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Free Radical Production by Azomethine H: Effects on Pancreatic and Hepatic Tissues

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The antimalarial properties of azomethine H represent the basis for its use as a chemotherapeutic agent. This work was carried out in order to verify the biological side effects of azomethine H and to clarify the contribution of reactive oxygen species (ROS) in this process. It was shown that azomethine H increased serum activities of amylase, alanine transaminase (ALT) and the TBARS concentrations, in rats. No changes were observed in glutathione peroxidase and catalase activities. The drug-induced tissue damage might be due to superoxide radicals ($O_2^{\bullet -}$), since Cu-Zn superoxide dismutase activities were increased by azomethine H treatment. This study allows tentative conclusions to be drawn regarding which reactive oxygen metabolites play a role in azomethine H activity. We concluded that ($O_2^{\bullet -}$) maybe produced as a mediator of azomethine H action.

Keywords: Azomethine H, superoxide radical, toxicity, Cu-Zn superoxide dismutase, glutathione peroxidase

INTRODUCTION

Azomethine H (4-hydroxy-5 (salicylidene-amino) 2,7 naphthalene disulfonic monosodium (salt) ($C_{17}H_{12}NO_8S_2Na$) (Fig. 1) is a thiosemicarbazone derivative compound which possesses a broad spectrum of potentially useful chemotherapeutic properties such as antitumor, antibacterial, antiviral and antimalarial.[1] On the other hand, several studies have shown that in response to malaria infection, phagocytes such as macrophages and neutrophils engage in the respiratory burst as a host cell-mediated immune reaction. Superoxide ($O_2^{\bullet -}$), the first product of this respiratory burst, is formed by one-electron reduction of molecular oxygen, catalyzed by the phagocyte's membrane NADPH oxidase. The superoxide free radical further reacts to yield the other reactive oxygen species (ROS), hydrogen peroxide and hydroxyl radical, which function in the microbicidal event.[2] So, it may be reasonable to assume that azomethine H action should be related to ROS generation.

Excess of ROS is normally eliminated by the mammalian natural scavenger molecules. The enzymes, Cu Zn superoxide dismtase (SOD-E.C.1.15.1.1.), catalase (E.C. 1.11.1.6) and glutathione peroxidase (GSH-PX-E.C.1.11.1.9.)

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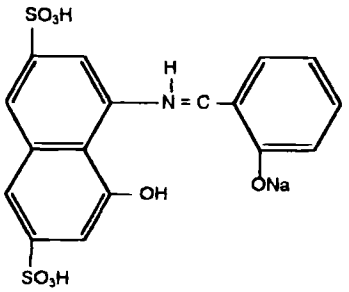

FIGURE 1 Chemical structure of azomethine H.

complete the reduction of ROS to H_2O and also prevent them from interacting to produce hydroxyl radical and singlet oxygen through an Fe-dependent Fenton reaction.[3] However in the enhanced generation of ROS, as may be the case in some chemotherapeutic agents,[4] these natural scavengers may be inadequate and there is likely to be an optimal level of production of these ROS which is compatible with azomethine H microbicidal function, without causing deleterious side effects on the host. Since unscavenged superoxide radical causes toxic effects such as nickel induced toxicity,[5] cataract formation and aging, inflammatory tissue necrosis, tumor proliferation and neurodegenerative disorders,[6] in the prevention of malaria a careful risk-benefit is required to balance the risk of acquiring potentially serious malaria, against the risk of harm from the prophylactic agent. Sometimes the biological side effects of the drugs are more deleterious than the antimalarial activity.

In order to test the biological side effects of azomethine H, it seemed of interest to investigate which reactive oxygen species play a role in azomethine H activity.

MATERIALS AND METHODS

Azomethine H. Azomethine H was readily available as the condensation product of H—acid (8 amino-1 naphthol-3,6-disulfonic acid) and salicylaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in the Department of Chemistry, IB, UNESP, Botucatu, São Paulo, Brazil. This azomethine H (purity > 96%) has the same purity and physical properties as the Merck reagent.[7]

Animals. Male Wistar rats weighing 180–200g (60 animals) were used. Food (Purina Ltd., Campinas, Brazil) and water were provided ad libitum. The azomethine H treated rats received a single intraperitoneal (i.p.) injection of 1g/kg.[7] The animals were divided into four groups of fifteen rats each. The controls groups (A) and (C) received only 1.0 mL of HCl 0.1 N, and 1.0 mL of ascorbic acid (1.5 g: 10.0 mL H_2O) respectively. The group (B) received 1.0 mL of HCl-azomethine H, and the group (D) 1.0 mL of ascorbic acid-azomethine H. Rats were sacrificed by decapitation at time intervals of 3,7 and 60 days after injections.

