Comparative *in vitro* effects of closantel and selected β-ketoamide anthelmintics on a gastrointestinal nematode and vertebrate liver cells

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PNU-87407 and PNU-88509, β-ketoamide anthelmintics that are structurally related to each other and to the salicylanilide anthelmintic closantel, exhibit different anthelmintic spectra and apparent toxicity in mammals. The basis for this differential pharmacology was examined in experiments that measured motility and adenosine triphosphate (ATP) levels in larval and adult stages of the gastrointestinal nematode, *Haemonchus contortus*, and in a vertebrate liver cell line and mitochondria. PNU-87407 and PNU-88509 both exhibited functional cross-resistance with closantel in larval migration assays using closantel-resistant and -sensitive isolates of *H. contortus*. Each compound reduced motility and ATP levels in cultured adult *H. contortus* in a concentration- and time-dependent manner; however, motility was reduced more rapidly by PNU-88509, and ATP levels were reduced by lower concentrations of closantel than the β-ketoamides. Tension recordings from segments of adult *H. contortus* showed that PNU-88509 induces spastic paralysis, while PNU-87407 and closantel induce flaccid paralysis of the somatic musculature. Marked differences in the actions of these compounds were also observed in the mammalian preparations. In Chang liver cells, ATP levels were reduced after 3 h exposures to $\geqslant 0.25$ μM PNU-87407, $\geqslant 1$ μM closantel or $\geqslant 10$ μM PNU-88509. Reductions in ATP caused by PNU-88509 were completely reversible, while the effects of closantel and PNU-87407 were irreversible. PNU-87407, closantel and PNU-88509 uncoupled oxidative phosphorylation in isolated rat liver mitochondria, inhibiting the respiratory control index (with glutamate or succinate as substrate) by 50% at concentrations of 0.14, 0.9 and 7.6 μM, respectively.

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INTRODUCTION

PNU-87407 and PNU-88509 (Fig. 1) are novel β-ketoamides which possess anthelmintic properties (Lee & Clothier, 1992). These compounds are structurally related to closantel, an anthelmintic which is believed to act by disrupting proton gradients across mitochondrial membranes in nematodes (Van den Bossche, 1972) and other parasitic helminths (Van Miert & Groeneveld, 1969). Campbell & Montague (1981) showed that a good correlation exists between uncoupling and anthelmintic activities for a wide range of uncoupling agents, including closantel, during *in vitro* and *in vivo* exposures of the liver fluke, *Fasciola hepatica*. Other studies, however, have shown that the effects of salicylanilide anthelmintics on motility (Coles, 1977) and electrochemical gradients across muscle and tegumental membranes (Thompson *et al.*, 1984; Pax & Bennett, 1989, 1990) in trematodes occur faster and at lower concentrations than their effects on ATP levels, suggesting that other mechanisms may underlie their anthelmintic actions.

The clinical utility of closantel and other salicylanilide anthelmintics is limited by several factors. These compounds possess narrow anthelmintic spectra and their effectiveness in the field is eroding due to the emergence of resistant strains of parasites (Van Wyk *et al.*, 1989; Rolfe *et al.*, 1990). In addition, a number of host toxicity problems have been reported (Brown *et al.*, 1972; Van Cauteren *et al.*, 1977) and extended withdrawal times are required for these compounds (Hall *et al.*, 1981). The concept that PNU-87407 and PNU-88509 act by a mechanism similar to that of salicylanilide anthelmintics was suggested, based on structural similarities among these compounds.

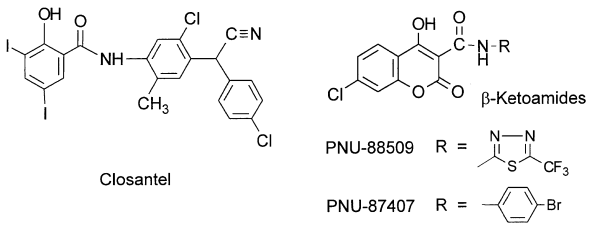

Fig. 1. Structures of PNU-87407, PNU-88509 and closantel.

However, in nematode-infected rodent and sheep models, these compounds exhibit different anthelmintic spectra, with PNU-87407 being more effective against species that reside in the stomach (including *H. contortus*) and PNU-88509 more effective against *Trichostrongylus colubriformis*, which resides in the small intestine (Table 1; Lee & Clothier, 1992).

PNU-87407 and PNU-88509 exhibit markedly different physicochemical properties (Ho *et al.*, 1994), and it is possible that differences in their *in vivo* anthelmintic activities may be attributable to, for instance, the 10-fold greater lipophilicity of PNU-87407. To determine if the actions of PNU-87407 and PNU-88509 on gastrointestinal nematodes are similar, and include metabolic uncoupling activity like that of salicylanilide anthelmintics, we examined the effects of these compounds on physiological and biochemical endpoints which are known to be affected by therapeutic concentrations of closantel and other salicylanilide anthelmintics, including motility and muscle function (Coles, 1977; Skuce & Fairweather, 1990) and ATP levels (Van den Bossche, 1972; Kane *et al.*, 1980). Using these endpoints, we determined the *in vitro* potencies and kinetics of action for PNU-87407 and PNU-88509 on adult *H. contortus*, a gastrointestinal nematode that is sensitive to these two compounds both *in vitro* and *in vivo*, and also on motility of 4th-stage larvae of closantel-resistant and susceptible isolates of *H. contortus*. For comparative purposes, the effects of these compounds were further characterized using Chang (vertebrate liver) cells and isolated rat liver mitochondria.

