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In Vitro Efficacy of Lufenuron against Filamentous Fungi and Blood Concentrations after PO Administration in Horses

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Lufenuron is a benzoylphenyl urea-derived insecticide that has been recently introduced as a novel treatment for fungal infections in horses. The purposes of this study were to determine (1) the in vitro efficacy of lufenuron against *Aspergillus* and *Fusarium* spp. and (2) the ability of lufenuron to reach efficacious blood concentrations after PO administration in horses. Fungal colonies isolated from diseased equine corneas were tested against lufenuron solutions up to 700 μg/mL. Twenty-one adult horses received 1 of 3 PO lufenuron treatment regimens: 5 mg/kg body weight (BW) q24h for 3 days, 20 mg/kg BW q24h for 3 days, or 60 mg/kg BW q24h for 1 day. Blood samples were collected up to 96 hours after drug administration and analyzed by high-performance liquid chromatography. Statistical analyses of lufenuron blood concentrations were performed by analysis of variance and Fischer's Least Significant Difference test, with statistical significance set at $P < .05$. Lufenuron showed no effect on the in vitro growth of *Aspergillus* or *Fusarium* spp. Lufenuron was detected in the blood of all but 1 horse and showed no adverse effects. The maximum blood lufenuron concentration (83.5 ± 58.7 μg/L) was lower than the concentrations proven to be ineffective in vitro in this study. Further therapeutic use of lufenuron as an antifungal agent in horses should be based on proven efficacy against specific strains of clinically relevant fungi with pharmacokinetic data demonstrating sufficient lufenuron concentrations in target tissues.

Key words: Antifungal; *Aspergillus;* Endometritis; Equine; *Fusarium;* Keratitis; Sinusitis.

Lufenuron is a benzoylphenyl urea-derived insecticide that has been recently introduced as a novel treatment for fungal infections in horses.[1] The insecticidal properties of lufenuron are attributed to its ability to inhibit chitin synthesis, polymerization, and deposition,[2] making it a popular pesticide in the agricultural industry and a commonly used method of flea control in dogs and cats. Lufenuron has also been advocated for the management of dermatophytosis in cats, dogs, and chimpanzees,[3,4] presumably due to its ability to disrupt chitin within fungal cell walls. Veterinarians have been using lufenuron for the treatment of various fungal infections in horses, including endometritis,[1] guttural pouch mycosis, and paranasal sinusitis. Most recently, it has been instituted as a potential treatment for keratomycosis, a prevalent and potentially sight-threatening condition for which reliable medical therapy is currently lacking.[a,b] For this as well as for paranasal sinusitis, lufenuron has been PO administered to horses at 60 mg/kg q2wk and at 5 mg/kg q24h, respectively. Lufenuron has also been used topically on the cornea for equine keratomycosis at 70 mg/mL q24h.[b]

There is a paucity of data regarding the efficacy of lufenuron against any fungal species. Lufenuron has demonstrated noncytotoxic in vitro efficacy against microsporidial species of fungi,[5] but its in vitro efficacy against filamentous fungi has not been reported. Furthermore, the only published, prospective, controlled in vivo study of lufenuron's antifungal activity reported no effect on the time from peak dermatophytic infection to disease resolution in cats treated with lufenuron PO.[6]

Lufenuron has been shown to be safe when administered either PO or parenterally in cats, dogs, and rats.[7,8] Lufenuron achieves measurable concentrations in blood and skin after PO and parenteral administration in dogs and cats, with transient accumulation in adipose tissues due to its highly lipophilic nature.[8,9] To the authors' knowledge, there is no analogous information regarding the distribution of lufenuron in horses. The main objectives of this study were to determine (1) the in vitro efficacy of lufenuron against common filamentous fungal infectious agents in horses and (2) the ability of lufenuron to reach efficacious blood concentrations after PO administration in horses. These objectives serve ultimately to determine lufenuron's potential as a therapeutic agent for mycotic infections in horses. This investigation represents the first controlled analysis of lufenuron's efficacy against filamentous fungi and the first controlled comparison of the treatment regimens currently in use for equine mycoses.

