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ROLE OF REACTIVE OXYGEN SPECIES IN THE CHEMOPROPHYLACTIC ACTION OF METHYL [5-[[4-(2-PYRIDINYL)-1-PIPERAZINYL]-CARBONYL]-1*H*-BENZIMIDAZOL-2 YL] CARBAMATE IN HAMSTER AGAINST *ANCYLOSTOMA CEYLANICUM**

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Abstract—To delineate mechanisms involved in the prophylactic action of methyl [5-[[4-(2-pyridinyl)-1-piperazinyl]-carbonyl]-1*H*-benzimidazol-2 yl] carbamate (compound 81/470) in hamster against *Ancylostoma ceylanicum* infection, plasma level of the compound and status of reactive oxygen metabolites in jejunum at different periods of the drug treatment were examined. The compound was found to enhance the generation of both O_2^- and H_2O_2 by the jejunum possibly by activating xanthine oxidase. This stimulation was found to be both time and dose dependent. At 100 mg/kg dose the increase in O_2^- production could be recorded at least upto 50 days, whereas at 25 mg/kg the stimulation remained effective upto 20 days only, and at 5 mg/kg there was no change in the activity. This correlated well with the reported prophylactic pattern of the compound i.e. upto 45 and 7 days by 100 and 25 mg/kg doses, respectively. Plasma level of the compound also exhibited dose dependent variation. The compound given at 100 mg/kg dose could be detected in significant concentration upto at least 42 days while that given in 25 and 5 mg/kg doses was present in equivalent concentration upto 14 days and 1 day, respectively. It is concluded that the activation of respiratory burst in the jejunum induced by the persistent presence of compound 81/470 may represent one of the important mechanisms for the chemoprophylactic activity of this anthelmintic.

Key words: compound 81/470; chemoprophylaxis; *Ancylostoma ceylanicum*; xanthine oxidase; reactive oxygen species; plasma level

Compound 81/470§ exhibits fairly good activity against a variety of helminth parasites [1, 2]. The compound is quite safe and exhibits a high safety margin [3, 4]. Interestingly, a single i.m. dose of 100 mg/kg protects hamsters against *A. ceylanicum* upto 45 days suggesting prophylactic use of the compound [5]. This compound at present is under comprehensive investigation with an aim to develop it as a broad spectrum anthelmintic for veterinary and human use.

A compound can exert prophylactic effects in two ways: (1) by its direct action on the parasite and, (2) by enhancing host resistance. Metabolic disposition studies have indicated that compound 81/470 when administered intramuscularly, forms a depot at the site of application from which it is released into circulation at a very slow rate. Consequently the i.m. dose is easily detectable in blood upto 7 weeks whereas the oral dose is almost completely eliminated within 3 days (Srivastava, J. K. *et al.* unpublished data). Recently, anthelmintic action of this compound against *Nippostrongylus brasiliensis* has been attributed to its capacity to enhance the production of ROS by rat intestines and to depress antioxidant defenses of the parasite [6]. Whether a similar mechanism is also responsible for the prophylactic action of the compound against *A. ceylanicum* forms the basis of the present study.

MATERIALS AND METHODS

Drug treatment and infection. Hamsters (40–60 g) were divided into four groups each of 18 animals. The animals of groups C and D were intramuscularly injected with single doses (100 mg/kg) of compound 81/470 whereas those of groups A and B received saline only. Six animals each from groups B and D on days 15, 30 and 45 were challenged orally with 60 ± 5 infective larvae of *A. ceylanicum*. Similar numbers of animals from groups A and C simultaneously ingested saline. After 15 days of the challenge five animals from each group were subjected to deep anaesthesia with ether and jejunum portion of the intestine was excised. The tissue was thoroughly cleaned with normal saline and blotted dry. By this time the animals reached a weight range of 70–80 g.