MATERIALS AND METHODS

Test substances

PNU-87407 and PNU-88509 were prepared as described by Lee and Clothier (1992). Closantel, 2,4-dinitrophenol (2,4-DNP) and the carbonyl cyanide phenylhydrazones, CCCP (carbonylcyanide *m*-chlorophenylhydrazone) and FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) were obtained from Sigma Chemical Company (St. Louis, MO). The molecular weights of PNU-87407, PNU-88509 and closantel are 394.6, 391.7 and 663.1 Da, respectively, and their logPC values (n-octanol/buffer at pH 7.5) are 3.65, 2.54 and 7.55, respectively (Ho *et al.*, 1994). All test substances for *in vitro* testing were dissolved in a DMSO:triethylamine cocktail (99:1) to 10 mM. These stock solutions were serially diluted (1:9 in the same vehicle) to yield less concentrated samples prior to addition to test incubates. Drug solutions were added to test incubates so that the final concentration of vehicle never exceeded 0.1%.

Table 1. Percent reduction* of *Haemonchus contoruts* or *Trichostrongylus colubriformis* in a dual infection jird model following oral dosing with PNU-87407, PNU-88509 or closantel at 2.75 mg *per os*

Drug	*H. contortus*	*T. colubriformis*
PNU-87407	100 ± 0	3 ± 1
PNU-88509	97 ± 2	94 ± 5
closantel	100 ± 0	21 ± 3

*Values reported are the mean (± SEM) percent reductions in worm burdens recorded in 3 separate studies (5 jirds/study) 3 days following oral dosing of drug ($n = 3$). Each drug was prepared in CMC/DMSO (Conder *et al.*, 1991).

Anthelmintic assays

Larval migration assay

Compounds were tested blindly at the University of Sydney using a larval migration assay (Rothwell & Sangster, 1993). Briefly, 4th-stage larvae of closantel-resistant (H41) and -susceptible (McM) isolates of *H. contortus* from Australia were exposed in culture to the drugs over 48 h, and viability assessed by counting the number of larvae either passing through or retained by a 50 μm aperture nylon mesh at 37°C. Concentrations required to inhibit migration of 50% of the worms (MIC_{50}) were determined. For each compound, larval migration assays were performed in triplicate.

Motility and ATP assays using adult *H. contortus*

Methods for collecting adult female *H. contortus* from sheep and measuring their responses to drugs using an automated motility recording system and a commercially available assay for ATP levels were previously described (Geary *et al.*, 1993). Individual data points in motility and ATP figures represent the mean ± 1 SEM of at least three separate experiments (four culture tubes/treatment group/experiment). In the ATP assay, differences between drug-treated and 0.1% DMSO control incubates were compared in time-matched incubates using Student's *t*-test, nonpaired (Geary *et al.*, 1993). Protocols used for maintaining infected sheep and collecting parasites for *in vitro* studies were approved by the Corporate Animal Use Committee at Pharmacia & Upjohn, Inc.

Somatic muscle tension recordings from isolated neuromuscular segments

Segments (neuromuscular strips) of parasite tissue were prepared by excising a section, 2–3 mm long, from the anterior end of adult female *H. contortus*. Segments prepared by this method continued to contract spontaneously in a manner similar to intact parasites for several hours. Following excision, calibrated muscle tension recordings were obtained from tissue segments using a suction pipette/balance beam system (Atchison *et al.*, 1992). Following attachment, tissue segments were allowed to equilibrate 5–10 min prior to testing. All recordings followed the

same protocol for time course of treatments, with continuous recordings obtained for 10 min following exposure to each drug treatment. Fresh tissue preparations were used for each test. Although continuous recordings were obtained on the physiograph, time points selected for data analysis included only 0 min (just prior to drug addition), 1, 5 and 15 min after drug addition. Individual data points in somatic muscle tension experiments are the mean values obtained from at least four separate preparations. Tension changes (normalized to the 0 min value for each preparation) in drug-treated and 0.1% DMSO controls were compared at the 1, 5 and 15 min time points using Student's *t*-test, nonpaired (Atchison *et al.*, 1992).

Chang cell and isolated rat liver mitochondria assays

Chang cell culture conditions and cell treatments

Chang cells were obtained from the American Type Culture Collection (ATCC, passage 257) and used for up to 10 passages from a cryopreserved cell stock. Cells were maintained in complete medium consisting of 90% Basal Medium Eagle (BME) with Hanks' Balanced Salt Solution (HBSS), 10% foetal bovine serum (FBS), supplemented with 20 mN N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and 50 μg/mL gentamicin sulfate. Cultures were exposed for 4 h to PNU-87407 (0.1, 0.25, 0.5, 1, 5 or 10 μM), PNU-88509 (1, 5, 10, 50 or 100 μM) or closantel (0.1, 1, 5 or 10 μM) in a humidified incubator at 37°C, 95% air: 5% CO_2. Following the incubation, the cultures were either assayed immediately or the dosing solutions were removed and replaced with complete medium; after an additional 3 or 24 h, these cultures were assayed. Triplicate cultures were used in each experiment.

