at 0.01% and 4% HSA confirmed the aforementioned *in vitro* findings. This study demonstrates that brain uptake of DLM under normal physiological conditions appears to be a passive, non-saturable process, limited by the high protein binding of the pyrethroid.

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1. Introduction

Pyrethroids are the most frequently utilized insecticides in the U.S., since the phase out of organophosphates (Williams et al., 2008). Pyrethroids are used outdoors in many agricultural, urban construction and landscaping settings, as well as indoors in homes and other structures. Certain pyrethroids, such as permethrin, are used to treat ticks, mites and lice on pets and humans (Frankowski et al., 2010). Thus, it is not surprising that large segments of the population are exposed to this class of pesticide. In fact, 3-phenoxybenzoic acid, a metabolite common to many pyrethroids, was detected in the urine of the majority of over 5000 persons monitored in the general U.S. population (Barr et al., 2010). Pyrethroid exposures most frequently result from the inadvertent ingestion of dusts, hand-to-mouth activity with pets and consumption of very low levels in foods (Lu et al., 2010; Morgan, 2012).

Although most pyrethroids exert relatively low mammalian toxicity, high doses can be acutely neurotoxic. Their primary mechanisms of action are interference with the closure of neuronal voltage-gated calcium and sodium channels (Cao et al., 2011;

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Soderlund, 2012). The duration of the closure delay and modification of sodium currents by deltamethrin (DLM) may last for several seconds, resulting in stimulus-dependent nerve depolarization and blockage. This results in typical toxic signs of salivation and hyperexcitability, possibly progressing to choreoathetosis (i.e., CS syndrome).

It would be anticipated that these highly lipophilic compounds should readily enter and accumulate in the brain. However, DLM peak brain concentrations and areas under plasma concentration-time curves were found to be only 15–20% of plasma and blood values (Kim et al., 2008, 2010). This suggests that the CNS deposition of DLM is governed by other parameters that are not well understood. It has been well established that binding to albumin and other plasma proteins can significantly reduce the free fraction (f_u) of drugs available for absorption into the CNS. *In situ* brain extraction of a series of benzodiazepines in rats, for example, is significantly influenced by their extent of plasma binding (Jones et al., 1988; Lin and Lin, 1990). Metabolism and systemic clearance also reduce the amount of free chemical available for CNS uptake. DLM and other pyrethroids are extensively oxidized by rat and human hepatic microsomal cytochrome P450s and hydrolyzed by hepatic and plasma carboxylesterases (Anand et al., 2006; Ross et al., 2006; Scollon et al., 2009). Transport mechanisms are prevalent within BBB endothelial cells, including a number of Solute Carrier and ATP Binding Cassette transporters. P-gp, an ATP-dependent efflux transporter, is located on the apical membrane of endothelial cells of the BBB. Although recent studies from our laboratory demonstrated that P-gp does not appreciably limit DLM intestinal permeability using the Caco-2 cell line model (Zastre et al., 2013), it is unknown if P-gp limits CNS deposition via the BBB. To our knowledge, no one has evaluated potential determinants of flux for DLM or other pyrethroids across the BBB.

The overall objective of this investigation was to gain an understanding of the transfer of the pyrethroid, DLM, from the blood into the brain. Emphasis was placed on clarifying the role of processes generally believed to limit access, in particular, protein binding and whether it is a substrate for influx or efflux transporters, notably P-gp. A human brain microvessel endothelial cell line, hCMEC/D3, was selected as a model *in vitro* system. These cells display many characteristics of brain endothelium *in vivo*, including tight junction formation and expression of transporters (Weksler et al., 2005; Zastre et al., 2009). This *in vitro* model was supplemented by *in situ* brain DLM uptake experiments in rats.

2. Materials and methods

2.1. Materials

Cell culture media and supplements, which include EBM-2 media and vascular endothelial growth factor, insulin-like growth factor 1, epidermal growth factor, fibroblast growth factor, hydrocortisone, ascorbate, and gentamycin supplements, were obtained from Lonza (Allendale, NJ). Rat tail collagen type 1 and trypsin/EDTA were purchased from BD Biosciences (San Jose, CA) and Mediatech (Manassas, VA), respectively. Fetal bovine serum (FBS), mannitol, and human serum albumin (HSA) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture flasks, plates, and dishes were obtained from Greiner Bio-one (Monroe, NC). Cyclosporine A (CSA) was purchased from Amresco (Salon, OH). Radiolabeled [^{14}C]-DLM (54.1 mCi/mmol) was kindly donated by Bayer CropScience (Research Triangle Park, NC). [^{3}H]-Paclitaxel (PTX) (23Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), while iso-octane *iso*-octane and acetonitrile were purchased from EMD Chemicals (Billerica, MA).

