

## Effects of S-Ethyl Hexahydro-1H-azepine-1-carbothioate (Molinate) on Di-n-butyl Dichlorovinyl Phosphate (DBDCVP) Neuropathy

Angelo Moretto,\* Giulio Gardiman,\* Susi Panfilo,\* Marie-Anne Colle,† Edward A. Lock‡, and Marcello Lotti\*<sup>1</sup>

\*Dipartimento di Medicina Ambientale e Sanità Pubblica, Università degli Studi di Padova, Padova, Italy; †Laboratoire de Neuropathologie R Escouffe, INSERM U306, Paris, France; and ‡Zeneca Central Toxicology Laboratory, Alderley Park, Cheshire, United Kingdom

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Certain esterase inhibitors protect from organophosphate-induced delayed polyneuropathy (OPIDP) when given before a neuropathic organophosphate by inhibiting neuropathy target esterase (NTE). In contrast, they can exaggerate OPIDP when given afterwards and this effect (promotion) is associated with inhibition of another esterase (M200). *In vitro* sensitivities of hen, rat, and human NTE and M200 to the active metabolites of molinate, sulfone, and sulfoxide, were similar. NTE and M200 were irreversibly inhibited (> 78%) in brain and peripheral nerve of hens and rats given molinate (100–180 mg/kg, sc). No clinical or morphological signs of neuropathy developed in these animals. Hens and rats were protected from di-n-butyl dichlorovinyl phosphate neuropathy (DBDCVP, 1 and 5 mg/kg, sc, respectively) by molinate (180 or 100 mg/kg, sc, 24 h earlier, respectively) whereas 45 mg/kg, sc molinate causing about 34% NTE inhibition offered partial protection to hens. Hens treated with DBDCVP (0.4 mg/kg, sc) developed a mild OPIDP; molinate (180 mg/kg, 24 h later) increased the severity of clinical effects and of histopathology in spinal cord and in peripheral nerves. Lower doses of molinate (45 mg/kg, sc), causing about 47% M200 inhibition, did not promote OPIDP whereas the effect of 90 mg/kg, sc (corresponding to about 50–60% inhibition) was mild and not statistically significant. OPIDP induced by DBDCVP (5 mg/kg, sc) in rats was promoted by molinate (100 mg/kg, sc). In conclusion, protection from DBDCVP neuropathy by molinate is correlated with inhibition of NTE whereas promotion of DBDCVP neuropathy is associated with > 50% M200 inhibition.

**Key Words:** OP neuropathy; protection; promotion; molinate; organophosphate; esterase; pathology; inhibition.

Certain esterase inhibitors including sulfonyl halides, carbamates, and organophosphinates, protect from an axonopathy known as organophosphate-induced delayed polyneuropathy (OPIDP) when they are given before an effective dose of an organophosphate (OP) (Johnson, 1990). However, when the

same inhibitors are given after the OP, they cause exacerbation of OPIDP (Lotti *et al.*, 1991; Pope and Padilla, 1990). Axonopathies other than OPIDP are also promoted but not protected by these chemicals (Moretto *et al.*, 1992a, 1993). Both initiation of and protection from OPIDP are related to interactions with neuropathy target esterase (NTE). The difference lies in the molecular rearrangements of inhibited NTE produced by initiating but not by protecting inhibitors (Johnson, 1990; Lotti *et al.*, 1993). Promotion is associated with interactions with another target, although all promoters so far identified are potential NTE inhibitors. A phenyl valerate (PV) esterase activity similar to NTE, named M200, has been identified and its inhibition has been associated with promotion (Lotti and Moretto, 1999). This activity resembles, at least in part, that previously described in the soluble fraction of peripheral nerve (Escudero and Vilanova, 1997; Escudero *et al.*, 1997), which was postulated to be a target for promotion (Vilanova *et al.*, 1999).

