Anthelmintic activity of a benzimidazoline compound in sheep by abomasal infusion

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Summary. In vitro and in vivo data on the benzimidazoline compound indicate anthelmintic potential when introduced directly into the abomasum.
Key words: Anthelmintic; abomasal infusion; *Ascaris;* EL-979; *Haemonchus; Nematodirus;* perfluoroalkylbenzimidazoles; *Trichostrongylus.*

Perfluoroalkylbenzimidazole compounds and their aminoanilide precursors are broad-spectrum systemic external parasiticides when given parenterally to animals[1-3]. Certain bezimidazoles have shown anthelmintic activity via oral and injectable routes, but none have insecticidal activity[4]. We wish to report that the benzimidazoline compound EL-979 [4-nitro-2-(1,1,2,2-tetrafluoroethyl)-6-(trifluoromethyl)-1H-benzimidazol-2-,01, sodium salt] has anthelmintic activity. Efficacy has been demonstrated against mature and immature stages of helminths in experimentally infected sheep treated by continuous abomasal infusion.

Materials and methods. Anthelmintic activity of the perfluoroalkylbenzimidazole compounds and their precursors was discovered in an in vitro *Haemonchus contortus* third-stage larval screen[5]. The most active compounds were further evaluated by oral and parenteral routes of administration to mice experimentally infected with *Ascaris suum* and *Nematospiroides dubius* according to Baker[6] and Boisvenue et al.[7]. Compounds exhibiting activity were then evaluated in sheep infected with *H. contortus* and *Trichostrongylus colubriformis* as outlined in the anthelmintic guidelines presented by Powers et al.[8].

EL-979 was first evaluated in sheep with established nematode infections at therapeutic doses of 10–100 mg/kg using p.o., ruminal and abomasal routes of administration. The benzimidazoline compound was then administered by abomasal infusion to a group of six lambs having established infections of both nematode species. Infusion with EL-979 was initiated in eight worm-free lambs 48 h prior to challenge with 4000 *H. contortus* and 6000 *T. colubriformis* benzimidazole susceptible larvae per lamb daily for five days. A proposed infusion rate was determined by multiplying the weight of the lamb by the therapeutic dose (mg/kg) and dividing the total amount of EL-979 by 30 to provide a daily miligram dose range for 30 days. Compared to most of the benzimidazole derivatives, EL-979 is extremely soluble in water with an aqueous solubility of 56% and is freely soluble in oxygenated organic solvents. The total amount of active ingredient needed for 24-h infusion was added to 720 ml of 0.85% saline in a graduated cylinder and placed in a styrofoam cooler for protection from light. The treated saline solution was dispensed by a Harvard peristaltic pump (model no. 1203) at the rate of 0.5 ml of solution per minute. The intramedic tubing used for infusion was surgically anchored in the upper left part of the abomasum. The number of worm eggs per gram of feces (EPG) was determined for test animals by microscopic examinations prior to and throughout the 30-day infusion period to observe inhibition of ova production. At the termination of the trial, all lambs, including nontreated controls, were euthanized and total worm recoveries were made. Parasites recovered were identified, sexed, sized, and ova in female worms were examined for development.

Results. EL-979 exhibited in vitro activity against third-stage exsheathed *H. contortus* larvae at a concentration of 0.0005% and in vivo activity against *A. suum* larvae and *N. dubius* in mice fed a 0.001% diet concentration for seven days or given a single 5 mg/kg p.o. dose by gavage. Anthelmintic activity was achieved in sheep given a single dose of 50 mg of dry technical

Table 1. Anthelmintic activity of EL-979 in sheep with established worm infections and treated by abomasal infusion for 30 days

Animal number	Sex	Initial weight (kg)	Daily dose (mg/kg)	Eggs per gram of feces pre- H.c.	T.c.	final H.c.	T.c.	Total worm count[1] H.c. A	I	T.c.	Percent activity[2] H.c. A	I	T.c.
821	M	41.8	1	500	167	0	0	0	0	0	100	100	100
827	F	34.1	1	2967	367	100	100	68	116	38	80	72	82
812	F	37.7	2	400	133	0	0	13	41	27	97	90	88
822	F	35.0	2	200	67	0	0	0	11	0	100	97	100
825	F	28.6	0	467	200	200	200	216	445	186	—	—	—
816	M	50.0	0	467	167	300	1200	478	391	248	—	—	—

M, male; F, female; * saline placebo; [1]at termination of infusion; [2]compared to nontreated sheep; H.c., *Haemonchus contortus*; T.c., *Trichostrongylus colubriformis*; A, adult worms; I, immature worms.

Table 2. Anthelmintic activity of EL-979 against developing nematodes in sheep treated by abomasal infusion at 2 mg/kg/day

Animal number	Weight (kg)	Infusion days	Patency EPG Day	H.c.	T.c.	Final EPG[1] H.c.	T.c.	Total worm counts[1] H.c.	T.c.	Percent activity[2] H.c.	T.c.
873	34.1	31	NA	0	0	0	0	0	0	100	100
876	31.8	31	29	0	100	0	0	0	37	100	88
877	30.1	33	NA	0	0	0	0	0	6	100	98
880	31.8	37	20	0	100	0	0	0	24	100	92
882	35.9	31	NA	0	0	0	0	0	0	100	100
886	29.5	37	23	0	100	0	0	0	41	100	87
875	29.5	37	24	200	100	500	200	394	445	—	—
879	31.8	37	28	100	200	500	200	339	168	—	—

* All male sheep; ** saline placebo for control sheep; [1]at termination of infusion; [2]compared to nontreated sheep; H.c., *Haemonchus contortus*; T.c., *Trichostrongylus colubriformis*; EPG, worm eggs/g of feces; NA, patency not attained.

powder per kg b.wt in a gelatin capsule surgically implanted in the abomasum or by abomasal injection of EL-979 in liquid preparation. Ruminal routes of administration by direct injection, drench, or gelatin capsule implantation were ineffective. Inactivity was due to conversion in the rumen by microflora of the nitro group at the four position to an amine compound i.e. 2-(1,1,2,2,tetrafluoroethyl)-6-(trifluoromethyl)-1H-benzimidazol-4-amine. This compound lacks anthelmintic activity.