Evaluation of cytotoxicity

The basal cytotoxic potential was evaluated 24 h post-treatment using cell count data and lactate dehydrogenase (LDH) leakage. The medium was removed from the cultures and stored at 4°C for LDH analysis at a later time. The cultures were then washed with 2 mL HBSS and aspirated. Two mL of 0.25% trypsin was added to detach the remaining cells. A 1 mL portion of the cell suspension was diluted 20-fold into an isotonic solution and counted on a model Zf Coulter Counter (Coulter Inc., Hialeah, FLA). LDH leakage was assessed in the culture medium by measuring the conversion of NAD^+ to NADH in the presence of pyruvate according to the method of Wroblewski & LaDue (1955). Protein content of the cultures was measured by dissolving the cells in 0.1 M NaOH and using the Pierce BCA protein assay kit.

ATP measurements in Chang cell cultures

The effects of compounds on Chang cell mitochondria were evaluated in treated cultures either immediately following the 4 h dosing regimen or 3 h post-treatment. Triplicate cultures were used in parallel plates to measure cell count and ATP levels. Cell counts were determined as described above. 2,4-DNP (2 mM) was included as a positive control. ATP levels were evaluated by removing the dosing solutions at the completion of the treatment period and washing the cultures with HBSS. In order to extract ATP from the cells, 1.5 mL of 0.4 M $HClO_4$ was added to cultures for 15 min at 27°C. The extracted ATP solution was then removed and stored at −20°C until assayed. ATP was assayed using a Sigma ATP bioluminescent assay kit (FL-AA). The extracted solution was neutralized with 0.4 mL 1 M K_2CO_3 and diluted 10-fold into 50 mM HEPES buffer, pH 7.8. A 0.2 mL portion of the dilution was added to 0.2 mL of the Sigma ATP assay mix and bioluminescence was recorded with a Lab-Line ATP Photometer (Melrose Park, IL).

Respiratory control index (RCI) of coupled rat liver mitochondria

Rat liver mitochondria were isolated by the method of Johnson & Lardy (1967). Deionized water was double distilled before use. All laboratory apparatus and utensils that came into contact with the mitochondria were thoroughly cleansed with phosphate-free soap (MICRO™, International Products Corp., Trenton, NJ) before use. Ultra pure, ribonuclease-free sucrose (Schwartz-Mann, Spring Valley, NY) was used throughout. The respiratory control index (RCI) was determined by the method of Chance & Williams (1955) as outlined in Gazzotti *et al.* (1979) using a polarographic technique employing Clark-type oxygen electrodes. Oxygen consumption was followed on a YSI Model 5300 Oxygen Monitor System (Yellow Springs Instrument Co., Yellow Springs, OH) attached to a Gould 3200 2-channel chart recorder (Gould Electronics, Cleveland, OH). The medium used for the measurement of respiration consisted of 5 mM KH_2PO_4, 120 mM KCl and 20 mM Tris-HCl, pH 7.4. Glutamate (2.0 mM final concentration) and succinate (2.5 mM final concentration) were used as mitochondrial substrates. Temperature was controlled at 30 ± 0.2°C. High sensitivity oxygen membranes (Yellow Springs Instrument Co.) were used and tested regularly for proper response. Mitochondria were preincubated with PNU-87407, PNU-88509, closantel, FCCP, CCCP or 2,4-DNP for 1 min before determinations were made; 6–12 concentrations for each compound were tested. Controls contained equal volumes of uncoupler-free DMSO; DMSO in the amounts used did not affect the RCI. Mitochondrial protein (1 mg/mL, final concentration) was determined by the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978).

RESULTS

Effects on H. contortus

Migration of 4th-stage larvae

The *in vitro* larval migration assay showed clear evidence that PNU-87407 and PNU-88509 were cross-resistant with closantel, each being about 3-fold less potent against resistant worms than susceptible worms (Table 2). Against this stage of the parasite, the rank-order of apparent potency for these com-

Table 2. Minimum inhibitory concentration (MIC_{50}) of PNU-87407, PNU-88509 or closantel for migration *in vitro* of 50% of closantel-susceptible (McM) and -resistant (H41) isolates of *Haemonchus contortus* 4th-stage larvae following 48 h incubations

Drug	MIC_{50} (μM)* McM	H41
PNU-87407	15	41
PNU-88509	1.4	4.8
closantel	13	35.6

*Mean concentration that reduced migration in a single study performed in triplicate ($n = 3$).

pounds was similar for both isolates, with PNU-88509 being about 10-fold more potent than PNU-87407 or closantel (Table 2).