In another study aimed at dose dependent effect of compound 81/470, animals were divided into four

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§ Abbreviations: ROS, reactive oxygen species; XO, xanthine oxidase; SOD, superoxide dismutase; GPx, glutathione peroxidase; compound 81/470, methyl [5-[[4-(2-pyridinyl)-1-piperazinyl]-carbonyl]-1*H*-benzimidazol-2 yl] carbamate.

Table 1. Alterations in enzyme activities in the jejunum of hamsters at various periods of *A. ceylanicum* infection and treatment with compound 81/470 (100 mg/kg i.m.)

Enzymes	Days	Units/mg protein			
		A, Normal	B, Infected	C, Drug treated	D, Treated challenged
Xanthine	15 + 15 = 30	0.90 ± 0.016	0.35 ± 0.007§	1.35 ± 0.009§	0.98 ± 0.010¶
Oxidase*	30 + 15 = 45	0.91 ± 0.017	0.36 ± 0.009§	1.22 ± 0.007§	0.98 ± 0.019¶
	45 + 15 = 60	0.91 ± 0.011	0.37 ± 0.009§	1.12 ± 0.010‖	0.96 ± 0.033¶
Superoxide	30	1.98 ± 0.019	2.44 ± 0.016‖	2.29 ± 0.060¶	2.07 ± 0.039¶
dismutase‡	45	2.00 ± 0.034	2.47 ± 0.014‖	2.17 ± 0.090¶	2.39 ± 0.049¶
	60	2.03 ± 0.039	2.59 ± 0.019‖	2.11 ± 0.092¶	2.36 ± 0.050¶
Catalase*	30	4.83 ± 0.140	6.88 ± 0.11§	5.90 ± 0.16‖	5.99 ± 0.21‖
	45	4.87 ± 0.190	6.69 ± 0.12§	5.09 ± 0.46¶	5.90 ± 0.16‖
	60	4.92 ± 0.160	6.73 ± 0.19§	5.11 ± 0.92¶	5.92 ± 0.11‖
Glutathione	30	74.1 ± 4.3	67.2 ± 8.7¶	70.9 ± 5.1¶	71.2 ± 1.1¶
peroxidase†	45	76.8 ± 2.1	69.3 ± 9.8¶	78.8 ± 4.1¶	70.4 ± 1.7¶
	60	76.3 ± 6.7	70.1 ± 9.6¶	80.3 ± 4.2¶	71.7 ± 1.9¶

Data are means ± SD of five experiments.
* μmol/min and,
† nmol/min as units.
‡ One unit is the amount of protein that inhibits the auto-oxidation of epinephrine by 50%.
§ $P < 0.005$; ‖ $P < 0.05$; ¶ $P > 0.05$ with respect to normal (Group A).

groups and were injected intramuscularly with 100, 25 and 5 mg/kg of the compound and saline, respectively. After 20 and 50 days, the animals were killed and the jejunum were collected for various assays.

Determination of O_2^- *and* H_2O_2 *production.* The rate of release of O_2^- from the jejunum was measured by the reduction of cytochrome *c* at 550 nm [7], while that of H_2O_2 was determined by the phenol red method [8].

Preparation of homogenate and assay of enzymes. Remaining portion of the jejunum was homogenized in isotonic KCl (1.15% w/v) and centrifuged at 900 *g* for 10 min. The supernate was respun at 105,000 *g* for 60 min and the cytosole assayed for XO [9], SOD [10], catalase [11] and GPx [12] as described elsewhere [6, 13].

Protein content was measured colorimeterically using bovine serum albumin as a standard [14].

Plasma level of [^{3}H]*compound* 81/470. Five hamsters (75–80 g) were each administered intramuscularly with single doses of 100, 25 and 5 mg/kg, respectively, of compound 81/470 (each containing 4 μCi of the tritiated compound). The animals were housed separately in metabolic cages fabricated of wire mesh with free access to water and standard rodent food pellets (Lipton India Ltd, Chandigarh, India). At specified periods 0.2–0.3 mL blood were drawn from retro-orbital plexus into heparinized tubes. Plasma was collected by centrifugation and counted for radioactivity in a Packard Tricarb Scintillation spectrometer using PPO (0.4% w/v) and POPOP (0.02% w/v) in toluene–methoxy ethanol (1:1) as the cocktail. This gives a total estimate of the compound and its metabolites present in plasma.

