Biochemical Pharmacology, Vol. 44, No. 4, pp. 727–731, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECT OF 2,2′-DICARBOMETHOXYLAMINO-5,5′-DIBENZIMIDAZOLYL KETONE ON ANTIOXIDANT DEFENSES OF *ACANTHOCHEILONEMA VITEAE* AND ITS LABORATORY HOST *MASTOMYS NATALENSIS*

SANJAY BATRA, SHARDA P. SINGH, NIGAR FATMA,* SATYAVAN SHARMA,† RANJIT K. CHATTERJEE* and VISHWA M. L. SRIVASTAVA‡

Divisions of Biochemistry, *Parasitology and †Medicinal Chemistry, Central Drug Research Institute, Lucknow 226001, India

(*Received* 6 *January* 1992; *accepted* 14 *May* 1992)

Abstract—The effect of the macrofilaricidal agent of 2,2′-dicarbomethoxylamino-5,5′-dibenzimidazolyl ketone (C.D.R.I. compound 82/437), on the metabolism of reactive oxygen species (ROs) in *Acanthocheilonema viteae* and *Mastomys natalensis* was measured following intraperitoneal administration at therapeutic doses. The recovered worms possessed substantially reduced levels of catalase and glutathione peroxidase (GPx), and thus were less able to detoxify H_2O_2. Nonetheless, the subcutaneous and adjoining muscle tissues, in which the parasites were lodged, exhibited elevated levels of antioxidant enzymes and reduced glutathione. It is concluded that compound 82/437 kills the filariid by paralysing its H_2O_2 detoxifying capacity without altering ROs metabolism in the tissue in which the parasite resides. Furthermore, since catalase and GPx of the liver and lungs do not show sign of inhibition, a difference appears to exist in the enzymes of the parasite and the host.

Acanthocheilonema viteae becomes established and survives for considerable periods in its laboratory host, *Mastomys natalensis*. Amongst several possible mechanisms for the evasion of host attack, neutralization of oxidative assault has been proven to be the most important. *A. viteae* achieves this defence in two ways: one, the parasite modulates host metabolism in favour of lower accumulation of reactive oxygen species (ROs§) in the tissue in which it dwells ([1], Batra *et al.*, unpublished data); and two, the filariid too like many other organisms [2–5] scavenges host generated oxidants through its antioxidant enzymes [6]. Interestingly, compound 82/437, 2,2′-dicarbomethoxylamino-5,5′-dibenzimidazolyl ketone, has already been shown to exert macrofilaricidal action by severely depriving filariids of their power to detoxify H_2O_2 by inhibiting their catalase and glutathione peroxidase (GPx) [6, 7]. Since no parallel effect on the antioxidant enzymes of the host tissues is observed, differences in catalase and GPx of the host and the parasite may exist and catalase has been proposed as a target for filarial chemotherapy [6].

Nonetheless, before initiating comprehensive studies on catalase it was considered important to examine validity of the *in vitro* findings *in vivo*. The present report deals with alterations in the activities of antioxidant enzymes in *A. viteae* and mastomys tissues at the minimum effective dose of compound 82/437. Changes in the levels of H_2O_2, GSH and vitamin E in the host tissues were also determined.

‡ Corresponding author.

§ Abbreviations: ROs, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; MDA, malonyldialdehyde; compound 82/437, 2,2′-dicarbomethoxylamino-5,5′-dibenzimidazolyl ketone.

MATERIALS AND METHODS

Host and drug treatment. Six mastomys of the same group harbouring 10–12 weeks old infection of *A. viteae* were divided into three groups. Animals of one group were injected subcutaneously with 12.5 or 25.0 mg/kg dose of compound 82/437 for 5 consecutive days. After 1 week the second group of animals was treated likewise. Mastomys of the third group, which served as controls, received normal saline only. At the end of the second week all six animals were killed under deep anaesthesia with ether. Liver, lungs and subcutaneous tissue containing adjoining muscle, in which the parasites were lodged, were immediately excised. The tissues were cleaned, washed three times with saline and blotted dry. The worms and the tissues obtained from two animals of the same group were pooled. This constituted one set of experiment. Three such experiments were carried out each with 12.5 and 25.0 mg/kg doses.