The thiocarbamate herbicide molinate is an inhibitor of esterases *in vivo* (Jewell *et al.*, 1998) and its testicular toxicity in rats is thought to be related to inhibition of Leydig cell neutral cholesterol ester hydrolase activity (Ellis *et al.*, 1998; Wickramaratne *et al.*, 1998). Esterase inhibition is due to molinate sulfoxide and molinate sulfone, the oxidation metabolites of molinate known to be formed by NADPH-dependent liver microsomal enzymes (Jewell and Miller, 1998). We report here *in vitro* studies with molinate oxides on NTE and M200 activities from hen, rat, and human nervous tissues and the effects of molinate in both rats and hens when tested for protection from and promotion of neuropathy induced by di-n-butyl dichlorovinyl phosphate (DBDCVP).

### MATERIALS AND METHODS

**Chemicals.** Technical molinate, 96.9% pure (3.1% butylated hydroxy toluene), was supplied by Zeneca Central Toxicology Laboratory (Alderley Park, Cheshire, UK); the commercial formulation of molinate (Erbitor Giavone, Siapa, Italy, 72.6% purity, with unknown coformulants) was purchased from a local dealer. Molinate sulfoxide (pure) and molinate sulfone (pure) were synthesized at Zeneca Central Toxicology Laboratory (Casida *et al.*, 1975). Paraoxon (O,O-diethyl *p*-nitrophenyl phosphate) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified according to Johnson (1977).

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<sup>1</sup> To whom correspondence should be addressed at Dipartimento di Medicina Ambientale e Sanità Pubblica, Medicina del Lavoro, via Giustiniani 2, 35128 Padova, Italy. Fax: +39 049 8242550. E-mail: lottitox@ux1.unipd.it.

Mipafox (N,N-diisopropyl phosphorodiamidofluoridate) and phenyl valerate (PV) were purchased from Oryza Laboratories, Inc. (Chelmsford, MA). DBDCVP was purchased from Chemsyn Science Labs (Lenexa, KS).

**Animals and dosing.** Adult hens (1.7–2.5 kg bw) were purchased from a local breeder. Male Wistar rats (1-month-old, 0.1–0.2 kg bw and 6-month-old, 0.5–0.7 kg bw) were purchased from Charles River Italia, (Calcio, LC, Italy). Animals were allowed food and water *ad libitum*. Molinate and DBDCVP were dissolved in glycerol formal immediately before use and administered sc in the anterothoracic region (hens) or in the nape of the neck (rats) at volumes of 0.2 ml/kg (hens) or 0.5 ml/kg (rats). Controls were injected with a corresponding volume of vehicle. Experiments in hens were conducted with either technical molinate or the commercial formulation whereas rats were treated with commercial formulation only, because the purpose of the latter experiment was only confirmatory. Hens and rats were observed for clinical signs of peripheral neuropathy every other day from day 7 through 21 after treatment. Assessment of hens was based on a 0 to 8 point scale (Lotti *et al.*, 1991) and assessment of rats was based on 0 to 3 point scales for walking and rod test (Moretto *et al.*, 1992b). Scores observed on day 21 are reported in the results.

**Tissues.** Brains and sciatic nerves of hens and rats were excised from animals after decapitation and stored at  $-80^{\circ}\text{C}$  until assay. Samples of human nucleus caudatus and cerebral cortex were obtained during routine postmortem examinations performed within 36 h of death from the bodies of 2 male subjects who died of non-neurological diseases.