Effects on motility and ATP levels in adult *H. contortus*

Motility and ATP levels in *H. contortus* were reduced by PNU-87407 and PNU-88509 in a concentration- and time-dependent manner (Fig. 2). In the motility assay, at the 24 h time point, the apparent potency of all three compounds was similar, each inducing near complete paralysis at 3 and 10 μM, and each inducing reductions of 25% or more at concentrations ⩾0.1 μM. However, at each concentration tested, the onset kinetics of paralysis for PNU-88509 was about 3-fold more rapid than that for PNU-87407 or closantel (Fig. 2). Motility levels were also reduced by 25% or more by the uncouplers CCCP (0.03 μM at 24 h), FCCP (0.1 μM at 24 h) and 2,4-DNP (1 μM at 24 h) (data not shown).

During the first 4 h of exposure, neither PNU-87407 nor PNU-88509 reduced ATP levels in *H. contortus*, while the effects of closantel on ATP were apparent at both 1 and 10 μM (data not shown). Following 24 h incubations, PNU-87407 and PNU-88509 reduced ATP levels by 20–30% at 1 μM ($P \leqslant 0.05$) and by 75% at 10 mM (Fig. 2). At this time point, closantel reduced ATP in the parasite tissue by 75% and 78% at 1 μM and 10 μM, respectively; lower concentrations were ineffective. The effects of CCCP, FCCP and 2,4-DNP on ATP levels in *H. contortus* following

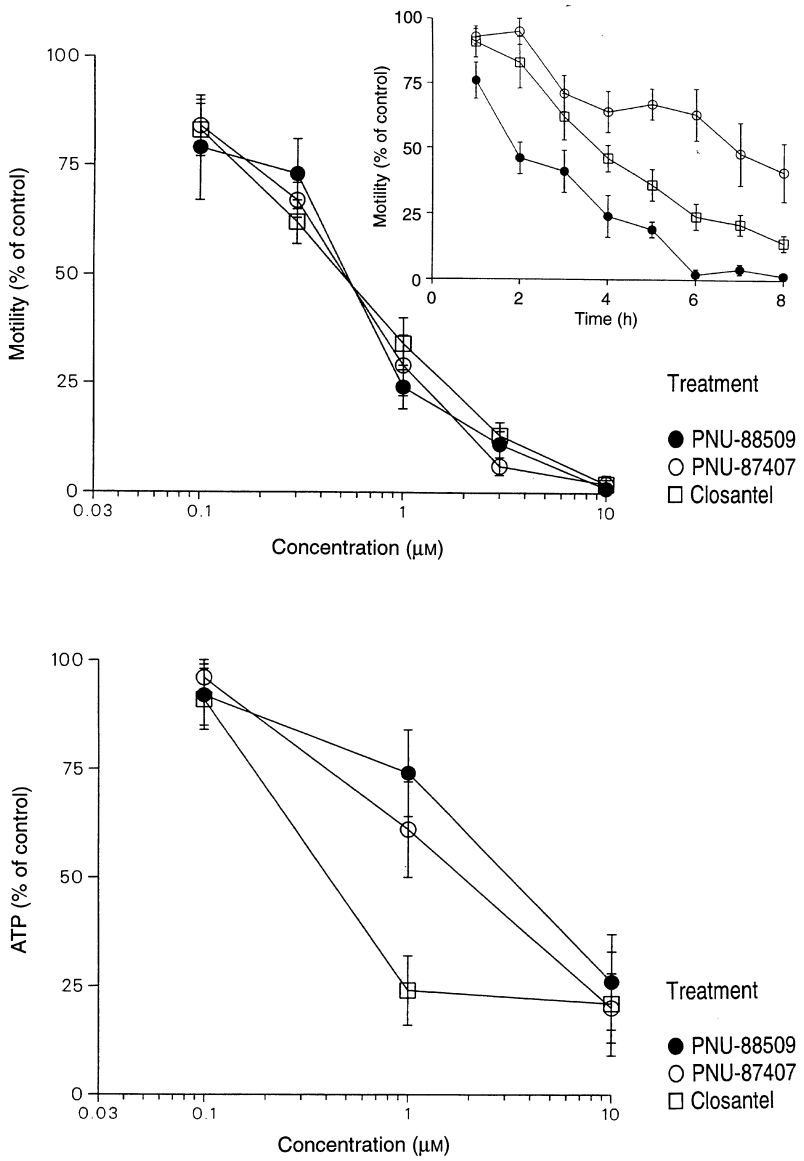

Fig. 2. Motility (top) and ATP (bottom) levels in adult female *Haemonchus contortus* following 24 h incubations in medium containing 0.1–10 μM PNU-87407, PNU-88509 or closantel. Inset shows onset kinetics of each compound when tested at 10 μM.

24 h exposures titrated to 0.1, 0.1 and 1 μM, respectively (data not shown).

Effects on somatic muscle tension

Muscle tension recordings from 2–3 mm anterior segments isolated from adult *H. contortus* showed that PNU-88509 induced a spastic contraction of the somatic musculature (Fig. 3). Significant increases in tension occurred within 1 min and 5 min following bath application of PNU-88509 at 10 μM and 1 μM concentrations. These effects were markedly different from those of PNU-87407, which induced a flaccid paralysis (Fig. 3). The reduction in somatic muscle tension induced by PNU-87407 was significant by 15 min only at concentrations $\geqslant$ 10 μM. Based on the patterns of physiographic data recorded, the type of flaccid paralysis induced by PNU-87407 was qualitatively similar to that which occurred following bath application of closantel (Fig. 3), CCCP, FCCP or 2,4-DNP (data not shown).