H_2O_2 *level.* For this approximately 500 mg portion of the tissues immediately after their separation from the animal were dropped in trichloroacetic acid. After mincing the tissue properly, the suspension was centrifuged at 9000 *g* for 30 min and the supernate was assayed for H_2O_2 [8].

Preparation of homogenate and assay of antioxidants. Remaining portion of the tissues was homogenized in 1.15% KCl (10% w/v) using a Potter–Elvehjem tissue grinder fitted with a Teflon pestle. The worms (60–80 mg) were homogenized to the strength of 5% (w/v). The homogenates were spun at 900 *g* for 10 min followed by recentrifugation at 9000 *g*

Table 1. Level of antioxidant enzymes in *Acanthocheilonema viteae* recovered from drug-treated *Mastomys natalensis*

	Dose (mg/kg)	Control	Day 7 Specific activity	Day 7 Change %	Day 14 Specific activity	Day 14 Change %
SOD*	12.5	6.77 ± 0.48	7.28 ± 0.33	+7.5^{c}	7.76 ± 0.22^{i}	+14.6^{b}
	25.0	6.44 ± 0.31^{f}	7.82 ± 0.43^{f}	+21.4^{b}	8.55 ± 0.38^{e,i}	+32.7^{a}
Catalase†	12.5	1.32 ± 0.19	0.86 ± 0.15	−34.8^{b}	0.63 ± 0.18^{i}	−52.3^{b}
	25.0	1.49 ± 0.11^{f}	0.75 ± 0.07^{f}	−50.0^{a}	0.44 ± 0.04^{f,g}	−70.5^{a}
GPx‡	12.5	0.19 ± 0.02	0.15 ± 0.02	−21.1^{b}	0.13 ± 0.03^{i}	−31.6^{b}
	25.0	0.23 ± 0.03^{f}	0.17 ± 0.03^{f}	−26.1^{c}	0.14 ± 0.02^{f,i}	−39.1^{b}
MDA§	25.0	24.75	—	—	38.3	+54.7

* One unit corresponds to the amount of protein that inhibits auto-oxidation of epinephrine by 50%.
† μmol/min/mg protein.
‡ nmol/min/mg protein.
§ nmol/mg worm (means of two experiments).
Other data are means ± SD of three experiments.
^{a,d,g} $P < 0.005$ (highly significant); ^{b,e,h} $P < 0.05$ (significant); ^{c,f,i} $P \geq 0.05$ (insignificant). ^{a,b,c} with respect to control; ^{d,e,f} between doses; ^{g,h,i} between days.

Table 2. Effect of *in vivo* treatment with compound 82/437 on antioxidant defences of subcutaneous tissue (muscle) of *M. natalensis*

Scavenger	Dose (mg/kg)	Untreated	Day 7 Specific activity	Day 7 Change %	Day 14 Specific activity	Day 14 Change %
SOD	12.5	5.43 ± 0.34	6.57 ± 0.46	+20.9^{b}	7.29 ± 0.29^{i}	+34.3^{a}
	25.0	6.03 ± 0.49^{f}	7.83 ± 0.54^{e}	+29.9^{b}	8.31 ± 0.78^{f,i}	+37.8^{b}
Catalase	12.5	3.17 ± 0.26	4.05 ± 0.33	+27.7^{b}	4.23 ± 0.0.32^{i}	+33.4^{b}
	25.0	3.02 ± 0.24^{f}	4.12 ± 0.48^{f}	+36.4^{a}	4.38 ± 0.25^{f,i}	+45.0^{a}
GPx	12.5	7.82 ± 0.38	8.42 ± 0.41	+7.7^{c}	9.24 ± 0.38^{i}	+18.2^{b}
	25.0	7.35 ± 0.33^{f}	8.89 ± 0.37^{f}	+20.9^{b}	10.93 ± 0.84^{f,h}	+48.7^{a}
GR*	12.5	36.4 ± 2.8	29.2 ± 2.1	−19.8^{b}	26.1 ± 1.7^{i}	−28.3^{b}
	25.0	37.6 ± 2.1^{f}	25.8 ± 1.4^{f}	−31.4^{a}	21.9 ± 3.3^{f,i}	−41.8^{a}
Vitamin E†	12.5	45.1 ± 3.3	52.2 ± 4.5	+15.7^{c}	56.7 ± 4.6^{i}	+25.7^{b}
	25.0	51.4 ± 3.6^{f}	58.6 ± 5.4^{f}	+14.0^{c}	62.6 ± 5.2^{f,i}	+21.8^{b}
GSH‡	12.5	14.4 ± 1.11	12.8 ± 1.56	−11.1^{c}	10.8 ± 1.23^{i}	−25.0^{b}
	25.0	15.7 ± 2.63^{f}	11.9 ± 1.08^{f}	−24.2^{c}	9.3 ± 1.42^{f,i}	−41.0^{b}