Chemicals and reagents. Glutathione reductase, horse radish peroxidase, SOD and phenol red were procured from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Reduced glutathione and cytochrome *c* were purchased from SISCO Research Laboratories (Bombay, India). Epinephrine was the product of Romali (Bombay, India).

Compound 81/470 was obtained from the Medicinal Chemistry Division of the Institute. The compound was received in tritiated form from Bhabha Radiation and Isotope Technology (Trombay, India) and was purified by repeated solublization in ethanol and precipitation with water followed by TLC on silica gel.

RESULTS

A. ceylanicum infection markedly depressed XO level in the jejunum whilst compound 81/470 increased the enzyme activity appreciably (Table 1). On the contrary, when the infection was given after the drug treatment, practically no change in the enzyme level could be observed. SOD showed greater activity only in the infected animals. Catalase, on the other hand, expressed elevated levels in all the cases except in the drug treated animals on days 30 and 45. GPx levels remained almost unchanged in all the cases. The magnitude of alterations occurred in all the enzyme activities was time dependent and showed a decreasing trend with time.

The rates of release of O_2^- and H_2O_2 by the jejunum were accelerated in the drug treated (group C) as well as in the treated challenged (group D) hamsters, but were retarded in the infected animals (group B) (Table 2). The change in H_2O_2 production was, however, significant in the case of the drug treated animals only.

In further experiments the enhancement in the ROS production was found to be both time and dose dependent (Table 3). For instance, the increase in O_2^- production on day 15 which figured as 207% for 100 mg/kg, showed a value of 6.6% only for 5 mg/

Table 2. Rates of release of O_2^- and H_2O_2 by the jejunum of hamsters infected with *A. ceylanicum* and/or treated with compound 81/470 (100 mg/kg, i.m.)

ROS	Group	Rate of release (μmol/hr/g tissue) on day 15 + 15 = 30	45 + 15 = 60
O_2^-	A. Normal	4.54 ± 0.51	4.76 ± 1.02
	B. Infected	1.89 ± 0.91*	1.71 ± 0.71*
	C. Treated	13.57 ± 4.08*	7.70 ± 1.63*
	D. Treated & infected	7.21 ± 1.64*	7.58 ± 1.73*
H_2O_2	A. Normal	1.38 ± 0.36	1.47 ± 0.09
	B. Infected	0.93 ± 0.38‡	1.10 ± 0.19‡
	C. Treated	2.49 ± 0.62†	2.26 ± 0.21*
	D. Treated & infected	1.60 ± 0.12‡	1.69 ± 0.11‡

Data are means ± SD of three sets of two animals each. * $P < 0.005$; † $P < 0.05$; and ‡ $P > 0.05$ with respect to normal (Group A).

Table 3. Rates of O_2^- and H_2O_2 release by the jejunum of hamsters intramuscularly administered with various doses of compound 81/470

ROS	Dose (mg/kg)	Rate of release (μmol/hr/g tissue) on day 15 + 5 = 20	45 + 5 = 50
O_2^-	0	4.78 ± 0.07	4.91 ± 0.50
	5	5.10 ± 0.09†	5.02 ± 0.32†
	25	8.57 ± 0.18*	5.04 ± 0.73†
	100	14.68 ± 0.26*	8.97 ± 0.66*
H_2O_2	0	1.42 ± 0.06	1.51 ± 0.05
	5	1.54 ± 0.07†	1.50 ± 0.10†
	25	2.39 ± 0.07*	1.73 ± 0.12†
	100	2.57 ± 0.03*	2.49 ± 0.07*

Data are means ± SD of three sets of two animals each. * $P < 0.005$; and † $P > 0.05$ with respect to control.

kg dose. Time too had a significant impact on ROS production. Thus, the accelaration in the rate of O_2^- and H_2O_2 release went down to 61.6 and 53.4% on day 45 from 207 and 81% on day 15, respectively.