**Biochemistry.** NTE activity was determined according to Johnson (1977) and Moretto *et al.* (1989). AChE activity in whole brain (hens and rats) or nucleus caudatus (humans) was determined according to Ellman *et al.* (1961), with modifications of volumes and tissue concentrations to obtain adequate absorbances while maintaining linearity of color development. M200 is defined as the activity resistant to paraoxon (40  $\mu\text{M}$ ) plus mipafox (50  $\mu\text{M}$ ) and sensitive to mipafox (1 mM) (Lotti and Moretto, 1999). It was determined using the same procedure of NTE assay with slight modifications of tissue concentration (13.3 and 80 mg/kg for brain and peripheral nerve, respectively) and time of hydrolysis (35 min) in order to obtain adequate absorbances. Nerves of rats were pooled (6 nerves from 3 animals) due to the small amount of tissue. Rates of reappearance of NTE and M200 activities in hen brain and peripheral nerve after treatment with molinate (180 mg/kg, sc) were calculated from the semilog plot of days after dosing vs. log % recovery. The recovery was calculated as percentage of inhibition found on day 1 after dosing. The  $I_{50}$  (concentration that inhibits 50% of enzyme activity) for both NTE and M200 in hen, rat, and human (mixture of cerebral cortex and nucleus caudatus) homogenates for molinate sulfone and sulfoxide were derived as follows: Inhibitors were dissolved in acetone and mixtures were incubated for 20 min at  $37^{\circ}\text{C}$  before addition of PV.  $I_{50}$  values were calculated by regression analysis of the semilog plots of percent remaining activity vs. inhibitor concentrations (7–10 concentrations up to  $5\text{--}10 \times I_{50}$ ). Time courses (up to 180 minutes) of inhibition were also performed with inhibitor concentrations corresponding to  $0.5\text{--}2 \times I_{50}$  at 20 min.

The reversibility of brain NTE inhibition was examined as follows: Homogenates of hen, rat, and human brain (6.6 mg/ml) were incubated with each molinate metabolite (at concentrations corresponding to  $2\text{--}4 \times I_{50}$ ) for 20 min. Inhibition was halted by adding 30 volumes of ice-cold Tris buffer followed by centrifugation at  $30,000 \times g$  for 30 min. Pellets were resuspended in the initial volume and NTE activity was determined immediately or after 120 min incubation at  $37^{\circ}\text{C}$ . Because M200 activity is in the soluble fraction, a dilution procedure to assess reversibility was applied. Thus, homogenates (100 mg/ml) were incubated with molinate sulfone or sulfoxide (at concentrations corresponding to  $0.5 \times I_{50}$  for 20 min) for 60 min at  $37^{\circ}\text{C}$ . Then 15 volumes of Tris were added and M200 activity measured immediately or after 120 min incubation. In a concurrent control, part of the sample was diluted with Tris containing the same concentrations of molinate sulfone or sulfoxide. In addition, the reversibility of NTE activity was also measured in these conditions.

**Pathology.** Sampling was performed 21–22 days after treatment. Hens were anesthetized with ketamine and halothane, and perfused through the heart with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer.

**TABLE 1**  
*In Vitro* Sensitivity of Hen, Rat, and Human Brain NTE and M200 to Molinate Metabolites

Metabolite	Hen		Rat		Human	
	NTE	M200	NTE	M200	NTE	M200
Molinate sulfoxide	7 (10)	4 (5)	8	4	8	5
Molinate sulfone	1 (3)*	1 (1)*	2	2	2	2

*Note.* Sensitivity is shown as  $I_{50}$  values (20 min,  $37^{\circ}\text{C}$ , pH 8.0), given in  $\mu\text{M}$ . Data in parentheses refer to peripheral nerve. The ranges of the  $r^2$  of the semilog plots (inhibitor concentrations vs. log % remaining activity) were 0.904–0.992.

\*Significantly different from corresponding  $I_{50}$ s in rat and human brain.

Brain and medulla, cervical, thoracic and lumbosacral spinal cord, and sciatic, peroneal, and tibial nerves from both limbs were collected. Paraffin sections were prepared from 6 transverse sections of the brain and medulla, and stained with hematoxylin and eosin. Spinal cord samples were sectioned transversely and peripheral nerves were sectioned both transversely and longitudinally. Tissues were then embedded in methacrylate resin, sectioned, and stained with toluidine blue. Axonal degeneration was found in the spinal cord and peripheral nerve and was scored as: 0 = none, 1 = minimal, 2 = slight, 3 = moderate, 4 = marked degeneration.