The anthelmintic activity of EL-979 in sheep established nematode infections and treated with 1 and 2 mg/kg/day by abomasal infusion is presented in table 1. Inhibition of ova production was observed in both species at seven and 30 days of infusion, especially at the higher dose rate. Comparing total number of retained worms at necropsy, there was a 90% and 86% reduction in *H. contortus* mature and immature worm populations in sheep treated at the 1 mg/kg level. Approximately 91% of the *T. colubriformis* populations were reduced. Total worm reductions in sheep receiving the higher infusion rate were 98.5% and 93.5% for mature and immature *H. contortus* and 94% for *T. colubriformis* populations. Developing nematode data from sheep infused with 2 mg/kg/day indicate that the new infections were inhibited by the benzimidazoline treatment (table 2). The long term infusion resulted in a 94% reduction of *T. colubriformis* populations in the six treated sheep. Also, complete elimination of nematode ova in the feces and of both worm populations in the intestinal tract occurred. In vitro and in vivo data on the benzimidazoline compound indicate anthelmintic potential when introduced directly into the abomasum. The authors realize that the abomasal route of administration is impractical for control of helminths in the field. This method and the rumen infusion method provide basic dosage data needed to determine compound feasibility in control release rumen devices as described by Ludwig and Boisvenue[9].

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Occurrence of tetrodotoxin in the starfish *Astropecten latespinosus*[1]

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Summary. A toxin causing paralysis was detected in the starfish *Astropecten latespinosus*. The toxin was purified by a method consisting of charcoal treatment and chromatography on CM-Sephadex C-25 and Bio-Rex 70. The toxin was identified as tetrodotoxin by its behavior in thin-layer chromatography and electrophoresis and its ^{1}H-NMR spectrum.
Key words. Astropecten latespinosus; starfish; tetrodotoxin.

Tetrodotoxin (TTX) is one of the most potent neurotoxins. It was first isolated from the pufferfish[4], and then from several vertebrates (newts of the genus *Taricha,* a goby *Gobius criniger* and frogs of the genus *Atelopus*)[4] and an invertebrate (the blue-ringed octopus *Octopus maculosa*)[5]. Recently, we have detected TTX in some gastropod mollusks (a trumpet shell *Charonia sauliae*[6], the Japanese ivory shell *Babylonia japonica*[7] and a frog shell *Tutufa lissostoma*[8]). We have also revealed the presence of TTX in a starfish *Astropecten polyacanthus*[9], débris of which is often found in the trumpet shell. In the course of elucidating the mechanisms involved in the toxification of Japanese ivory shell, we detected paralysis-producing toxicity in another starfish, *A. latespinosus* (fig.). The present work was performed to demonstrate that this starfish also contains TTX.

Experimental procedure and results. A total of 1.5 kg of *A. latespinosus* specimens (average b.wt, 28.8 ± 6.7 g) were collected in June 1983 from Sakajiri Bay, Fukui Prefecture, Japan. They were repeatedly homogenized with 3 vol. of 1% acetic acid in methanol. The extracts were combined, evaporated in vacuo and defatted with dichloromethane. The aqueous layer (total toxicity: 6000 mouse units (MU)[10]) was concentrated to 500 ml, adjusted to pH 5.2 with 1 N NaOH, and treated batchwise with 300 g of activated charcoal (Wako Pure Chem.). The charcoal was washed with water. The toxin (5000 MU) was eluted with 1% acetic acid-20% ethanol and evaporated to dryness. The solid obtained was dissolved in a small amount of 0.1 M ammonium acetate (pH 6) and applied to a column (2.6 × 34 cm) of CM-Sephadex C-25 (NH_4^+ form, Pharmacia Fine Chem.). The column was developed by a linear gradient of 0.1 to 0.4 M ammonium acetate (pH 6). The toxic fractions were combined and lyophilized. The lyophilizate (4400 MU) was dissolved in a small amount of water and applied to a column (0.8 × 95 cm) of Bio-Rex 70 (H^+ form, Bio-Rad Lab.). The column was developed by a linear gradient of 0 to 0.1 M acetic acid. The toxic fractions were combined and freeze-dried to afford 0.8 mg white powder. The starfish toxin thus obtained exhibited a specific toxicity of 4600 MU/mg, the value which is almost comparable to that of an authentic TTX isolated from a pufferfish ovary by Goto's method[11].

Attempts were made to identify the starfish toxin by thin-layer chromatography and electrophoresis. The former was carried out on Whatman LHP-K high-performance plates, with a solvent system of pyridine-ethylacetate-acetic acid-water (15:5:3:4). Electrophoresis was conducted on Chemetron cellulose acetate membranes under a constant current of 0.8 mA/cm for 30 min, using 0.08 M Tris-HCl buffer (pH 8.7). The starfish toxin was detected as a pink spot with the Weber reagent, or as a yellow fluorescent spot under UV light (365 nm) after spraying the plate with 10% KOH and heating at 110 °C