The pattern of plasma concentration of tritiated compound 81/470 at different periods of observation at three dose levels has been depicted in Fig. 1. As expected the dose size expressed a significant effect on plasma level. Thus 100 mg/kg dose was detectable in the blood even on day 49 whereas 25 and 5 mg/kg doses became undetectable on days 42 and 28, respectively.

DISCUSSION

Following oral administration in hamster infective larvae of *A. ceylanicum* accumulate in the stomach, from where they gradually pass into the intestines. These larvae subsequently undergo molting and develop into adults within 14–17 days. This is the time when establishment or rejection of the infection can judiciously be determined [15]. Taking this point into consideration all observations in the present study concerning chemoprophylactic action of compound 81/470 have been recorded on day 15 post infection.

A. ceylanicum infection markedly depressed XO and elevated SOD and catalase activities in the jejunum (Table 1). The overall impact of these changes, as reported earlier [16], would seem to conclude in the lower production of ROS. This interpretation gets strong support from the observed depression in the release of both O_2^- and H_2O_2 by the infected tissue (Table 2). The altered conditions would provide a better opportunity for the infection to establish.

Treatment of the animals with compound 81/470 produced an opposite effect. It increased ROS production (Table 2) by stimulating XO without altering SOD and catalase activities (Table 1). Hence, the compound by virtue of enhancing ROS levels would not allow any infection to survive in the jejunum. Interestingly, when the drug treated hamsters were challenged with the hookworm larvae, no noticeable alterations, possibly due to nulifying effect of each other, in the enzyme activities could be recorded (Table 1). Nonetheless, the production of O_2^- in greater amounts possibly produces an injurious effect on the parasite and thereby imparts prophylactic activity to compound 81/470.

The increase in O_2^- production as a result of stimulated XO appears consistent qualitatively, however, when judged quantitatively the relationship looks less convincing. Interestingly, intestines are known to elicit a mixed inflammatory response against helminth parasites. This includes plasma cells, lymphocytes, leucocytes, eosinophils, macrophages, mast cells and goblet cells. An enormous increase in the latter two cell types is well documented in the case of *N. brasiliensis* [17, 18]. Since mast cells, eosinophils and macrophages on stimulation are known to produce respiratory burst, the alterations in ROS production by jejunum in the present study may be attributed to the impact of the hookworm infection and/or compound 81/470 on these cells. Furthermore, since NADPH oxidase in these cells is known to contribute significantly to the formation of O_2^-, the greater increase in O_2^- production compared to that in XO activity might be accounted for by the extra amount of O_2^- generated through NADPH oxidase. This possibility remains to be examined comprehensively.

Interestingly, compound 81/470 is reported to display dose dependent variation in its prophylactic effect [4]. Consistent with this view, data presented in Table 3 also exhibit dose and time dependent variation in the magnitude of elevation produced by the compound in the release of ROS. Since the reaching of all the administered larvae to the jejunum may require 3–4 days, the toxic effect of the drug in order to eliminate the given infection should last for at least 5 days post infection. Hence, for explaining prophylactic action upto 15 or 45 days, the effect on ROS production has been recorded on days 20 and

