Closantel: Myoneural Activity and Mechanism of Fasciolicidal Activity

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Abstract ❑ Closantel, a recently introduced fasciolicidal drug, was tested on a number of skeletal muscle preparations in a trial to explore its effect on the neuromuscular transmission and to explain the mechanism of its fascioticidal activity. On the chick biventer cervicis muscle, closantel induced irreversible neuromuscular blockade accompanied by slowly developing contracture. Closantel, however, slightly reduced carbachol induced contracture. On the rat hemi-diaphragm, closantel potentiated the electrically evoked muscle twitches followed by irreversible neuromuscular blockade. Neither neostigmine nor calcium chloride could reverse the established neuromuscular blockade. Closantel induced significant contracture of the diaphragm muscle which was not affected by pretreatment with dantrolene, TMB-8, diltiazem or tubocurarine. On the frog rectus abdominis muscle, closantel induced a well defined contracture which was not affected by tubocurarine. At the same time, closantel failed to affect ACh-induced contracture of the muscle. Closantel also caused initial stimulation of the motility of *Fasciola gigantica* worms manifested as increase in the tone and frequency of contraction. This was followed by irreversible spastic paralysis. The drug also significantly inhibited the activity of total cholinesterase enzyme obtained from *Fasciola gigantica* homogenates. It was concluded that closantel possesses a pronounced non-specific neuromuscular blocking activity, which might be related to its reported capacity to uncouple mitochondrial oxidative phosphorylation

Keyphrases ❑ Closantel, Neuromuscular transmission, Cholinesterase activity.

The mode of action of many anthelmintic drugs depends on affecting the parasite neuromuscular system, leading to either muscle paralysis or their death and disintegration. Closantel (Flukiver®) is a new derivative of salicylanilide arthelmintic that is highly effective in sheep and cattle against both mature and blood sucking nematodes as haemonchus species (1-3). The lack of thorough pharmacological studies on the neuromuscular transmission prompted the investigation of the effect of closantel on the neuromuscular transmission in a number of ***in vitro*** skeletal muscle preparations as well as on the motility and cholinesterase activity of ***Fasciola gigantica*** worms in a trial to elucidate its mechanism of action.

Methods:

Preparation of isolated toad rectus abdominis muscle: The muscle was isolated according to the method of Burn (4). It was suspended in an organ bath of 10 ml capacity containing Ringer's solution at R.T. and aerated with bubbles of air generated from an air pump. The muscle was left to equilibrate for 15 min., under 1 g tension before the addition of drugs. Submaximal contractures were obtained by ACh for 1 min. The contractures were recorded on a Grass polygraph (model 7D).

Preparation of isolated chick biventer cervicis muscle: This was prepared according to the method described by Ginsborg and Warriner (5). The muscle was suspended in a 50 ml organ bath. The lower end of the muscle was fixed at the bottom of the electrode holder, whereas the thread from the upper end was carefully passed through the electrode and attached to a Grass force displacement transducer (model FT-03C) connected to a Grass Polygraph (model 7D). The bathing solution was Krebs' solution kept at 37°C aerated with carbogen (95% O_2+ 5% CO_2). The electrode was made from platinum in the form of two rings through which the tendonous part of the muscle containing the nerve was passed. The nerve was stimulated by rectangular pulses of supramaximal voltage (1-4 V) and a duration of 0.5 msec. at a frequency of 0.2 Hz using a Grass electronic stimulator (model 548).

Preparation of isolated rat phrenic nerve hemidiaphragm: The preparation was performed as described by Bulbring (6). The muscle was mounted in a 50 ml organ bath and the nerve was placed on a pair of platinum electrodes. The thread attached to the apex of the diaphragm was connected to a Grass force displacement transducer (model FT-03C). The bathing fluid was Krebs' solution (mM): NaCl (95.5); KCl (4.69);

$CaCl_2$ (2.5); $MgSO_4$. $7H_2O$ (1.18); KH_2PO_4, (2.2); $NaHCO_3$ (24.9) and glucose (10.6). The indirect muscle twitches were evoked by stimulating the nerve with rectangular pulses of supramaximal voltage (1-5 V) and pulse width of 0.5 msec. at a frequency of 0.1 Hz using a Grass electronic stimulator (model S48). Tetanic stimulation, when required, was obtained by stimulating the nerve at a frequency of 60 Hz for 5 sec. every 5 min. Direct muscle twitches were obtained by direct stimulation of the diaphragm muscle through the fixed pines (holder) with rectangular pulses of supramaximal voltage (60-70V), and a pulse width of 0.5 msec. at a frequency of 0.1 Hz in the presence of 4 µg/ml tubocurarine.