* nmol/min/mg protein.
† ng/mg protein.
‡ μg/mg protein.
Other details are the same as given in Table 1.

for 30 min. The supernate was used for the determination of reduced glutathione (GSH) [9], vitamin E [10], superoxide dismutase (SOD) [11], catalase [12], GPx [13] and glutathione reductase (GR) [14] as described elsewhere [6]. Malondialdehyde (MDA) levels in the recovered worms (30–40 mg) after 1 hr incubation in normal saline containing 50 mM glucose was also assayed [15]. Protein content was measured colorimetrically using bovine serum albumin as a standard [16]. Statistical analysis of alterations occurring in various parameters due to time, dose and drug treatment has been carried out by Student's *t*-test.

Chemicals. Catalase, GR, cytochrome *c*, NADPH and bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). H_2O_2, GSH and oxidized glutathione were the products of SISCO Research Laboratories (Bombay, India). Epinephrine and thiobarbituric acid were purchased from Romali (Bombay, India) and Loba Chemie (Bombay, India), respectively.

Table 3. Effect of *in vivo* treatment of compound 82/437 on antioxidant defences of liver and lungs of *M. natalensis*

Scavenger	Dose (mg/kg)	Liver			Lungs		
		Untreated	Day 7	Day 14	Untreated	Day 7	Day 14
SOD	12.5	17.1 ± 1.56	15.9 ± 0.87^{c}	15.0 ± 1.33^{c,i}	28.8 ± 2.31	30.0 ± 3.15^{c}	30.9 ± 3.21^{c,i}
	25.0	18.1 ± 2.21^{f}	16.2 ± 1.38^{c,f}	15.0 ± 2.48^{c,f,i}	25.9 ± 1.82^{f}	26.2 ± 2.16^{c,f}	27.0 ± 2.02^{c,f,i}
Catalase	12.5	17.9 ± 1.23	19.2 ± 1.89^{c}	23.2 ± 1.32^{b,h}	3.2 ± 0.22	3.4 ± 0.27^{c}	3.7 ± 0.19^{b,i}
	25.0	18.8 ± 1.54^{f}	20.5 ± 2.08^{c,f}	26.3 ± 2.71^{b,f,h}	2.9 ± 0.33^{f}	3.6 ± 0.37^{c,f}	4.0 ± 0.45^{b,f,i}
GPx	12.5	5.3 ± 0.32	5.5 ± 0.29^{c}	6.4 ± 0.39^{b,h}	3.2 ± 0.20	3.6 ± 0.16^{c}	4.0 ± 0.33^{b,i}
	25.0	5.3 ± 0.23^{f}	5.5 ± 0.40^{c,f}	6.2 ± 0.31^{b,f,i}	2.9 ± 0.26^{f}	3.9 ± 0.29^{b,f}	4.4 ± 0.35^{a,f,i}
GR	12.5	37.1 ± 2.43	35.6 ± 1.82^{c}	31.3 ± 1.80^{b,h}	47.7 ± 3.42	59.9 ± 2.23^{b}	64.1 ± 3.13^{a,i}
	25.0	43.6 ± 2.11^{e}	39.9 ± 2.34^{c,e}	37.7 ± 1.92^{b,e,i}	53.6 ± 4.84^{f}	69.6 ± 3.70^{b,e}	80.4 ± 7.34^{b,e,i}
Vitamin E	12.5	37.3 ± 4.14	42.7 ± 3.36^{c}	49.9 ± 3.43^{b,h}	56.4 ± 5.26	58.8 ± 2.63	54.3 ± 4.44^{c,i}
	25.0	33.2 ± 2.28^{f}	38.7 ± 3.11^{c,f}	48.2 ± 5.20^{b,f,i}	61.1 ± 3.51^{f}	58.8 ± 3.37^{f}	63.5 ± 4.23^{c,e,i}
GSH	12.5	18.6 ± 1.29	23.8 ± 1.84^{b}	25.5 ± 2.01^{b,i}	15.5 ± 1.57	18.8 ± 2.00^{c}	25.6 ± 3.62^{b,h}
	25.0	17.1 ± 2.23^{f}	24.2 ± 1.64^{b,f}	25.9 ± 1.88^{b,f,i}	16.7 ± 1.96^{f}	20.6 ± 2.71^{c,f}	27.8 ± 3.38^{b,f,h}