**Statistical analysis.** Comparison of *in vivo* inhibitions, clinical and morphological scores were performed by either the *U*-test or the Kruskal-Wallis test and significance set at  $p < 0.05$ . Differences between  $I_{50}$ s and *in vivo* recovery of activities were tested by comparing regression coefficients and significance was set at  $p < 0.05$ .

## RESULTS

Table 1 shows the  $I_{50}$ s of molinate sulfoxide and sulfone for both NTE and M200 activities. No significant difference was found among  $I_{50}$ s when measured in different species except for molinate sulfone whose  $I_{50}$  for hen brain NTE and M200 was slightly, but statistically significantly, lower than that for rat and human enzymes. Molinate sulfone was a better inhibitor of both NTE and M200 than the sulfoxide. After either centrifugation (inhibited NTE) or dilution (inhibited NTE and M200) no reactivation was found of either activity inhibited with both metabolites, indicating that inhibition was irreversible (data not shown). Time courses of hen brain NTE and M200 inhibitions by molinate sulfone show that NTE inhibition was linear whereas M200 inhibition did not proceed further in a linear fashion after about 40 min of incubation (Fig. 1). Similar time courses have been observed with molinate sulfoxide. However, linear time courses of NTE and M200 inhibition have been observed with either metabolite in rat and human brain (data not shown). In hen nervous tissues, a fraction (about 25%) of M200 activity was also found to be less sensitive to inhibition with either metabolite, when determined by fixed-time incubation, indicating that this activity is likely not to be due to a single enzyme. In these tissues, M200  $I_{50}$ s refer to the more sensitive activity (75% of the total). Molinate

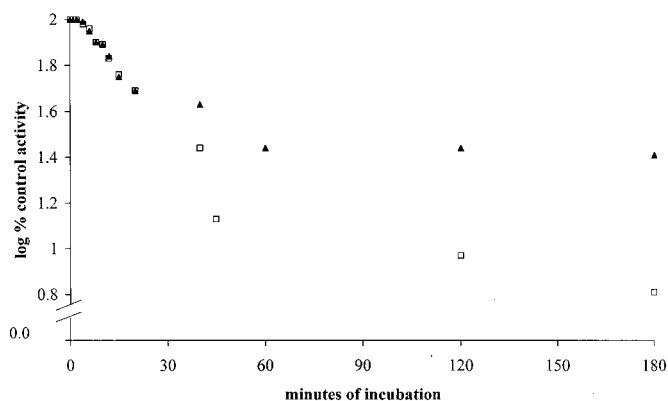


FIG. 1. Time courses of hen M200 (closed triangle) and NTE (open square) inhibition by molinate sulfone (0.75  $\mu$ M, pH 8.0, 37°C). Control activities were 382 and 2315 nmoles/min/g of tissue, respectively.

caused no inhibition of either NTE or M200 when tested at up to 1 mM (data not shown).

Brain and peripheral nerve NTE and M200 were inhibited to a similar extent by molinate (180 mg/kg bw) given to hens and rats, suggesting a similar disposal of molinate in both species and at different ages in rats (Table 2). No significant differences were detected in hens treated with either technical or commercial formulation except for higher inhibition of M200 in peripheral nerve by the commercial formulation. We do not have an explanation for this observation. Control activities were (nmoles/min/g of tissue, mean  $\pm$  SD,  $n = 6$ ) as follows: NTE: 2232  $\pm$  76 and 91  $\pm$  10 in hen brain and peripheral nerve; 986  $\pm$  33 and 1059  $\pm$  132 in brain of 1-month-old and 6-month-old rats; 59 and 42 in peripheral nerves of 1-month-

