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FENTRIFANIL: A DIARYLAMINE ACARICIDE WITH POTENT MITOCHONDRIAL UNCOUPLING ACTIVITY[a]

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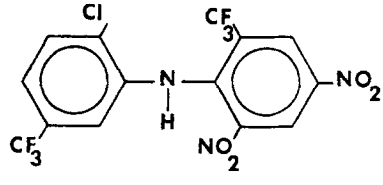
SUMMARY: The acaricide fentrifanil (2'-chloro-2,4-dinitro-5',6-di(trifluoromethyl)diphenylamine) is a new and very potent type of uncoupler of respiratory chain phosphorylation. It stimulates oxygen consumption, reduces the respiratory control index, lowers the ADP/O ratio and releases the oligomycin block of ADP-stimulated respiration in mouse liver mitochondria. In reducing the respiratory control index by 50%, fentrifanil is about 4,500-fold more potent than 2,4-dinitrophenol and slightly more potent than carbonylcyanide m-chlorophenylhydrazone (MCCP).

Fentrifanil (Fusilade, PP-199) is a new selective acaricide with a novel diarylamine structure. It was developed by ICI Co. Ltd. in 1974 and has given good results against phytophagous mites in field trials (1). Fentrifanil has a fairly high acute toxicity to vertebrates. The acute oral LD_{50} to male mice is 30 to 40 mg/kg and the signs of poisoning include hyperthermia, labored respiration, and death followed by rapid rigor mortis which are typical of mitochondrial uncouplers (Nizamani and Hollingworth, unpublished data). Also, the substituted primary arylamine, 2,6-dichloro-4-nitroaniline, has been shown to inhibit electron transport and uncouple oxidative phosphorylation in rat liver mitochondria in vitro (2). For these reasons we decided to investigate the effects of fentrifanil on mitochondrial energetics in a mammalian system.



Fentrifanil

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METHODS: Mitochondria were isolated from fresh livers of male Swiss mice by the method of Katyare et al. (3). Non-fasted mice were killed by a sharp blow to the head. The livers were removed, washed, and gently homogenized in ice-cold 250 mM sucrose/4 mM Tris-HCl (pH 7.4) in a Potter-Elvehjem glass vessel fitted with a teflon pestle. From this homogenate, mitochondria were isolated at 4°C by differential centrifugation at 600g for 10 minutes to remove the nuclear material, and then at 10,300g for 10 minutes to separate the mitochondrial pellet. This pellet was washed by resuspension in isolation medium and recentrifugation at 10,300g for 10 minutes. Finally, the pellet was resuspended in isolation medium to give approximately 10 mg protein per ml.

Oxygen uptake was measured polarographically using a Clark oxygen electrode (Yellow Spring Instruments) in an assay mixture containing 100 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4, 5 mM glutamic acid and 1 mM EDTA at pH 7.4 and 30°C as described by Luciani et al. (4). The total volume of assay mixture was 3 ml containing about 6 mg mitochondrial protein. Small amounts of ADP were added from time to time to the polarographic medium by means of a syringe driven by an ISCO Model M microapplicator. Test compounds were dissolved in ethanol and in each case 5 µl of the solution were added to the incubation medium with glass disposable micropipets. This quantity of ethanol did not affect the respiration of the mitochondria. The ADP/O ratio, respiratory control index (RCI), and stimulation of state 4 respiration were calculated by the method of Estabrook (5). Mitochondrial protein was determined colorimetrically (6) using bovine serum albumin as the standard.

Fentrifanil (primary analytical standard, 99.7% purity) was kindly provided by ICI Co. Ltd., England. MCCP (Carbonylcyanide m-chlorophenylhydrazone), oligomycin (mixture of 15% oligomycin A and 85% oligomycin B) and ADP (disodium salt) were obtained from Sigma Chemical Co., St. Louis, MO. 2,4-Dinitrophenol was purchased from Eastman Kodak Co., Rochester, NY. MON-0858 (3'-(p-chlorophenyl)-4,5-dichlorosalicylanilide) was obtained from Monsanto Chemical Co., St. Louis, MO.

RESULTS: The oxygen electrode tracing in Figure 1 shows that fentrifanil stimulated oxygen uptake in state 4, reduced the RCI, and lowered the ADP/O ratio in coupled mouse liver mitochondria. The tracing shows that there was a slow rate of oxygen consumption in the presence of glutamate, molecular oxygen and inorganic phosphate (A). The addition of small amounts of ADP to this reaction mixture (B) increased the oxygen uptake rate sharply from about 0.80 to 4.10 µmoles O_2/mg protein/hour. However, this higher rate slowed to the original level (0.84 µmoles) as available ADP was phosphorylated to ATP, giving an RCI of 4.88. The ADP/O ratio in this experiment was 2.95. The subsequent addition of 0.0053 nmoles of fentrifanil/mg protein (10^{-8}M final concentration) (C) increased oxygen consumption by 112%. After a second addition of ADP to this partially uncoupled preparation (D) the RCI was now 2.03 and the ADP/O ratio was reduced to 1.78.