Materials and Methods

In Vitro Susceptibility

Susceptibility to *Aspergillus* and *Fusarium* spp. was tested with both chemical-grade lufenuron and a commercial topical suspension,[c] according to standard recommendations for filamentous fungi.[10] All fungal organisms tested were isolated from corneas of horses affected with keratomycosis. Lufenuron was diluted in isopropyl alcohol and further diluted to final working concentrations in RPMI-1640 with L-glutamine without bicarbonate and buffered with 4-morpholinopropanesulfonic acid. Fungal colonies were overlaid with sterile distilled water. A conidial suspension was prepared by gently scratching the surface of the colony with a Pasteur pipette. Larger particles were allowed to settle out of the suspension for 5 minutes. The final test inoculum was adjusted spectrophotometrically to achieve a suspension of 5,000 to 50,000 colony-forming units/mL. The inoculum was added to media containing different drug concentrations and incubated at

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35°C for 24 hours. One *Aspergillus* isolate was tested over concentrations of 0.125 to 64.000 μg/mL of the chemical grade lufenuron; 2 *Aspergillus* and 2 *Fusarium* isolates were tested over concentrations reaching 700 μg/mL of the commercial topical suspension. Fungal growth was compared to drug-free control tubes containing equivalent initial *Aspergillus* or *Fusarium* concentrations.

Drug Preparation

Technical-grade lufenuron in a powder formulation was obtained directly from the manufacturer[d] and stored (according to its recommendations) at room temperature until administration. The commercial topical lufenuron suspension[c] (70 mg/mL) was obtained from a retail pharmacy and was also stored, according to the manufacturer's recommendations, at room temperature.

Horses

Twenty-one healthy adult horses (16 males and 5 females) were obtained from the University of Missouri College of Veterinary Medicine's donation and blood donor herds. The horses ranged in age from 2 to 24 years (mean age = 12 years) and ranged in weight from 414 to 623 kg (mean weight = 535 kg). All horses were housed in the Veterinary Medical Teaching Hospital throughout their participation in the study. An acclimation period of at least 48 hours was allotted to all horses before the administration of lufenuron. Each horse was deemed healthy based on results of a physical examination performed during the acclimation period. All procedures were approved by the University of Missouri's Animal Care and Use Committee.

Complete ophthalmic examinations, including slit lamp biomicroscopy and indirect ophthalmoscopy, were performed on each horse during the acclimation period. Each horse was sedated with either xylazine hydrochloride[e] (0.6 mg/kg IV) or detomidine hydrochloride[f] (0.01 mg/kg IV), and mydriasis was obtained with 25 μg tropicamide[g] topically in each eye. Follow-up ophthalmic examinations were performed similarly between 12 and 14 weeks after the first dose of lufenuron in available horses (n = 3; 1 horse from group 2 and 2 horses from group 3).

All horses were fed a ration consisting of 2 flakes of an alfalfa-grass hay mixture twice daily and water ad libitum; in addition, horses were provided with a daily bran mash at 8:00 AM each morning (50% moist bran, 50% Omolene 100® Active Pleasure[h]). At the time of drug dosing, lufenuron was thoroughly mixed into the bran mash immediately before feeding. The bran mash buckets were checked within 30 minutes of dosing to ensure that the entire contents had been consumed.

Experimental Protocol

Horses were divided into 3 groups. The time at which lufenuron was administered was designated as hour 0. Group 1 horses (n = 9) received lufenuron at 5 mg/kg body weight (BW) PO at hours 0, 24, and 48. Group 2 horses (n = 6) received lufenuron at 20 mg/kg BW PO in the same fashion. Group 3 horses (n = 6) received a single 60 mg/kg BW PO dose of lufenuron at hour 0. All horses received physical examinations (including evaluations of rectal temperature, heart rate, respiratory rate, mucous membrane color, capillary refill time, intestinal sounds, and digital arterial pulses) twice daily and were monitored for signs of colic or discomfort at least every 2 hours until hour 96. Blood was drawn from each horse from the jugular vein into ethylenediaminetetraacetic acid–containing tubes and stored at −20°C for up to 4 weeks, until evaluated. Samples were collected at hours 0, 1, 2, 3, 6, 12, 24, 48, 72, and 96.