Effect on Fasciola gigantica activity: active and mature ***Fasciola gigantica*** worms were obtained from bile ducts of livers of infected slaughtered cattle from the abbatoir. Worms were transferred in their bile medium to the laboratory. The flukes were washed with warm Tyrode's solution at 38°C. Then, one worm was mounted in a 50 ml organ bath containing Tyrode's solution at 38°C. Normal tracings of the motility were recorded on a smoked paper on a drum for 5 min. The effect of different doses of closantel were then investigated.

Effect of closantel on cholinesterase activity: Cholinesterase enzyme was prepared by homogenization of 5 g ***Fasciola gigantica*** worms in 15 ml of ice cold phosphate buffer (pH 7.4) using a polytrone homogenizer (type PT 45/80, Switzerland) at a high speed for 1 min. The homogenate was then spinned at 5000 r.p.m. for 10 min., using ultracentrifuge (Prepsin 75, MSE). All operations were carried out at 0-4°C. For colorimetric studies, acetylcholine iodide (28.29 mg/100 ml dist. H_2O) and DTNB (39.6 mg/100 ml of sodium dibasic buffer pH 7.4) were prepared.

For measuring the activity of the enzyme, the supernatent fluid after centrifugation was collected and the colorimetric method described by Ellman, ***et. al.*** (7), and modified by Brownson and Watts (8), was adopted. U.V. double beam spectrophotometer (model SP8-100) was used. The method of Lowery, ***et. al.*** (9), was used for measuring the amount of protein in each sample. The specific activity was expressed as activity/mg protein/min.

Statistical analysis: The results were statistically analyzed using the Stucent's t-test after Sendecor (10).

Drugs: Closantel (Flukiver), acetylcholine chloride, carbachol chloride and diltiazem HCl were purchased from Sigma chemical company; TMB-8 (8-(diethylamino)octyl 3,4,5-trimethoxybenzoate) from Aldrich chemical company, dantrolene (Norwich Eaton). All other compounds and reagents were obtained from standard commercial sources.

RESULTS

A) Effect of closantel on the direct and indirect muscle twitches of the rat diaphragm: Closantel

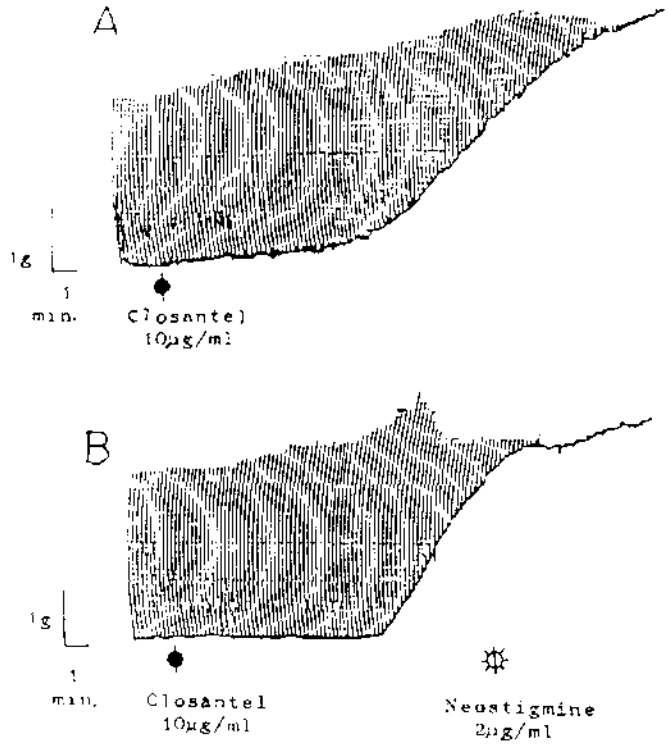

Figure 1: **Effect of closantel on direct (A) and indirect (B) muscle twitches of the rat diaphragm. Muscle twitches were elicited by stimulating the muscle (direct) or the phrenic nerve (indirect) with impulses of supramaximal voltage, 0.5 msec. duration and at a frequency of 0.1 Hz.**