Effects on Chang cells and isolated rat liver mitochondria

Cytotoxic ranking in Chang cell assays

The cell count data obtained 24 h post-treatment demonstrated that the cytotoxicity ranking of the three compounds was PNU-87407 > closantel $\gg$ PNU-88509 (Fig. 4). PNU-87407 and closantel induced a concentration-dependent decrease in the cell number at 24 h at concentrations > 1 and 5 μM, respectively (Fig. 4). In contrast, there was no observed toxic response associated with PNU-88509 at $\leqslant$ 100 μM. LDH results mirrored the cell count data, showing a large LDH leakage from PNU-87407- and closantel-treated cells at 24 h post-treatment (Table 3). PNU-88509 induced only minimal leakage of LDH. At the 4 h time point, closantel caused only a slight elevation in LDH, whereas treatment with 5 or 10 μM PNU-87407 caused substantial increases in LDH leakage.

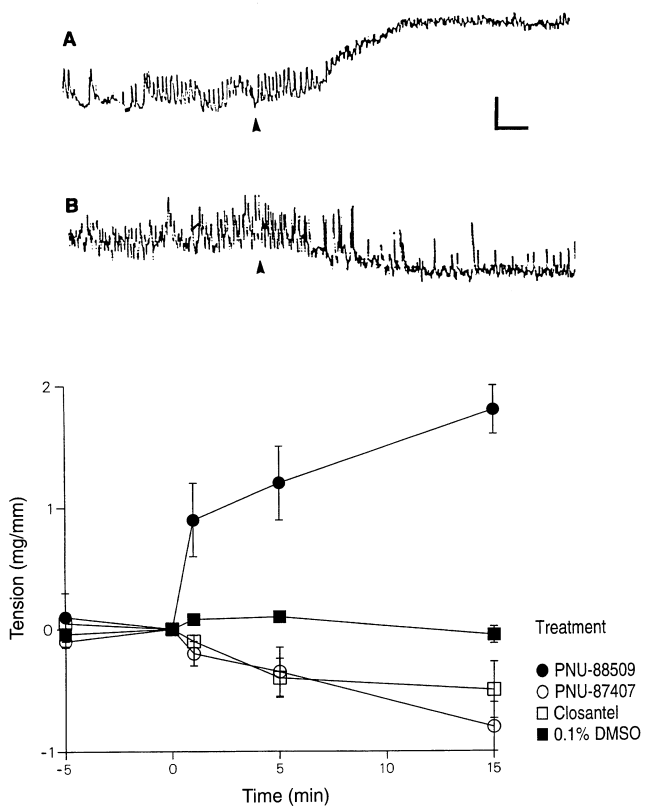

Fig. 3. Muscle tension recordings from 2–3 mm anterior segments of *Haemonchus contortus* showing responses to bath application of 10 μM PNU-88509 (A) or 10 μM PNU-87407 (B); drugs added at arrows, calibration bar = 1 mg per 1 min. Data points in the plot (below) are the average tension change ± SEM (n = 4), following bath application of 10 μM drug or vehicle at 0 min.

Chang cell mitochondrial function

Chang cell cultures were evaluated either immediately after the 4 h treatment period or 3 h post-treatment in order to detect changes in mitochondrial function in the absence of major cell damage (as indicated by cell count and LDH leakage). Closantel and PNU-87407 reduced ATP content, even at concentrations that did not affect cell count (Fig. 5). A 20–35% reduction in ATP was recorded following 4 h exposure to 10–100 μM PNU-88509; this effect was not concentration-dependent over the range of drug levels tested. When cultures were assayed in drug-free medium 3 h post-treatment, ATP levels were restored to control levels after removal of PNU-88509; however, ATP levels in Chang cells treated with PNU-87407 or closantel remained below control values (Fig. 5).

Effects on isolated rat liver mitochondria

To further test the hypothesis that the mode of action of PNU-88509 and PNU-87407 is related to the uncoupling of oxidative phosphorylation from electron transport, the effects of these two compounds and closantel on the RCI of coupled rat liver mitochondria were determined and compared to those of the

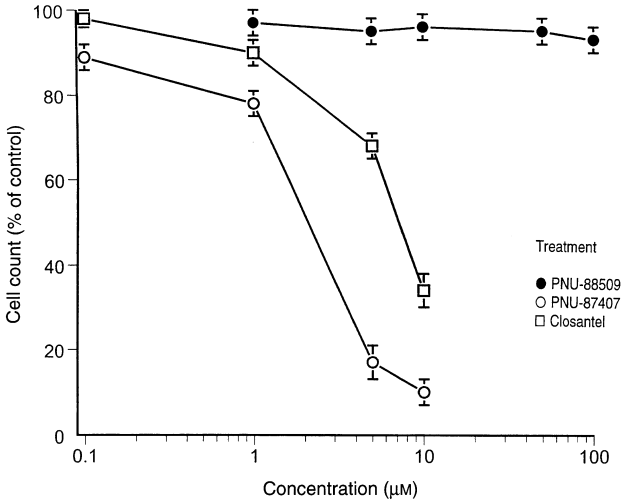

Fig. 4. Concentration-dependent responses, at 24 h, in Chang cell cultures treated with PNU-87407, PNU-88509 or closantel. Attached cells were counted and expressed as percent of 0.5% vehicle control ± SEM (n = 3).