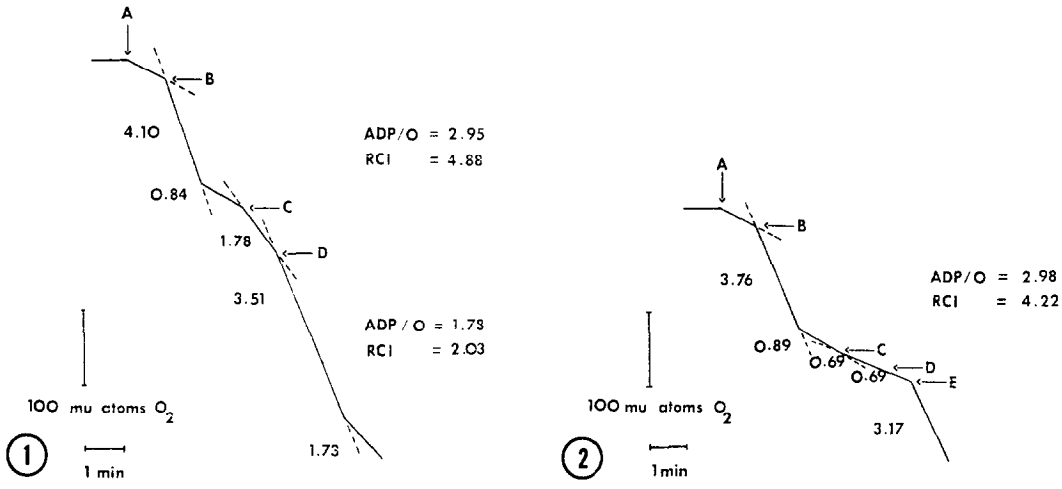

Fig. 1. Uncoupling action of fentrifanil on coupled respiration of mouse liver mitochondria. The line represents the output from an oxygen electrode. The numerical values on the curve represent the respiration rate expressed in μmoles of O_2/mg protein/hr. Additions: at A, 0.5 ml mitochondrial suspension (about 1.9 mg protein per ml reaction medium); at B, 5.0 μl (0.4 μmoles) ADP; at C, 5.0 μl of fentrifanil (0.0053 nmoles/mg protein or 10^{-8}M); at D, a further 5.0 μl (0.4 μmoles) ADP.

Fig. 2. Release by fentrifanil of the oligomycin inhibition of coupled respiration. Conditions as described in Fig. 1a, except at C, oligomycin (7.14 nmoles/mg protein or 10^{-5}M); at D, 0.4 μmoles of ADP; and at E, fentrifanil (0.0714 nmoles/mg protein or 10^{-7}M) were added, with mitochondrial protein of 1.4 mg/ml.

Figure 2 shows the release by fentrifanil of the oligomycin-inhibited respiration of coupled mitochondria. The oxygen tracing shows that the addition of 7.14 nmoles of oligomycin/mg protein at C was able to completely block the ability of ADP to stimulate oxygen uptake (D). However, the subsequent addition of fentrifanil (0.0714 nmoles/mg protein) (E) stimulated oxygen uptake by 359%.

After establishing that fentrifanil behaves as an uncoupler, further experiments were conducted in order to establish a concentration/uncoupling activity relationship. The data from these experiments are shown in Table 1 with comparable values for three standard mitochondrial uncouplers. For fentrifanil, maximum stimulation of state 4 respiration (301% of control) was noted at the concentration of 0.0265 nmoles/mg protein. At this con-

Table 1. Effect of Fentrifanil on ADP/O Ratio, State 4 Respiration and RCI, using Glutamate as Substrate[a]

Treatment	Concentration (μmole/mg Protein)	Percent of Control		
		ADP/O Ratio	State 4 Respiration[b]	RCI
Control[c]	---	100.1	100.0	100.0
Fentrifanil	5.30×10^{-7}	87.4 ± 1.0	100.0 ± 2.4	73.4 ± 2.8
	2.65×10^{-6}	71.4 ± 4.4	149.1 ± 9.1	61.5 ± 4.4
	5.30×10^{-6}	60.3 ± 1.5	212.0 ± 6.2	41.5 ± 0.5
	2.65 ± 10^{-5}	---	300.5 ± 8.0	34.1 ± 2.8
	5.30×10^{-5}	---	263.1 ± 10.5	36.9 ± 0.6
Control[d]	---	100.0	100.0	100.0
2,4-Dinitrophenol	7.10×10^{-3}	88.7 ± 9.7	175.0 ± 10.6	67.2 ± 7.7
	3.55×10^{-2}	---	219.2 ± 8.6	26.5 ± 0.1
	7.10×10^{-2}	---	225.9 ± 19.7	28.1 ± 2.8
Control[e]	---	100.0	100.0	100.0
MCCP	4.97×10^{-6}	63.8 ± 5.9	133.8 ± 2.1	53.3 ± 8.4
	9.94×10^{-6}	50.8 ± 9.1	168.6 ± 44.2	41.8 ± 6.2
	2.49×10^{-5}	55.8 ± 8.0	136.6 ± 10.3	44.8 ± 6.6
Control[f]	---	100.0	100.0	100.0
MON-0858	4.38×10^{-5}	88.3 ± 1.6	138.1 ± 12.6	71.9 ± 8.6
	2.19×10^{-4}	66.9 ± 2.4	250.8 ± 4.5	49.3 ± 1.7
	4.38×10^{-4}	50.1 ± 0.9	290.5 ± 17.2	36.7 ± 3.2

[a] The results are averages of at least three independent experiments ± standard deviation.