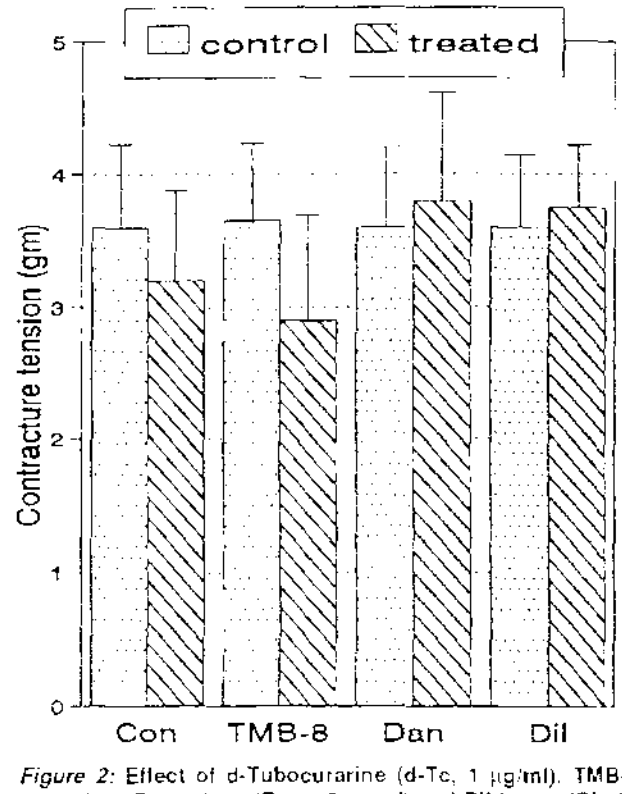

Figure 2: **Effect of d-Tubocurarine (d-Tc, 1 µg/ml), TMB-8 (10 µg/ml), Dantrolene (Dan, 10 ug/ml) and Diltiazem (Dil, 20 ug/ml) on closantel-induced contracture of the rat diaphragm.**

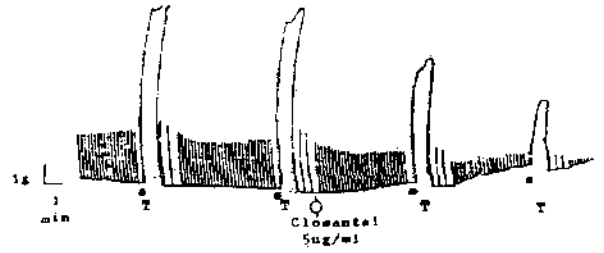

Figure 3: **Effect of closantel on tetanic tension curve and post-tetanic potentiation of the rat phrenic nerve hemidiaphragm muscle preparation. Indirect muscle twitches were elicited by stimuli of supramaximal voltage, 0.5 msec. duration at a frequency of 0.1 Hz. Tetanic stimulation (T) was performed for 5 sec. with pulses of 0.5 msec duration at a frequency of 60 Hz repeated every 5 min.**

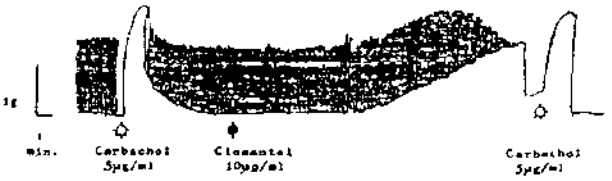

Figure 4: **Effect of closantel on electrically-evoked muscle twitches and carbachol-induced contracture of the chick biventer cervicis muscle preparation. Muscle twitches were elicited by stimuli of supramaximal voltage, 0.5 msec duration at a frequency of 0.2 Hz.**

(5,10 µg/ml) caused irreversible neuromuscular blockade of both direct and indirect muscle twitches. The blockade was preceded by a transient phase of twitch augmentation which was more prominent for the indirect twitches. Addition of calcium chloride (0.26 mg/ml) or neostigmine (2 µg/ml) could not reverse such blockade. At the same time, closantel (10 µg/ml) induced a significant contracture which amounted to 3.6±0.8g after 15 minutes during which muscle twitches were completely abolished *(Fig. 1).*

Prior to the addition of d-tubocurarine (d-Tc, 1 µg/ml), TMB-8 (10 µg/ml), dantrolene (15 µg/ml) or diltiazem (20 µg/ml) did not significantly affect closantel (10 µg/ml) induced contracture *(Fig. 2).* However, closantel partially antagonized d-Tc-induced neuromuscular blockade.