Table 3. LDH leakage in Chang cell cultures following treatment with PNU-87407, PNU-88509 or closantel

Treatment*	4 h	24 h
PNU-87407 (μM)		
10	362.9 ± 18.3	1598 ± 102.5
5	218.9 ± 25.4	735.6 ± 55.2
1	54.2 ± 6.5	306.3 ± 37.8
0.1	25.4 ± 2.4	183.2 ± 14.6
PNU-88509 (μM)		
100	27.0 ± 1.6	234.5 ± 7.7
50	30.5 ± 2.4	221.6 ± 11.0
closantel (μM)		
10	47.6 ± 4.3	1108.9 ± 245.4
5	35.2 ± 2.7	531.0 ± 10.1
1	27.4 ± 2.9	266.7 ± 13.6
0.1	25.0 ± 3.6	148.6 ± 7.4
0.5% DMSO	24.4 ± 2.0	183.6 ± 8.5

*Cultures were treated for 4 h in serum-free medium containing drug and assayed either immediately following the removal of dosing solutions or 24 h post-treatment. Results are expressed as mean LDH units/mg protein ± SEM ($n = 3$).

santel ⩾ PNU-88509; RCI_{50} concentrations for these compounds were 0.14, 0.9 and 7.6 μM, respectively.

DISCUSSION

We examined the effects of PNU-87407, PNU-88509 and closantel, a structurally-related compound with known anthelmintic properties, on the motility of 4th-stage larvae of closantel-resistant and -susceptible isolates of *H. contortus*, and on motility, muscle function and ATP levels using the adult stage of a closantel-susceptible isolate of the same parasite. For comparative purposes, and in order to gain additional insights to the physiological and biochemical effects of these compounds, we also examined their *in vitro* effects on cell viability and mitochondrial function using a transformed vertebrate cell line and isolated rat liver mitochondria. The endpoints selected were known from previous studies (Van den Bossche, 1972; Coles, 1977; Kane *et al.*, 1980; Skuce & Fairweather, 1990; Rothwell & Sangster, 1993) to be affected by therapeutically relevant concentrations of closantel.

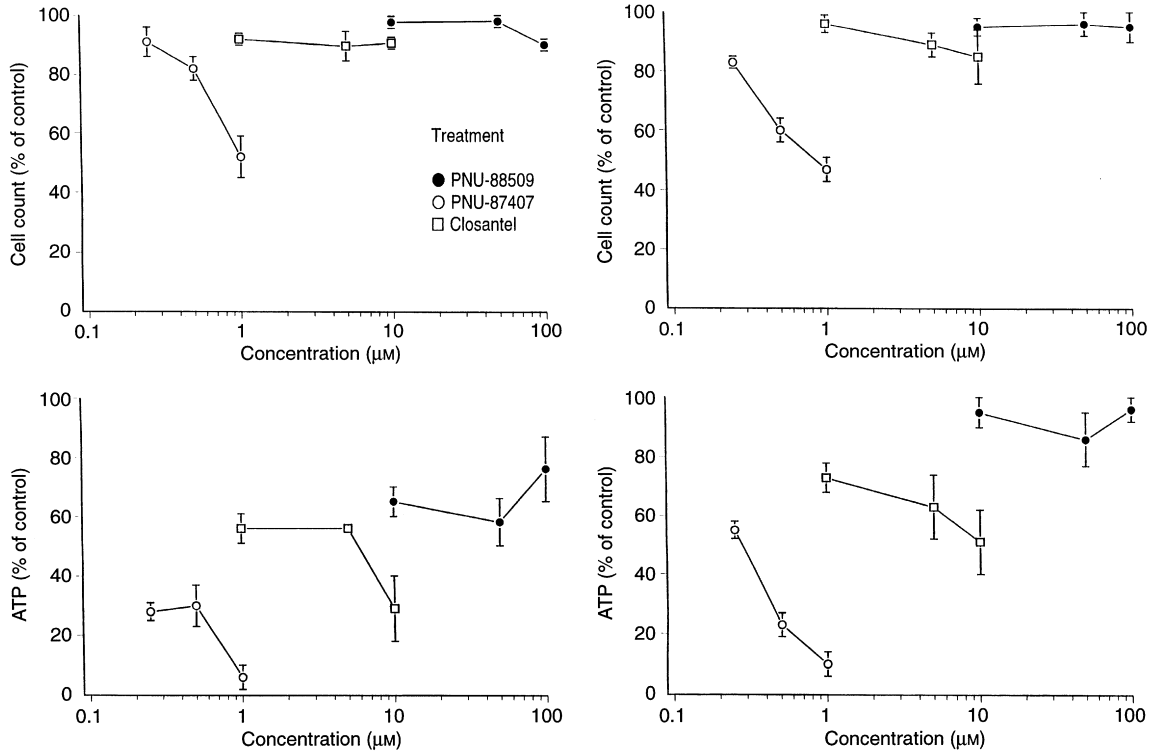

Fig. 5. Concentration-dependent responses in Chang cells treated with closantel and evaluated for cell count (upper plots) and ATP (lower plots) immediately following 4 h exposure to PNU-87407, PNU-88509, or closantel (left panels), or following 3 h post-recovery in unamended medium (right panels). Results are expressed as means ± SEM ($n = 3$).