Details are the same as given in Table 2.

RESULTS

A. viteae recovered from the drug-treated mastomys showed altered levels of major antioxidant enzymes (Table 1). The worms possessed greater SOD activity but catalase and GPx were lowered. The changes recorded in the activities of these enzymes were both time and dose dependent. For instance, treatment with 12.5 mg/kg dose caused 35 and 52% reduction in catalase activity on days 7 and 14, respectively. However, when the dose was raised to 25 mg/kg, the drop in the enzyme activity was found to be 50 and 70%, respectively. Likewise, in case of GPx the larger dose resulted in 26 and 39% depression compared to 21 and 32% by the lower dose. Nonetheless, MDA content in the treated worms was 55% higher than in the controls.

Subcutaneous and adjoining muscle tissues of treated mastomys possessed greater activities of all the antioxidant enzymes except GR. Among other scavengers, GSH was present in larger and vitamin E in lower amounts. Here again variation in the levels of both enzymatic and non-enzymatic scavengers was dose and time dependent (Table 2). Interestingly, all the changes including those which were statistically insignificant on day 7, became significant on day 14.

SOD levels in the liver remained virtually unaltered by drug treatment (Table 3). On the other hand, catalase and GPx were elevated while GR was depressed. The concentrations of both non-enzymatic scavengers GSH and vitamin E were elevated appreciably in this organ of the treated animals. All these changes irrespective of the dose became significant on day 14. Lungs of the treated animals showed a pattern similar to that of the liver. Thus, catalase, GPx and GSH expressed marked elevation on day 14, whereas SOD exhibited insignificant alteration. Vitamin E presented an exception and its concentration in the tissue remained virtually unchanged.

H_2O_2 concentration in lungs and subcutaneous tissue did not show noticeable change at any stage (Table 4) while in the liver it was elevated by the lower dose on day 14 and by the larger dose already on day 7.

DISCUSSION

The site of predilection of adult *A. viteae* is the subcutaneous tissue of *M. natalensis*, the experimental host. Treatment of this host with an intraperitoneal dose of 25 mg/kg of compound 82/437 daily for 5 days leads to the death of all the worms *in situ* after 21 days. Accordingly, to obtain maximal biochemical alterations in the parasite without causing its death, days 7 and 14 were chosen for measurements. Effect of the compound at a subcurative dose of 12.5 mg/kg daily for 5 days, which kills 90–95% of adult *A. viteae*, was also examined to give a comparative picture of alterations at two doses. The data presented in Tables 1–4 suggests that the changes in most of the parameters although apparent on day 7 post treatment, become significant on day 14 only. This is important because the worms are killed after 21 days and hence the injury to the worms may be expected to increase with time.

Treatment of mastomys with compound 82/437 enhanced the activities of the major antioxidant enzymes namely SOD, catalase and GPx in subcutaneous tissues, the abode of *A. viteae* adults (Table 2). This makes the tissue capable of quicker elimination of O_2^- and H_2O_2, the two major oxidants. The existence of a non-toxic environment in the tissue is substantiated by the presence of vitamin E at an elevated level. The depression in GSH content appears to result from its slow regeneration due to lower activity of GR. However, it would not affect the level of H_2O_2 because activated catalase which does not require any co-factor, directly destroys this peroxide. The unaltered level of H_2O_2 found in the muscle tissue is in accord with the above conjecture (Table 4).