Assays of dose-response relationships. To study the dose-response relationships of azomethine H, 25 rats (5 for each group) were injected i.p. with 1.0 mL of saline (0.9% NaCl- Merck Darmstadt, Germany) (control group saline), and with a single dose of azomethine H from 0.9% saline of 80, 160, 320 and 640 mg/kg (5 rats for each dose). This experiment was done to exclude the possibility that insoluble saline azomethine H itself might be as toxic as the acids soluble forms. The biochemical determinations were made at 14 days after the treatments.

Biochemical assays. Total protein,[8] amylase (E.C.3.2.1.1.),[9] alanine transaminase (ALT-E.C.2.6.1.2.),[10] glutathione peroxidase (GSH-PX-E.C.1.11.1.9.)[11] and catalase (E.C.1.11.1.6.)[12] were determined in serum. Cu-Zn superoxide dismutase activity (SOD E.C.1.15.1.1) was determined, based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT—Sigma Chemical Co., St. Louis, MO, USA) which was generated by hydroxylamine in alkaline solution.[13] Hydroxylamine (37.5 mM) (Carlo Erba, Italy) was utilized to generate a superoxide flux. The assay was performed in 0.5 M sodium

carbonate (pH 10.2) with 0.1 M EDTA. The reduction of NBT by ($O_2^{\bullet -}$) to blue formazan was measured spectrophotometrically at 560 nm. The rate of NBT reduction in the absence of sample was used as the reference rate. One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50% of maximum inhibition. All data were expressed in units of SOD per milligram of protein. Thiobarbituric acid reacting substances (TBARS) were measured colorimetrically by the thiobarbituric acid (TBA—Sigma Chemical Co., St. Louis, MO. USA)[14] test. The determined values are expressed in terms of μEquiv/ml malondialdehyde (MDA) used as reference by a standard curve. Following incubation at 37C for 90 min., the samples were poured into test tubes containing 1 mL of 30% trichloroacetic acid. One milliliter of 0.75% TBA, in 2M sodium sulfate, was added. Following 15 min. of boiling, and subsequent cooling, the samples were centrifuged 10 min. at 2000 rpm and the optical density of the supernatant fraction was read at 530 nm.

Statistical analysis. Analysis of variance was used to examine the effects of treatment, and comparison between the means was performed by Tuckey test. The probability of 0.05 was chosen as the significant level.

Determination of the median-effective dose (LD50). For mortality index LD_{50} determination, azomethine H from HCl and ascorbic solutions were injected i.p. to 4 groups of 5 rats (200g) as 1.0 mL of a single dose of 0.5, 1.0, 2.0 and 4.0g/kg. All rats were used for necropsy examination, to allow conclusions to be drawn regarding the appearance and damage of tissues.

RESULTS

The dose-response relationship studies of azomethine H in saline 0.9% are shown in Table I. Increased amylase was only observed at 14 days after a dose of 160 mg/kg.

TABLE I Effects of azomethine H concentration from 0.9% saline on serum activities of amylase and alanine transaminase (ALT) at 14 days after i. p. injection.

Azomethine H (mg/kg)	Amylase (U/dL)	ALT (U/mL)
0Control	60.2 ± 0.3	5.3 ± 0.2
80	65.3 ± 10.2	6.8 ± 0.3
160	97.2 ± 7.0*	7.3 ± 0.2
320	102.9 ± 16.6*	5.3 ± 0.5
640	143.3 ± 10.8*	6.8 ± 1.0

*Values are significantly different from those of the control group by Student's **t** test ($p < 0.05$).