TABLE 2  
Effects of Molinate on Hen and Rat Nervous Tissue  
NTE and M200

Species	Molinate (mg/kg, sc)	% Control activity			
		Brain		Peripheral nerve	
		NTE	M200	NTE	M200
Hen	45 <sup>a</sup>	67 $\pm$ 20	52 $\pm$ 22	65 $\pm$ 14	54 $\pm$ 14
Hen	90 <sup>a</sup>	44 $\pm$ 14	36 $\pm$ 5	54 $\pm$ 13	51 $\pm$ 11
Hen	180 <sup>a</sup>	17 $\pm$ 14	22 $\pm$ 13	20 $\pm$ 9	19 $\pm$ 4
Hen	180 <sup>b</sup>	18 $\pm$ 8	13 $\pm$ 7	16 $\pm$ 11	1 $\pm$ 1*
Rat (1 month old)	180 <sup>b</sup>	20 $\pm$ 4	20 $\pm$ 11	25 <sup>c</sup>	14 <sup>c</sup>
Rat (6 months old)	100 <sup>b</sup>	18 $\pm$ 3	21 $\pm$ 7	13 <sup>c</sup>	11 <sup>c</sup>
Rat (6 months old)	180 <sup>b</sup>	4 $\pm$ 2	22 $\pm$ 4	3 <sup>c</sup>	11 <sup>c</sup>

Note. Effects of molinate were measured 24 h after a single dose (mean  $\pm$  SD,  $n = 3-5$ ).

<sup>a</sup>Technical molinate.

<sup>b</sup>Commercial formulation.

<sup>c</sup>Pool of nerves from 3 animals.

\*Significantly different from peripheral nerve M200 in hens treated with the same dose of technical preparation.

TABLE 3  
Time Course of NTE and M200 Reappearance in Hen Nervous  
Tissues after a Single Dose of Molinate

Days after treatment	% Recovery			
	Brain		Peripheral nerve	
	NTE	M200	NTE	M200
2	12 $\pm$ 7	35 $\pm$ 7	0 $\pm$ 6	6 $\pm$ 24
4	36 $\pm$ 4	23 $\pm$ 3	17 $\pm$ 8	58 $\pm$ 19
7	73 $\pm$ 23	61 $\pm$ 23	61 $\pm$ 17	69 $\pm$ 21
20	90 $\pm$ 10	86 $\pm$ 12	83 $\pm$ 12	97 $\pm$ 6

Note. Hens were dosed with 180 mg/kg sc, technical; % recovery was calculated as percentage of the inhibition on day 1 reported in Table 2. Values are mean  $\pm$  SD;  $n = 3$ .

old and 6-month old rats; M200: 365  $\pm$  31 and 24  $\pm$  11 in hen brain and peripheral nerve; 167  $\pm$  14 and 163  $\pm$  10 in brain of 1-month-old and 6-month-old rats; 18 and 14 in peripheral nerves of 1-month-old and 6-month-old rats. No inhibition of brain AChE was ever found in hen or rat brain after treatment with molinate (data not shown). The rates of reappearance of NTE and M200 were similar in hen brain and peripheral nerves and activities recovered almost completely by day 20 when activities were not significantly ( $p > 0.05$ ) lower than control values (Table 3). Calculated half-life of recovery from the data reported in Table 3 were 4.3–6 days for both enzymes and in both tissues. The ranges of the  $r^2$  of the semilog plots (days after treatment vs. log % recovery) were 0.867–0.978. Comparison of the slopes did not show significant differences between enzymes or tissues ( $p > 0.05$ ). Data on NTE recovery *in vivo* were similar to those already reported after dosing with several irreversible inhibitors (Johnson, 1974).