Table 4. Effect of *in vivo* treatment of compound 82/437 on the level of H_2O_2 of liver, lungs and subcutaneous tissue

Tissue	Dose (mg/kg)	Infected control (specific activity)	Day 7		Day 14	
			Specific activity	% Change	Specific activity	% Change
Liver	12.5	35.5 ± 2.11	38.3 ± 1.56	+7.9[c]	41.8 ± 2.99[j]	+17.7[b]
	25.0	33.2 ± 1.12[f]	37.9 ± 1.50[f]	+14.2[b]	43.4 ± 3.24[f,i]	+30.7[b]
Lungs	12.5	223.8 ± 8.61	232.3 ±10.57	+3.8[c]	218.3 ± 7.88[h]	−2.5[c]
	25.0	191.9 ±14.84[e]	209.8 ± 9.37[f]	+9.3[c]	211.0 ±12.64[f,i]	+10.0[c]
Subcutaneous tissues	12.5	173.2 ± 8.92	160.5 ± 5.66	−7.3[c]	157.1 ±11.15[i]	−9.3[c]
	25.0	188.8 ± 9.11[f]	169.2 ± 8.84[f]	−10.4[c]	163.8 ± 8.21[f,i]	−13.2[b]

Units: nmol/gm tissue.
Data are mean ± SD of three experiments.
Other details as in Table 1.

The drug treatment, on the other hand, markedly depresses catalase and GPx activity in *A. viteae* (Table 1). This severely diminishes the filariids capacity to detoxify H_2O_2. The increased concentration of MDA in the treated parasite supports this view. Attempts were also made to measure H_2O_2 content, but because of unavailability of the worms in sufficient quantity this has not been possible. Interestingly, compound 82/437 does not alter H_2O_2 level in the viscinity of the parasite (subcutaneous and adjoining tissue of the host) (Table 4), but markedly depresses the activities of H_2O_2 metabolizing enzymes of *A. viteae*. This implies that the compound kills the filariid directly without any contribution from the host. This is substantiated by two *in vitro* observations: first, the drug *per se* damages the filariid (as judged by the increase in the leakage of preabsorbed radiolabelled adenine); and second, the compound markedly inhibits filarial catalase and GPx without causing an adverse effect on the host enzymes [6].

It is pertinent to add that by virtue of bearing a benzimidazole structure, compound 82/437 also competes with colchicine for *A. viteae* tubulin (Srivastava, unpublished data). According to an old concept, impairment of microtubules disrupts cytoplasmic transport of nutrients such as glucose. This depletes energy supply of parasites and leads to their death [17]. Inhibition of glucose uptake and transcuticular absorption of methylglucose by *A. viteae* after exposure to 50 μM concentration of compound 82/437 has been recorded by us also (see Ref. 6). Hence, the effects observed on the ROs defence system cannot be treated as the primary or the only mode of action of compound 82/437. Nonetheless, interference with the antioxidant system inducing H_2O_2 toxicity may be more important because this alone can alter the structure and function of numerous biomolecules including transport proteins and tubulin.

Subcutaneous tissue does not present any indication of toxicity of compound 82/437. In liver, although an increase in H_2O_2 concentration is induced (Table 4), it may be effectively detoxified by the elevated levels of several scavengers such as catalase, GPx, vitamin E and GSH (Table 3). Lungs, on the other hand, do not exhibit any change in H_2O_2 concentration and in addition, possess catalase, GPx, GR and GSH in greater amounts (Table 3). Thus, both liver and lungs appear not to show signs of ROs toxicity. This finding is in agreement with the observations recorded elsewhere that compound 82/437 has a high safety margin; its LD_{50} is 2500 mg/kg compared to MED dose of 25 mg/kg for *A. viteae* in *M. natalensis* ([6], Chatterjee, Unpublished data). Surprisingly, the compound induces alterations in GR and GSH levels in muscle and lungs. In liver, however, the changes show an adverse pattern i.e. a depression in GR and an enhancement in GSH content. In liver, therefore, the maintenance of GSH level does not appear to depend on GR. It may be because liver is the organ of an active metabolism, wherein GSH may be synthesized directly from its precursors.