Sample Analysis

Whole blood samples were analyzed by high-performance liquid chromatography (HPLC). The method was adapted from a protocol previously described[8] and employed a Hitachi L-7100 pump, L-7200 autosampler, L-7400 ultraviolet (UV) detector, D-7000 data handling system,[i] Supelco Discovery HS F5 C18 reverse phase column (150 mm × 4.6 mm),[j] mobile phase of methanol : water (85 : 15), flow rate of 1 mL/min, and UV detection at 260 nm. A primary standard of 1,000 mg/L lufenuron was prepared by dissolving 10 mg of the drug in 10 mL of methanol. Additional working standards of 100 μg/L and 50 μg/L were also prepared in methanol. The level of detection was 2 μg of lufenuron per liter of whole blood.

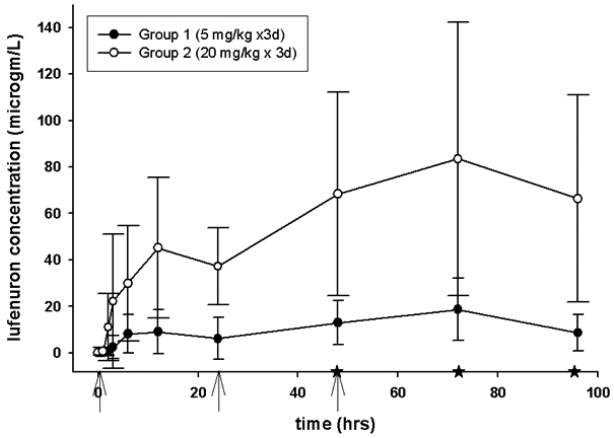

Fig 1. Mean lufenuron concentration versus time, groups 1 and 2. Error bars represent standard deviations. Asterisks mark the times for which statistically significant differences were detected between groups 1 and 2. Arrows mark the times at which lufenuron was administered.

Analysis of Data

Data for the PO absorption study were analyzed by a repeated measurement split-plot in time.[11] The areas under the blood lufenuron concentration-time curves were determined by statistical software by the trapezoidal rule, and statistical analyses were performed by analysis of variance. Mean differences were determined by Fischer's Least Significant Difference test, with statistical significance set at $P < .05$. Results were reported as mean ± standard deviation.

Results

In Vitro Susceptibility

Compared to the drug-free controls, there was no inhibition of *Aspergillus* spp. or *Fusarium* spp. growth for any concentration tested. There were also no susceptibility differences between the technical-grade and the commercial lufenuron suspensions.

Whole Blood Lufenuron Concentrations

There was marked variation in whole blood concentrations of lufenuron among horses within all treatment groups (Figs 1, 2). One horse in group 1 failed to achieve detectable lufenuron concentrations at any time point. In groups 1 and 2, there was an initial peak in the mean whole blood lufenuron concentration at hour 12 (Fig 1). Mean whole blood concentrations of lufenuron in these groups were progressively increased 24 hours after each lufenuron dose. The maximum mean whole blood lufenuron concentrations between hours 0 and 96 in groups 1 and 2 were 18.7 ±

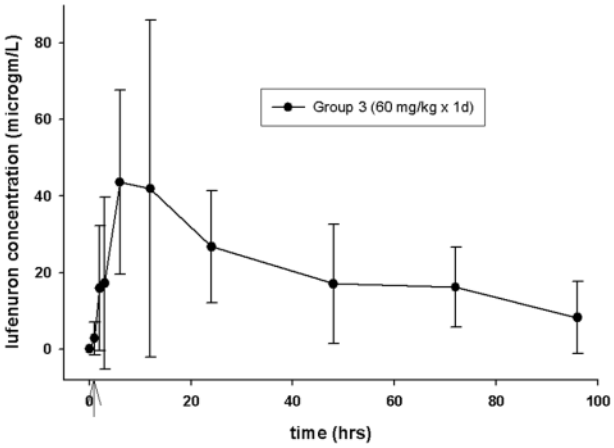

Fig 2. Mean lufenuron concentration versus time, group 3. Error bars represent standard deviations. The arrow marks the time at which lufenuron was administered.