Molinate given to hens (180 mg/kg, sc) or to 6-month-old rats (100 mg/kg) did not cause clinical or morphological signs of neuropathy. Molinate (180 mg/kg, sc) given 24 h before a high dose of DBDCVP (1 mg/kg, sc) almost completely protected hens from severe OPIDP (Table 4). The lower dose (45 mg/kg, sc), causing about 35% NTE inhibition (see Table 2), afforded only partial protection. Molinate (180 mg/kg, sc) promoted OPIDP when administered after a minimal neuropathic dose of DBDCVP (0.4 mg/kg, sc). Lower doses of molinate (45 and 90 mg/kg, sc) had no effect (Table 4). Nevertheless, the promotion effect of 180 mg/kg molinate was not maximal because the highest clinical score was 5. When comparing these results with those reported in Table 2, promotion was associated with 80–90% M200 inhibition. No differences in the clinical outcome were observed between animals treated with either the technical compound or the commercial formulation of molinate. These clinical data are consistent with the morphological data in hens (Table 5). DBDCVP (1 mg/kg, sc) caused severe axonal degeneration in the spinal cord and moderate degeneration in peripheral nerves

**TABLE 4**  
Effect of Molinate on DBDCVP Neuropathy in the Hen

Treatment (mg/kg, sc)		Clinical score	n
1st	2nd		
Vehicle	DBDCVP (1) <sup>a</sup>	4 (3–8)	7
Molinate (45) <sup>b</sup>	DBDCVP (1)	3 (2–3)*	5
Molinate (180) <sup>b</sup>	DBDCVP (1)	0 (0–1)**	5
Molinate (180) <sup>b,c</sup>	DBDCVP (1)	0 (0–1)**	5
DBDCVP (0.4) <sup>d</sup>	Vehicle	1 (0–1)	8
DBDCVP (0.4)	Molinate (45) <sup>b</sup>	0 (0–1)	5
DBDCVP (0.4)	Molinate (90)	1 (1–3)	5
DBDCVP (0.4)	Molinate (180) <sup>b</sup>	3 (2–5)***	7
DBDCVP (0.4)	Molinate (180) <sup>b,c</sup>	3 (1–4)***	7

*Note.* Technical molinate, except where noted. Compounds were administered 24 h apart. Clinical scores are presented as median (0–8), with range in parentheses.

<sup>a</sup>Activities measured 24 h after treatment with DBDCVP only were (mean % control activity  $\pm$  SD,  $n = 3$ ): NTE  $8 \pm 2$  and  $20 \pm 7$  in brain and peripheral nerve, respectively; M200  $54 \pm 3$  and  $44 \pm 8$  in the same tissues, respectively.

<sup>b</sup>When given alone no neuropathy developed ( $n = 5$ ).

<sup>c</sup>Commercial formulation.

<sup>d</sup>Activities measured 24 h after treatment with DBDCVP only were (mean % control activity  $\pm$  SD,  $n = 3$ ): NTE:  $13 \pm 9$  and  $29 \pm 13$  in brain and peripheral nerve, respectively; M200:  $75 \pm 6$  and  $61 \pm 20$  in the same tissues.

\*Significantly different from DBDCVP- only (1 mg/kg, sc) treated animals and from molinate (180 mg/kg, sc) + DBDCVP-treated (1 mg/kg, sc) hens.

\*\*Significantly different from DBDCVP- only (1 mg/kg, sc) treated animals and from molinate (45 mg/kg, sc) + DBDCVP- (1 mg/kg, sc) treated hens.

\*\*\*Significantly different from DBDCVP-only (0.4 mg/kg, sc) treated animals and DBDCVP (0.4 mg/kg, sc) + molinate (45 or 90 mg/kg, sc)-treated hens.

of hens. When animals were pretreated with molinate (180 mg/kg sc), lesions in the spinal cord caused by DBDCVP (1 mg/kg, sc) were significantly reduced and completely absent in the peripheral nerves. A lower dose of DBDCVP (0.4 mg/kg, sc) to hens caused less severe axonal degeneration in spinal cord, and no lesions were detected in the peripheral nerves.