In summary, compound 82/437 exerts its microfilaricidal action by directly paralysing the filariid's H_2O_2 scavenging capability through the inhibition of catalase and GPx. Furthermore, the compound does not adversely affect host's (mastomys) antioxidant defense, and thus is non-toxic. The present *in vivo* and the earlier *in vitro* [6, 7] results, suggest sharp differences in catalase and GPx of the filariid and the host tissues. Preliminary studies concerning relative sensitivity of *A. viteae* and mastomys muscle catalase to several triazole compounds supplement this concept. These findings therefore warrant comprehensive investigations with the objective to use filarial catalase/GPx as vulnerable target(s) for chemotherapy.

Acknowledgements—The authors S.B. and S.P.S. are grateful to the Council of Scientific and Industrial Research, New Delhi for the award of Senior Research Fellowships. This paper is C.D.R.I. Communication No. 4967.

REFERENCES

1. Batra S, Singh SP, Srivastava VML and Chatterjee RK, Xanthine oxidase, superoxide dismutase, catalase and lipid peroxidation in *Mastomys natalensis:* effect of *Dipetalonema viteae* infection. *Ind J Exp Biol* **27**: 1067–1070, 1989.
2. Meshnick SR and Eaton JW, Leishmanial superoxide

dismutase: a possible target for chemotherapy. *Biochem Biophy Res Commun* **102**: 970–976, 1981.
3. Le Trant N, Meshnick SR, Kitchener K, Eaton JW and Cerami A, Iron containing superoxide dismutase from *Crithidia fasciculata*. Purification, characterization and similarity to leishmanial and Trypanosomal enzymes. *J Biol Chem* **258**: 125–130, 1983.
4. Smith NC and Bryant C, The role of host generated free radicals in helminth infections: *Nippostrongylus brasiliensis* and *Nematospiroides dubius* compared. *Int J Parasitol* **16**: 617–622, 1986.
5. Batra S, Singh SP, Gupta S, Katiyar JC and Srivastava VML, Reactive oxygen intermediates metabolizing enzymes in *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis*. *Free Rad Biol Med* **8**: 271–274, 1990.
6. Batra S, Chatterjee RK and Srivastava VML, Antioxidant enzymes in *Acanthocheilonema viteae* and effect of antifilarial agents. *Biochem Pharmacol* **40**: 2363–2369, 1990.
7. Batra S, Chatterjee RK and Srivastava VML, Antioxidant system of *Litomosoides carinii* and *Setaria cervi*: effect of a macrofilaricidal agent. *Vet Parasitol*, in press).
8. Thurman RG, Ley HG and Scholz R, Hepatic microsomal ethanol oxidation: hydrogen peroxide formation and the role of catalase. *Eur J Biochem* **25**: 420–430, 1972.
9. Beutler E, Duron O and Kelly BM, Improved method for the determination of blood glutathione. *J Lab Clin Med* **61** : 882–888, 1963.
10. Desai ID, Vitamin E analysis methods for animal tissues. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 138–147. Academic Press, New York, 1984.
11. Misra HP and Fridovich I, The univalent reduction of oxygen by reduced flavins and quinones. *J Biol Chem* **247**: 188–192, 1972.
12. Aebi H, Catalase *in vitro*. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 121–126. Academic Press, New York, 1984.
13. Leopold F and Wolfgang AG, Assays of glutathione peroxidase. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 114–121. Academic Press, New York, 1984.
14. Racker E, Glutathione reductase from Baker's yeast Beef Liver. *J Biol Chem* **217**: 855–865, 1955.
15. Utley GH, Bernheim F and Hochstein P, Effect of sulphydryl reagents on peroxidation in microsomes. *Arch Biochem Biophys* **118**: 29–32, 1967.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **192**: 265–276, 1951.
17. Van den Bossche H, Peculiar targets in anthelmintic chemotherapy. *Biochem Pharmacol* **29**: 1981–1990, 1980.