13.3 μg/L and 83.5 ± 58.7 μg/L, respectively, at hour 72 (Fig 1; Table 1). These groups demonstrated a dose-dependent relationship between PO-administered lufenuron and whole blood lufenuron concentration, with statistically significant differences identified at hours 48, 72, and 96 (Fig 1) and nadirs at hour 24.

The single PO dose of lufenuron administered to group 3 resulted in a maximum whole blood lufenuron concentration of 43.6 ± 24.0 μg/L at hour 6 (Fig 2; Table 1). This peak blood lufenuron concentration was followed by a steady decline, reaching 8.23 ± 9.42 μg/L by hour 96 (Fig 2).

Between hours 0 and 24, during which only 1 dose of lufenuron was given to each treatment group, the only statistically significant differences in mean whole blood lufenuron concentrations were between groups 1 and 3, at hours 6 and 12. The whole blood lufenuron area under the curve (AUC) was significantly less in group 1 compared with groups 2 and 3 (Table 1).

Daily and follow-up examinations revealed no adverse effects associated with PO lufenuron administration. Follow-up ophthalmic examinations revealed no significant changes.

Discussion

The failure of lufenuron to inhibit in vitro growth of 2 common equine fungal pathogens, *Aspergillus* and *Fusarium*,[12] at the concentrations used in this study is noteworthy. Indeed, this is consistent with the inefficacy of other chitin synthase inhibitors against *Aspergillus*.[13] Potential explanations for this inefficacy are speculative, given that the structure of chitin synthase has yet to be completely determined and given that the mechanism of lufenuron's chitin synthase inhibition is also incompletely understood.[14,15] However, it is known that *Aspergillus fumigatus* possesses 7 distinct chitin synthase isoenzymes, the second highest number of all fungal organisms investigated to date.[16,17] Given that there is differential activity of chitin synthase inhibitors for specific chitin synthase isoenzymes,[18] it is possible that the inhibition mechanism(s) of lufenuron does not adequately target specific *Aspergillus* isoenzymes. It has also been postulated that chitin synthase inhibition fails in filamentous fungi due to an absence of peptide transporters, enzymes that (in yeasts) serve to deliver chitin synthase inhibitors across the cell wall to the active site of chitin synthase.[19] Lastly, evidence indicates that certain fungi may adapt to the inhibition of one cell wall component's synthesis (such as chitin) by compensatory production of another (such as glucan).[20] It should be noted that the highest concentration of lufenuron tested (700 μg/mL) in this study was less than that used in the experimental topical treatment of equine keratomycosis (70 mg/mL).[b] However, this concentration was the maximum testable in vitro due to the opacity of the formulation as well as the dilution effect that results from the drug being combined with the fungal suspension.

Whole blood lufenuron concentration was deemed more appropriate than serum or plasma lufenuron concentrations. This was a result of the suspicion that lufenuron concentrates heavily within blood cells and blood cell membranes. It also enables comparison to studies of whole blood lufenuron concentrations after PO administration in other species.[8,9]

The results of the oral absorption study reveal that only low concentrations of lufenuron are detected in the peripheral circulation of horses after PO administration. This may be due to limited lufenuron solubility within/absorption by the gastrointestinal tract of horses, significant breakdown of lufenuron in the gastrointestinal tract of the horse prior to absorption, marked first-pass metabolism of absorbed lufenuron by the liver,[21] or a high volume of distribution, or a combination of these factors.