known uncouplers 2,4-DNP, FCCP and CCCP (Table 4). With glutamate or succinate as the mitochondrial substrate, and RCI as an indicator of uncoupling activity, PNU-88509, PNU-87407 and closantel uncoupled oxidative phosphorylation at or below micromolar concentrations (Fig. 6). Among the anthelmintics, the rank-order of *in vitro* drug activity was PNU-87407 > closantel ⩾ PNU-88509; RCI_{50} concentrations for these compounds were 0.14, 0.9 and 7.6 μM, respectively.

PNU-87407 and PNU-88509 both exhibited clear signs of functional cross-resistance with closantel, based on the fact that they were about 3-fold less potent against the closantel-resistant isolate than the susceptible isolate of *H. contortus* used in the larval migration assay. The rank-order of potency of these compounds in the larval migration assay, however, was reversed

Table 4. Effects of various uncouplers on mitochondrial respiratory control in isolated rat liver mitochondria

Uncoupler	RCI_{50}*(μM)
PNU-88509	7.6
2,4-DNP	2.7
closantel	0.9
PNU-87407	0.14
CCCP	0.03
FCCP	0.01

*RCI_{50} concentrations were derived from 3 separate studies similar to that shown in Fig. 6, in which 2.0 mM glutamate was used as mitochondrial substrate.

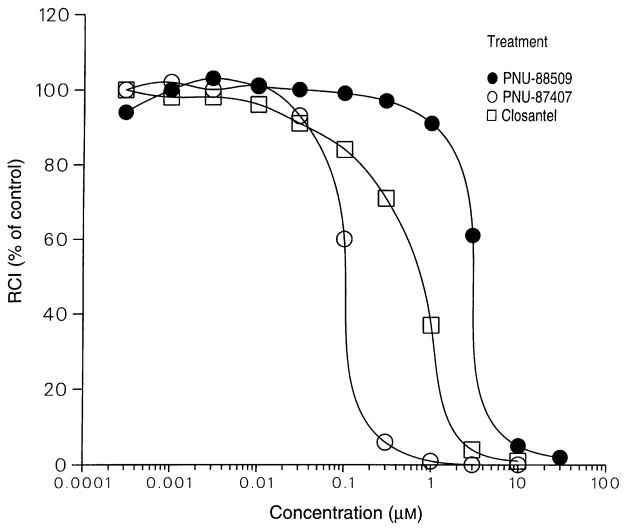

Fig. 6. Respiratory control index (RCI) in isolated rat liver mitochondria as a function of drug concentration following 3 min exposure to various concentrations of PNU-87407, PNU-88509 or closantel, using 2.0 mM glutamate as mitochondrial substrate. Results expressed are individual data points from a single study; similar results were obtained in two additional, separate studies using glutamate, and three separate studies in which 2.5 mM succinate was used as mitochondrial substrate.

from that observed *in vivo* (in jirds and sheep, see Table 1; Lee & Clothier, 1992) against a different isolate of closantel-sensitive *H. contortus*. The basis for this apparent difference in sensitivity, and whether it would also occur *in vivo* with the Australian isolate, has not been determined.

PNU-87407 and PNU-88509 appear to possess additional anthelmintic properties which mimic those of closantel and other salicylanilides; that is, among the physiological consequences of *in vitro* exposure to these β-ketoamides were paralysis and ATP depletion. These effects occurred rapidly and at micromolar concentrations. Similar effects were recorded following exposure to closantel and the known protonophores CCCP, FCCP and 2,4-DNP. In contrast to these compounds, other anthelmintics, including ivermectin and levamisole, paralyze *H. contortus* without affecting ATP levels; or, in the case of the benzimidazoles, affect neither parameter (Geary, *et al.*, 1993; Ho *et al.*, 1994). Ivermectin and levamisole act at neuromuscular receptors in nematodes, and it is not surprising that the paralysis they induce is not accompanied by ATP depletion, at least during the course of the short-term incubations tolerated by adult *H. contortus*.

While ATP depletion is one consequence of β-ketoamide exposure, it is not clear that energy generation is the only target for these compounds. That PNU-88509 may act, in part, by an unrelated mechanism is supported by our data showing that this β-ketoamide reduced *H. contortus* motility before significant depletion of ATP occurred, and by the type of paralysis recorded, which is clearly distinguishable from that induced by closantel, CCCP or the other metabolic poisons tested. These differences cannot be attributed to uptake kinetics, as previous studies by Ho *et al.* (1994) showed that the rates of absorption and partitioning for PNU-87407, PNU-88509 and closantel in *H. contortus* tissue are remarkably similar. It is intriguing to speculate that the thiadiazole ring structure of PNU-88509 imparts a second action mechanism to this compound. In this regard, it is noteworthy that analogues of PNU-87407, modified structurally in ways that eliminate the 'mobile protons' on this pharmacophore, are inactive against nematodes both *in vitro* and *in vivo* (Lee & Clothier, 1992). Furthermore, among salicylanilide uncouplers that are structurally related to these β-ketoamides, loss of uncoupling activity invariably accompanies chemical substitution at these positions (Heytler, 1979).