[b] Respiration in absence of exogenous ADP.

[c] The average control values (± S.D.) of ADP/O ratio, state 4 respiration, and RCI were 2.95 ± 0.04, 0.84 ± 0.16 μmoles O_2/hr/mg protein, and 4.88 ± 0.84, respectively. Final protein concentration was 1.89 mg/ml.

[d] The average control values (± S.D.) of ADP/O ratio, state 4 respiration, and RCI were 2.95 ± 0.02, 1.19 ± 0.05 μmoles O_2/hr/mg protein, and 3.80 ± 0.29, respectively. Final protein concentration was 1.41 mg/ml.

[e] The average control values (± S.D.) of ADP/O ratio, state 4 respiration, and RCI were 2.92 ± 0.09, 1.36 ± 0.02 μmoles O_2/hr/mg protein, and 4.12 ± 0.08, respectively. Final protein concentration was 2.01 mg/ml.

[f] The average control values (± S.D.) of ADP/O ratio, state 4 respiration, and RCI were 2.96 ± 0.00, 0.84 ± 0.00 μmoles O_2/hr/mg protein, and 4.43 ± 0.46, respectively. Final protein concentration was 2.28 mg/ml.

centration the RCI was decreased to 34.1% of control. An ADP/O ratio could not be measured because ADP-stimulated (state 3) respiration did not return to state 4. Any further increase in fentrifanil concentration resulted in the inhibition of state 4 respiration. In the case of the known uncouplers 2,4-dinitrophenol, MCCP, and MON-0858, a similar relation between concentration and uncoupling action was noted. Uncoupling potency was in the order fentrifanil > MCCP > MON-0858 > 2,4-dinitrophenol.

DISCUSSION: Oxygen electrode experiments show that fentrifanil is a true uncoupler of oxidative phosphorylation (7) in that it stimulates oxygen consumption, and lowers the RCI and ADP/O ratio in coupled mitochondria. Its uncoupling activity was confirmed by its ability to stimulate the oligomycin-inhibited respiration of intact mitochondria (8). The observed inhibition of state 4 respiration at higher concentrations is also characteristic of many uncouplers (9). When the results were compared with those obtained with 2,4-dinitrophenol, MCCP, and MON-0858, it was found that in terms of the concentration (nmoles/mg protein) required to decrease the RCI value by 50%, fentrifanil (0.00405) was more potent than MCCP (0.0056), MON-0858 (0.21) and 2,4-dinitrophenol (18.5) by 1.4, 52 and 4570-fold, respectively. Perhaps the most active mitochondrial uncoupler known is the experimental acaricide, malonoben (SF 6847, 3,5-di-tert-butyl-4-hydroxybenzylidene-malonitrile) (10). Using rat liver mitochondria with succinate as substrate, it has been shown (11) that this compound gives maximal stimulation of state 4 respiration at 2.9×10^{-8}M compared to 5×10^{-8}M for fentrifanil at a protein concentration similar to that used in this study. Comparisons of other uncoupling parameters for fentrifanil and malonoben give similar results. Thus, although direct comparisons of uncoupling potencies under differing experimental conditions should be regarded with caution, it is clear that fentrifanil is one of the most potent uncouplers yet discovered.

The uncoupling action of fentrifanil seems to be different from that of some related substituted monarylamines. For example, 2,6-dichloro-4-

nitroaniline does not act as a true uncoupler in rat liver mitochondria in that at optimum concentration levels it lowers the ADP/O ratio but inhibits oxygen utilization (2). A similar response is seen with N,N-dialkylnitroaniline herbicides which have been reported to be inhibitors of electron transport and uncouplers of oxidative phosphorylation in plant mitochondria (12). Compounds of this type have been termed inhibitory uncouplers by Moreland and Hilton (13). In a recent report Oettmeier (14) concluded that a series of substituted diphenylamines similar to fentrifanil are also inhibitory uncouplers of electron transport and photophosphorylation in spinach chloroplasts. Those compounds with electron withdrawing substituents on the aromatic ring(s) which made the >N-H group more acidic were most effective. The inhibition site was tentatively proposed to be localized between the two photosystems. However, our results indicate that, at least in mouse liver mitochondria, fentrifanil acts as a true uncoupler of oxidative phosphorylation in the same way as 2,4-dinitrophenol, MON-0858 and MCCP (15).

Several experimental and commercial acaricides are also potent mitochondrial uncouplers and it is probable that this type of action is a major factor in the toxicity of fentrifanil to mites as well as to mammals.

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