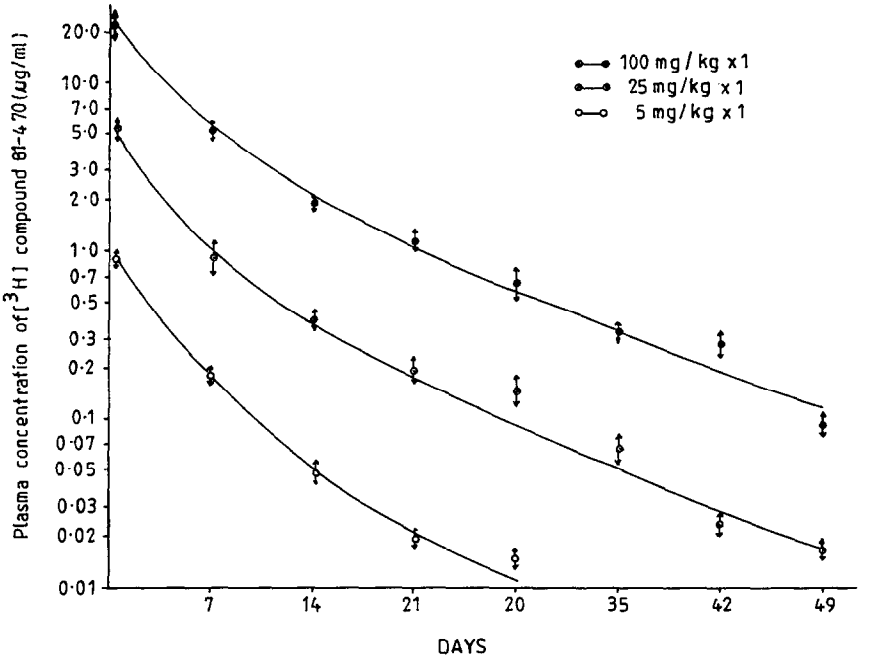

Fig. 1. Semilog plot for plasma concentration of [^{3}H]compound 81/470 following i.m. administration in hamster at three dose levels. The data are means ± SD of five animals.

50, respectively (Table 3). Furthermore, for examining the possibility that the prophylactic action of compound 81/470 is due to the virtual presence of the compound, plasma concentration of the tritiated compound was determined on different days. (Fig. 1). Although this Figure represents hybrid curves of parent drug and its metabolites, it certainly gives an indirect indication of the minimum effective concentration.

It can be observed from Table 3 and Fig. 1 that 5 mg/kg dose showing a plasma concentration of 0.18 μg/mL on day 7 neither modulates ROS production nor exhibits any protection against *A. ceylanicum* infection [4]. Hence, the minimum effective drug level for bringing these effects must be more than 0.18 μg/mL. On this basis detection of 100 mg/kg dose in good concentration (0.48 μg/mL ≅ 0.12 μM) upto 42 days correlates well with the substantial increase in ROS production and the protection of hamsters against the hookworm infection upto 45 days. In the case of 25 mg/kg dose, a similar plasma concentration was recorded upto 14 days and elevation in ROS production was noticed upto 21 days. However, the prophylactic effect is reported to occur upto 7 days only. This implies that in addition to ROS toxicity some other factor(s) is also necessary for the complete elimination of the infection. It is pertinent to mention that compound 81/470 at 10 and 25 μM concentrations did not produce any significant effect on SOD and catalase activities of either larval or adult forms of *A. ceylanicum* (data not included). The compound therefore, does not appear to weaken the parasite's antioxidant defence directly. Interestingly, almost all the benzimidazoles studied to date are known to act at the cytoskeletal system of parasites by disrupting microtubular assembly [19, 20]. Hence, by virtue of being a member of this group, compound 81/470 may also exert a similar effect on the hookworm parasite. This aspect therefore, needs proper attention.

Compound 81/470 appears to exert its action on the intestine and the parasite by its physical presence in the tissue possibly through a biliary route. It is pertinent to add that faecal excretion even of the intramuscular dose of the compound has been recorded throughout the observation period of 50 days (J. K. Srivastava *et al.* unpublished data).

It may, therefore, be summarized that compound 81/470 due to its continued presence in the body produces respiratory burst in the jejunum of hamsters. This possibly represents one of the important mechanisms for prophylactic action of the compound against *A. ceylanicum*. However, this alone does not appear sufficient and additional involvement of some other factor(s) seems necessary.

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