**TABLE 5**  
Morphological Lesions in Hens Induced by DBDCVP or by DBDCVP with Molinate Given in Different Sequences

Dose (mg/kg, sc)	Spinal cord			Peripheral nerve		
	Cervical	Thoracic	Lumbosacral	Sciatic	Tibial	Peroneal
Vehicle then DBDCVP (1)	4 (3–4)	2 (2–2)	3 (2–3)	1 (0–3)	1 (0–2)	1.5 (0–3)
Molinate (180) then DBDCVP (1)	1 (1–2)*	0 (0–1)*	0 (0–1)*	0 (0–0)	0 (0–0)*	0 (0–0)*
DBDCVP (0.4) then vehicle	3 (3–3)	0 (0–1)	2 (2–3)	0 (0–0)	0 (0–0)	0 (0–0)
DBDCVP (0.4) then molinate (180)	4 (4–4)**	4 (4–4)**	3 (3–4)**	2 (0–3)**	2 (0–2)**	2 (0–3)**

*Note.* Molinate (technical) was administered either 24 h before or 24 h after DBDCVP. Values represent median of scores (0–4 point scale,  $n = 4–5$ ), range in parentheses. Scores for animals treated with vehicle or molinate only (180 mg/kg, sc) were 0 throughout the nervous system.

\*Significantly different from DBDCVP-treated (1) hens.

\*\*Significantly different from DBDCVP-treated (0.4) hens.

**TABLE 6**  
Clinical Effects of Molinate on DBDCVP-Induced Polyneuropathy in 6-Month-Old Rats

Treatment	Clinical score, median (range) (n)	
	Walking	Rod test
DBDCVP	0 (0–1) (9)	1 (0–3) (9)
Molinate followed by DBDCVP	0 (0–0) (3)*	0 (0–0) (3)**
DBDCVP followed by molinate	1 (1–3) (4)**	2.5 (1–3) (4)**

*Note.* Commercial formulation of molinate, 100 mg/kg, sc; DBDCVP dose, 5 mg/kg sc. Compounds were administered 24 h apart.

\* $p < 0.06$  when compared to animals treated with DBDCVP alone.

\*\*Significantly different for animals treated with DBDCVP alone.

When molinate (180 mg/kg, sc) was given to hens after this dose of DBDCVP, animals displayed axonal degeneration in peripheral nerves and more severe degeneration in the spinal cord.

Table 6 shows the clinical responses after combined treatment of molinate and DBDCVP in 6-month-old rats. Pretreatment with molinate fully protected rats from OPIDP induced by DBDCVP, whereas when molinate was given after DBDCVP it promoted the neuropathy. The dose of molinate in rats was lowered from 180 to 100 mg/kg, sc because of general toxicity of the higher dose. Nonetheless, 2/5 and 1/5 rats died in the molinate (100 mg/kg, sc) plus DBDCVP and DBDCVP plus molinate (100 mg/kg, sc) group, respectively, with similar toxic effects. The toxic effects were not cholinergic, but rather unspecific with severe prostration and no other obvious clinical sign before death. Postmortem examination was not performed.

## DISCUSSION

Molinate is the first thiocarbamate found to protect from and to promote OPIDP, depending on the sequence of dosing, thereby sharing the same properties with chemicals belonging

to the groups of sulfonyl halides, carbamates, and phosphinates (Lotti and Moretto, 1999).

Protection from DBDCVP neuropathy given to both hens and rats by the pretreatment with molinate was correlated with NTE inhibition, probably through carbamylation of the active site that was shown to occur when a rat testicular esterase (hydrolase A) is inhibited by molinate (Jewell and Miller, 1998). Carbamylated NTE is known not to undergo the molecular rearrangements thought to be necessary to trigger OPIDP (Johnson, 1990; Lotti *et al.*, 1993). Therefore, when NTE is inhibited by molinate (> 75%), as shown in Table 2, no neuropathy develops and these animals are protected from challenging doses of the neuropathic DBDCVP (Tables 4 and 6).