Other lines of evidence, not included in this report, suggest that some β-ketoamides, including PNU-88509, possess other properties which distinguish them from closantel. These include expanded spectra among parasitic nematodes (i.e., the spectrum for some includes *T. colubriformis*), inactivity against the parasitic flatworm *Fasciola hepatica* (J.L. Bennett, personal communication), and the fact that some are up to 1,000-fold more potent than closantel against the free-living nematode *Caenorhabditis elegans* (E.M. Thomas, unpublished observations). Conversely, the fact that the β-ketoamides, like closantel, are uniformly inactive against *Ascaris suum in vitro* suggests that these compounds, despite some differences, may target the same receptor (i.e., one that is present in some nematodes, but not *A. suum*). Unfortunately, this observation also means that the one nematode large enough to study drug effects readily at the membrane level is not a relevant model for these compounds.

Rohrer *et al.*, (1986) have suggested that the apparent inactivity of closantel and other salicylanilides against *A. suum* may be a function of inadequate drug penetration. In trematodes, which are sensitive to a wide range of salicylanilides, there is no collagenous cuticle like that in the nematodes, which may act as a barrier to diffusion. However, in previous studies we detected no change in motility or appearance in *A. suum* following intrapseudocoelomic injection of closantel or 20 other β-ketoamides (including PNU-87407 and PNU-88509) to concentrations which exceed 20 μM. Furthermore, recent studies by Ho *et al.* (1994) have shown that PNU-87407 and PNU-88509 are absorbed rapidly across the cuticle of *H. contortus* and, based on the physicochemical properties of these molecules and closantel, transcuticular absorption by other species of parasitic nematode, including *A. suum* and *T. colubriformis*, should occur readily. In this regard, it is noteworthy that adult *T. colubriformis*

are equally sensitive to PNU-887407 and PNU-88509 *in vitro*, based on motility studies analogous to those included in this report (E.M. Thomas, unpublished studies). Still, it is possible that the weak activity exhibited by some β-ketoamides against several species of nematode *in vivo* (Lee & Clothier, 1992) may be a function of bioavailability. Closantel, for instance, binds tightly to serum albumen, and this probably limits its access to detritis feeding species such as *T. colubriformis*. Unfortunately, the pharmacokinetic properties of PNU-87407 and PNU-88509 have not been studied in a mammalian system.

There is little information in the literature on carbohydrate metabolism in *H. contortus*. Ward (1974) and Ward & Huskisson (1978, 1981) showed that, under aerobic conditions, adult *H. contortus* excrete significant amounts of CO_2 (10 mmol/g wet weight/h) for up to 4 h. Under anaerobic conditions, CO_2 excretion declined by over 95% and the worms became less motile over the same time course. However, excretion rates for the other major endproducts of carbohydrate metabolism, including propionate, propanol, acetate and ethanol were unaffected or increased slightly under anaerobic conditions. These results suggest that adult *H. contortus* utilize some variation of the tricarboxylic acid cycle under aerobic conditions, but are capable of surviving under anaerobic conditions as well. The observation by Ward & Huskisson (1978) that *H. contortus* are more active under aerobic conditions is consistent with our results that show motility levels decline rapidly during incubations under N_2 (unpublished observations) or in the presence of high levels of cyanide (as shown in this study). Bennett & Bryant (1984) showed that CO_2 production by *H. contortus* is cyanide-sensitive. In other systems, KSb-tartrate, KCN and CCCP interfere with ATP synthesis by specific inhibition at three distinct sites. The fact that each of these drugs induces a flaccid paralysis in *H. contortus* muscle segments at concentrations which also reduce ATP levels suggest that relaxation of the somatic muscle is a secondary consequence of energy depletion. The flaccid paralysis recorded following exposure to PNU-87407, together with data from ATP measurements, suggests that energy depletion underlies the anthelmintic action of this compound as well. The lack of published information regarding the effects of metabolic inhibitors on gastrointestinal nematodes other than *Ascaris spp.*, however, makes it difficult to draw meaningful conclusions about mechanism of action based on comparative pharmacology.

In summary, results of this study show that PNU-87407 and PNU-88509 reduce motility and ATP levels in adult *H. contortus*, with motility being a more sensitive indicator of drug action. Similar effects were recorded following *in vitro* incubations with closantel, and both experimental compounds were functionally cross-resistant to closantel in a 4th-stage *H. contortus* larval migration assay. The effects of PNU-87407 on somatic muscle tension in small segments of *H. contortus* were also similar to those induced by closantel and a wide range of other metabolic poisons, while the effects of PNU-88509 in that assay were qualitatively different. Marked differences between PNU-88509 and PNU-87407 were also recorded in each of the vertebrate cell and mitochondria assays, with effects of the former compound being reversible and less toxic by factors of 50–200. These observations suggest that PNU-88509 may possess a distinct mechanism of action relative to PNU-87407, closantel and other uncouplers of oxidative phosphorylation, and this may contribute to the different anthelmintic spectrum exhibited by this compound.

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