2.2. Cell culture

hCMEC/D3 cells were kindly donated by Dr. Babette Weksler, Weill Cornell Medical College. Cells were maintained at 37 °C, 5% CO_2, and 95% humidified air in EBM-2 media supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, fibroblast growth factor, hydrocortisone, ascorbate, gentamycin, and 2.5% FBS as previously described (Weksler et al., 2005). Cells were seeded onto rat-tail collagen type-1 coated flasks and 24-well plates.

2.3. Protein binding of DLM

Protein binding of DLM with HSA was measured using a modification of a procedure previously reported (Sethi et al., 2014). Briefly, 1.0 μM [^{14}C]-DLM was incubated with increasing concentrations of HSA (0.01, 0.1, 1.0, and 4.0% w/v) dissolved in PBS at 37 °C for 15 min. Two volumes of iso-octane were added and vortexed for 1 min, followed by centrifugation at 14,000*g* for 5 min to separate aqueous and organic layers. The organic layer was removed, and the free (unbound) fraction it contained was quantified using a liquid scintillation counter (LS 6500, Beckman Coulter, Brea, CA). The protein-bound fraction was quantified by subjecting the aqueous layer to protein precipitation using 3 vols of acetonitrile. The sample was vortexed for 1 min and centrifuged at 14,000*g* for 5 min. The supernatant was removed and quantified by liquid scintillation counting. To establish the effect of DLM concentration on protein binding, increasing concentrations of [^{14}C]-DLM ranging from 0.1–10 μM with either 0.01% or 4.0% HSA was assessed as described above.

2.4. Cellular uptake experiments

The uptake of 1.0 μM [^{14}C]-DLM by hCMEC/D3 cells from a series of HSA concentrations (0.01, 0.1, 1.0, and 4.0% w/v) was assessed at 37 °C for 3 min (Linear range of uptake—Suppl. Fig. 1). Prior to uptake, the cells were washed twice with pre-warmed HBSS (Mediatech) containing 10 mM HEPES pH = 7.2 (hereafter, referred to as transport buffer), and pre-incubated for 15 min with transport buffer. Subsequently, 1.0 μM [^{14}C]-DLM in HSA containing transport buffer was added to the wells. After the uptake time, the cells were washed 3 times with ice-cold PBS and lysed (1% Triton X–100, 50 mM Tris, 250 mM NaCl pH = 8.0). The amount of DLM was quantified using liquid scintillation counting, and the results were normalized to total protein using a BCA protein assay kit (Thermo Scientific, Rockford, IL).

A similar protocol was used to establish whether DLM is a substrate or inhibitor for P-gp-mediated efflux. Inhibitor properties were assessed by determining the uptake of the P-gp substrate [^{3}H]-PTX (4 nM) with or without the P-gp inhibitor, CSA (25 μM), and DLM (10 μM) at 37 °C for 30 min in transport buffer. For substrate properties of DLM for P-gp, the uptake of 1.0 μM [^{14}C]-DLM was monitored in the presence of the P-gp inhibitor CSA (25 μM) from 0.01% and 4% HSA (low and high protein binding extremes, respectively). The amount of PTX or DLM was quantified using liquid scintillation counting and normalized to total protein.

2.5. Kinetic analysis of DLM transport

The concentration-dependent uptake of DLM was determined to evaluate its transport kinetics for estimation of the Michaelis–Menton constant (K_m) and maximal transport velocity (V_{max}) in hCMEC/D3 cells. The uptake (3 min) of increasing concentrations of [^{14}C]-DLM (0.1–10 μM) in transport buffer containing 0.01% or 4% HSA was performed as described above. The total uptake rate into hCMEC/D3 cells is the sum of all saturable (specific; nonlinear) and

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