Tetanic stimulation elicited a triphasic response. First rapid twitch followed by a second slowly developing tonic phase till the end of stimulation. The third phase consisted of a transient increase in the amplitude of the first few twitches that follow cessation of the stimulus (post tetanic potentiation, PTP). Closantel (5 µg/ml), tested for 10 minutes, significantly reduced the first rapid phase, whereas the second tonic phase and PTP were not affected *(Fig. 3).*

Effect of closantel on the chick biventer cervicis muscle: Closantel (10, 20 µg/ml) caused complete neuromuscular blockade of the electrically evoked muscle twitches within 10 minutes. This blockade was preceded by a slight but insignificant potentiation of twitches. On the other hand, carbachol (5 µg/ml)-induced contracture was slightly reduced in the presence of closantel. The contracture was reduced from 2.2±0.3 to 1.9±0.4g (P>0.05) *(Fig.4), (n=3).*

Effect of closantel on the rectus abdominis muscle: Closantel (50 µg/ml) induced a significant contracture of the rectus abdominis muscle *(Fig. 5).* The contracture amounted to 1.6±0.3 g within 10 minutes. The addition of d-Tc (5 µg/ml) 5 minutes before closantel did not affect such contracture. On the other hand, ACh (2 µg/ml)-induced contracture was slightly reduced in the presence of 50 µg/ml closantel (2.5±0.4 vs. 2.2±0.3, P>0.05, n=4).

Effect of closantel on the activity of Fasciola gigantica worms: The worms showed marked myogenic activity. Addition of closantel (5,10,20 µg/ml) in single doses, produced dose-related increases in the amplitude and the frequency of the contraction. High concentrations of closantel (40, 80 µg/ml) resulted in a transient rapid potentiatory phase followed by complete abolition of the worm activity, along with a significant increase in the muscle tone (spastic paralysis) *(Fig. 6).*

Effect of closantel on cholinesterase activity: Closantel (40, 80 µg/ml) significantly inhibited cholinesterase enzyme as compared to the control values indicating a prominent anticholinesterase activity for closantel *(Table I).*

Table I: **Effect of Closantel on Cholinesterase Enzyme Activity of *Fasciola gigantica* Homogenates.**

group	Specific activity ($X10^{-4}$) (activity/mg protein/min)	%Cholinesterase activity
Control	11.00 ± 0.05	100
Closantel 40 µg/ml	6.30 ± 0.24*	57.27
Closantel 80 µg/ml	5.30 ± 0.25*	48.18

Values represent the mean ± S.E.M. of 12 observations. *P<0.05 vs. control value.

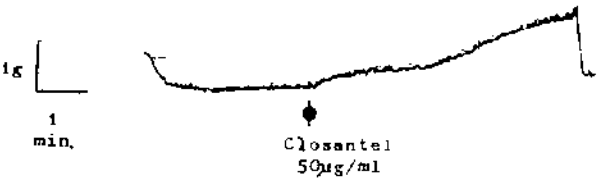

Figure 5: **Closantel-induced contracture of the isolated rectus abdominis muscle.**

DISCUSSION

The present study showed that closantel has pronounced effects on the neuromuscular junction. The drug augmented muscle twitches followed by irreversible neuromuscular blockade. The augmentatory phase was more prominent on the indirect muscle twitches of the rat diaphragm. Such augmentation could be attributed to the anticholinesterase activity reported in this and previous (11) studies. Also closantel effectively antagonized d-tubocurarine-induced neuromuscular blockade on the rat diaphragm, an effect that may support the anticholinesterase activity of closantel. However, closantel failed to potentiate ACh-induced contracture of the rectus abdominis muscle. This failure could be attributed to the blockade of postjunctional sites which could mask its anticholinesterase activity.

The present study showed that closantel evoked comparable inhibitory effects on both the directly and indirectly evoked muscle twitches of the rat diaphragm. This finding excludes a possible specific effect of closantel on ACh release or on the postjunctional nicotinic receptors. The inability of closantel to affect ACh release was evidenced from its failure to reduce the second and third phases of the tetanic response. It has been reported that drugs like d-tubocurarine and hexamethonium, which decrease ACh release from the motor nerve terminals, result in poorly maintained second phase (13, 14). Moreover, the PTP is known to be due to a transient increase in ACh quanta being released after tetanic stimulation (15), and agents which inhibit ACh release were reported to diminish it (16). The post-junctional blocking activity of closantel appears not to be mediated via blockade of nicotinic receptors or interaction with their ionic channels. This suggestion was evidenced from the inability of closantel to affect the contracture induced by the nicotinic receptor ligands, ACh and carbachol on both the frog rectus abdominis and the chick biventer cervicis muscles, respectively.