Groups 1 and 2 demonstrated a dose-dependent relationship with consecutive PO lufenuron dosing. A dose-dependent relationship was not identified for groups 2 and 3 over the applicable time period (the first 24 hours after lufenuron administration). Furthermore, the mean AUC of the whole blood concentration of lufenuron over the first 24 hours for group 3 is not statistically different from that of group 2.

Table 1. Whole blood lufenuron concentration data, hours 0 to 24.[a]

	Group 1	Group 2	Group 3
C_{max} (mg/L)	9.11 ± 9.36	45.2 ± 30.2	43.6 ± 24.0
AUC (mg*h/L)	162 ± 95	821 ± 467	787 ± 559
C_{max}/dose (mg/L/dose)	1.82 ± 1.87	2.26 ± 1.51	0.73 ± 0.40
AUC/dose (mg*h/L/dose)	32.3 ± 19.0	41.1 ± 23.3	13.1 ± 9.3

C_{max}, mean whole blood concentration maximum; AUC, mean whole blood area under the curve.
[a] Results show mean ± SD.

These comparisons suggest that either gastrointestinal absorption or transport of lufenuron within the blood compartment is saturated after a single PO administration of 20 mg lufenuron/kg BW.

There was considerable individual variation among horses in the concentrations of lufenuron that could be measured in whole blood. Of the dosing regimens tested, 20 mg lufenuron/kg BW appeared to approximate the upper limit of lufenuron that can be absorbed from the equine gastrointestinal tract or transported within the blood within a 24-hour period. The maximal mean whole blood concentration of lufenuron reached in horses by this treatment regimen (83.5 ± 58.7 μg/L) was shown to be ineffective in vitro against *Aspergillus* and *Fusarium* spp. in this study and is far below the concentrations known to be effective against other fungal organisms (5,000 μg/L for inhibition of *Encephalitozoon intestinalis* and 10,000 μg/L for inhibition of *Vittaforma corneae*).[5]

Lufenuron appears to be safe when administered PO to horses at the doses used in this study. This ''safety'' includes ophthalmic safety, because there were no ophthalmic changes detected in any of the horses examined in this study. However, results of this study demonstrated that lufenuron lacks in vitro efficacy against *Aspergillus* or *Fusarium* spp. at concentrations as high as 700 μg/mL and achieves very low concentrations in whole blood of horses after PO administration. These findings indicate that the current treatment regimens employing lufenuron for filamentous mycoses in horses are scientifically unsound. Further therapeutic use of lufenuron as an antifungal agent in horses should be based on proven efficacy against specific strains of clinically relevant fungi and pharmacokinetic data demonstrating sufficient lufenuron concentrations in target tissues.

Footnotes

[a] Scotty NC, Evans TJ, Johnson PJ, et al. Lufenuron: Measurement of blood. Concentrations after oral administration in horses and determination of antifungal activity in vitro. 34th Conference of the American College of Veterinary Ophthalmologists, Coeur D'Alene, ID, 2003

[b] Scotty NC, Evans TJ, Johnson PJ, et al. Lufenuron as a treatment for equine keratomycosis. Association for Research in Vision and Ophthalmology, Ft. Lauderdale, FL, 2004

[c] Topical lefenuron suspension (Program™), Novartis®, Greensborough, NC

[d] Technical-grade lefenuron powder, Novartis, Greensborough, NC

[e] Xylazine hydrochloride (Rompun®), Bayer Health Care, Bushy Park, SC

[f] Detomidine hydrochloride (Dormosedan®), Pfizer Animal Health, New York City, NY

[g] Tropicamide Ophthalmic Solution, Falcon Pharmaceuticals Ltd, Ft. Worth, TX

[h] Bran mash, Nestlé Purina® PetCare, St. Louis, MO

[i] L-7100 pump, L-7200 autosampler, L-7400 ultraviolet detector, D-7000 data handling system, Hitachi Instruments Inc, San Jose, CA

[j] Supelco Discovery HS F5 C18 reverse phase column, Supelco Inc, Bellefonte, PA

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