Promotion of DBDCVP neuropathy by molinate was associated with M200 inhibition higher than 50%, in both hens and rats, probably by carbamylation as well (Tables 2, 4 and 6). All compounds tested so far indicate that promotion is associated with inhibition of M200, whereas neuropathic OPs have negligible effect when given at doses causing high inhibition of NTE (> 70%) and OPIDP (Lotti and Moretto, 1999).

The recovery half-life of M200 activity after inhibition by a single dose of molinate was found to be about 5 days (Table 3). Within this time frame, another promoter (the phosphorothioic acid-O-(2-chloro-2,3-trifluoro-cyclobutyl)-O-ethyl S-propyl ester), not inhibiting NTE, was found to be effective when given before the initiating dose of a neuropathic OP (Moretto *et al.*, 1994). However, it is not known whether M200 inhibition lasting for a few days is required for promotion to occur or if a short-duration, higher inhibition is needed, similar to that of NTE for OPIDP development (Lotti and Johnson, 1980).

We previously mentioned that M200, as currently measured, is not the activity of a single enzyme and studies are underway to characterize the relevant protein. This protein is likely located in the soluble fraction of hen peripheral nerve where a PV esterase was found, approximately 60 kDa molecular mass (Escudero *et al.*, 1997; Escudero and Vilanova, 1997), which shares some biochemical characteristics with M200.

It is interesting that molinate causes testicular toxicity in rats through inhibition of Leydig cell hydrolase A, whose activity is sometimes determined as nonspecific esterase (NSE) using either  $\alpha$ -naphthyl acetate or *p*-nitrophenyl acetate as substrates (Ellis *et al.*, 1998; Jewell and Miller, 1998). This is a key enzyme in the synthesis of testosterone. It has a molecular mass of about 60 kDa and is also inhibited by PMSF, the prototype promoter, which causes testicular lesions in rats as well (Jewell and Miller, 1998). Tri-ortho-cresyl phosphate (TOCP) is also a known testicular toxicant in rats (Somkuti *et al.*, 1987a) and this effect is probably mediated by NSE inhibition in testes (Chapin *et al.*, 1990, 1991; Somkuti *et al.*, 1987b). Moreover, both hydrolase A and M200 are inhibited *in vitro* by the 2 oxide metabolites of molinate, sulfone and sulfoxide.

Exaggeration of DBDCVP-induced histopathological changes

was observed after molinate treatment causing more than 78% M200 inhibition, whereas protection from DBDCVP induced histopathological changes was observed with molinate pretreatment causing more than 80% (almost complete protection) or more than 33% (partial protection) NTE inhibition (Tables 2 and 5). The comparison of morphological lesions in spinal cord and peripheral nerves of hens displaying DBDCVP neuropathy, or DBDCVP neuropathy promoted by molinate confirmed that promotion causes an exacerbation of axonal degeneration, without other types of lesions or involvement of parts of the nervous system that are usually not affected by OPIDP (Harp *et al.*, 1997; Lotti *et al.*, 1991; Pope *et al.*, 1992; Randall *et al.*, 1997).

The data presented here also confirm that older rats do express clinical OPIDP (Moretto *et al.*, 1992b). Neuropathy can either be protected or promoted when molinate or other non-neuropathic NTE inhibitors are given before or after the neuropathic OP. Resistance of younger rats to OPIDP can not likely be ascribed to a difference in the disposal of compounds because the amount of inhibition of NTE was not significantly different across ages. Data in Table 2 confirm that inhibition of NTE and M200 is not different when molinate was given to either 1- or 6-month-old rats.

In conclusion, since human NTE and M200 have a sensitivity to the active metabolites of molinate comparable to that of hens and rats (Table 1), it is conceivable that protection from and promotion of OPIDP by molinate might occur in humans. However, since sulfur oxidation represents about 30% and 1% of molinate metabolism in rats and humans, respectively (Wickramaratne *et al.*, 1998), high exposures to molinate should be envisaged for such effects to occur in humans.

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