The non-specific blocking activity of closantel was accompanied by a depolarizing action resulting in an increase in the muscle tone of all the muscles tested. Such depolarization seemed to be a direct effect on the muscle rather than the net of an interaction with the nicotinic receptors since it was not inhibited by d-Tc. Further, this depolarization is unlikely to be due to mobilization of Ca^{++} from intracellular stores or an increase in intracellular Ca^{++} influx because the use of dantrolene and TMB-8,

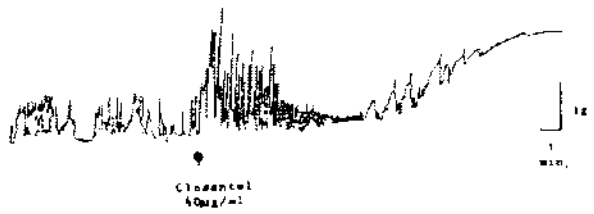

Figure 6: **Effect of closantel on the tone and motility of *Fasciola gigantica* worms suspended in Tyrode's solution.**

inhibitors of intracellular Ca^{++} release (17,18), or diltiazem, a Ca^{++} channel blocker (19), had no effect on closantel-induced contracture of the rat diaphragm.

The observed neuromuscular blockade, however, could be a consequence of interference with the production of the energy carrier, ATP, as a result of uncoupling oxidative phosphorylation (20). In support of this view is the observation that closantel inhibits the motility of ***Schistosoma mansoni in vitro*** by interfering with ATP production (21).

In conclusion, closantel showed a pronounced anticholinesterase activity. At the same time, it induced a non-specific depolarizing neuromuscular blockade of the skeletal muscles tested. This neuromuscular blockade might be related to the reported uncoupling of mitochondrial oxidative phosphorylation.

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Biological and Phytochemical Evaluation of Plants. II. *In vitro* Cytotoxicity Assays of Extracts from Twenty Three Yemeni Plants.

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Abstract □ **Twenty three ethanolic extracts of unselected higher plants, grown in Yemen, were screened for their possible cytotoxic activities. Cytotoxicity assays were performed on the two proliferating mouse cell lines, NIH3T3 and KA31T. Two out of the twenty three extracts exhibited potent cytotoxic activities as arbitrarily defined by IC_{50} of 10 μg/ml or less in both cell lines.**

Keyphrases □ **Higher plants, Cytotoxicity assays, Mouse cell lines.**

Only few drugs have been approved for the treatment of different types of cancer. Although, at present, there are many potential therapeutic agents in the drug development pipeline, the ideal anticancer drug has not yet emerged.

Seeking cytotoxic agents from natural sources has some advantages. Many compounds are being screened simultaneously in an extract, and agents identified in this way are more likely than synthetic compounds to be acting by novel mechanisms or have novel structures that act by one of the known mechanims. Even if novel natural products are not themselves useful as drugs, they may serve as lead compounds for medicinal chemists to do structural modifications in the way of development of useful drugs.

Although several hundreds of higher plant extracts have been screened for their possible cytotoxic activities, desert plants were almost not involved in these screening programs. The assumption that tropical plants are better sources to screen for bioactive compounds has been one reason for ignoring desert plants in different screening programs. In an earlier screening study which involved fifty Egyptian desert plants, we have observed that about 10% of these plants exhibited potent cytotoxic activities (1). In this study, we report on the results of an ***in vitro*** cytotoxicity screening of twenty three unselected Yemeni plants.

Experimental:

Plant materials:

The plants, or plant products, used in this study were collected in the spring and fall of 1992 and the spring of 1993 from different parts of Yemen ***(Table I)***. The plants

Table I: **Cytotoxicity of Some Yemeni Plant Extracts.**

Family/genus, species[*1]	Site/date[*2] of collection	IC_{50} μg/ml[*3] NIH3T3	KA31T
Agavaceae			
Dracaena cinnabari (resin)	Socotra/a		-
Apocynaceae			
Nerium oleander	Sana'a/c	75	35
Asclepiadaceae			
Gomphocarpus sinaicus	Sana'a/a	20	35
Asteraceae			
Artemisia abyssinca	Wadi Dhare/b	20	35
Pulicaria crispa	Tihama & Taiez/b	5	50