The biological side effects included, significantly increased serum Cu-Zn superoxide dismutase, amylase, alanine transaminase activities and increased TBARS concentrations. For comparison, increased TBARS concentrations and SOD activity were highest in rats treated with ascorbic-acid azomethine H. No toxic effect of azomethine H was observed at 60 days, since no significant difference was observed in biochemical parameters at this time in ascorbic-acid azometine H treated rats (group D) and the ascorbic-acid control group (C) (Table II). No changes in total protein concentrations, catalase and GSH-PX activities were observed throughout the experimental period. Azomethine H treatment significantly increased serum TBARS and SOD activity. These increases were highest on the 7th day and decreased to near control levels from day 60 onwards.

Mortality index LD_{50} for intraperitoneal administration of ascorbic acid-azomethine H was 0.95 ± 0.10 g/kg for rats weighing 200g. For comparison LD_{50} values for HCl-azomethine H by i.p. administration was 2.60 ± 0.40 g/kg. The necropsy of rats that died within 10 days after injection demonstrates at gross appearance hepatic hemorragic necrosis with disruption of cytosol in some cells of liver and pancreas tissues. Rats that survive at day 10 onwards were sacrificed 14 days after treatment and used for necropsy examinations. As with the biochemical parameters no alteration was observed in rats that survive at 14 days after treatment.

TABLE II Effects of Azomethine H on serum TBARS concentrations, alanine transaminase (ALT), amylase, glutathione peroxidase (GSH-PX), Cu-Zn superoxide dismutase, catalase activities. (A) HCl control group, (B) HCl-azomethine H, (C) ascorbic acid control group (D) ascorbic acid-azomethine H.

Biochemical Parameters		Time of treatment (days) 3	7	60
Total Protein (g/dL)	(A)	10.8 ± 0.8	11.2 ± 0.9	9.8 ± 0.9
	(B)	11.2 ± 0.6	10.7 ± 0.8	10.1 ± 0.9
	(C)	10.9 ± 0.91	10.9 ± 0.8	11.0 ± 0.8
	(D)	10.8 ± 0.7	11.1 ± 0.8	10.9 ± 1.1
TBARS (μEq/mL)	(A)	0.3 ± 0.1	0.3 ± 0.09	0.4 ± 0.2
	(B)	0.5 ± 0.02*	1.8 ± 0.1*	0.6 ± 0.3
	(C)	0.4 ± 0.1	0.4 ± 0.02	0.5 ± 0.2
	(D)	0.8 ± 0.1*	2.0 ± 0.01*	1.4 ± 0.6
Amylase (U/dL)	(A)	102.5 ± 14.2	90.8 ± 22.4	90.6 ± 23.6
	(B)	104.0 ± 10.3	94.3 ± 10.6	90.2 ± 16.9
	(C)	110.6 ± 19.2	79.6 ± 10.4	80.9 ± 16.2
	(D)	125.5 ± 3.4*	122.3 ± 3.4*	90.8 ± 20.6
ALT (U/mL)	(A)	3.9 ± 0.8	3.5 ± 0.9	3.2 ± 0.4
	(B)	5.0 ± 0.6*	5.5 ± 0.1	2.1 ± 0.1
	(C)	2.8 ± 0.3	3.2 ± 0.01	2.4 ± 0.3
	(D)	5.1 ± 0.2*	6.9 ± 0.02*	2.6 ± 0.4
GSH-PX (U/mg protein)	(A)	1.6 ± 0.3	1.1 ± 0.3	0.8 ± 0.2
	(B)	1.2 ± 0.1	1.2 ± 0.6	0.9 ± 0.2
	(C)	1.4 ± 0.3	1.4 ± 0.3	1.0 ± 0.4
	(D)	1.3 ± 0.2	1.5 ± 0.6	1.6 ± 0.9
SOD (U/mg protein)	(A)	27.3 ± 9.6	26.5 ± 8.6	26.3 ± 9.2
	(B)	34.7 ± 3.2	68.2 ± 8.7*	34.7 ± 9.8
	(C)	32.6 ± 3.2	34.3 ± 3.6	32.3 ± 3.1
	(D)	59.4 ± 10.3*	78.7 ± 2.9*	33.6 ± 2.0
Catalase (U/mg protein)	(A)	6.8 ± 0.6	6.1 ± 0.4	6.6 ± 0.5
	(B)	6.0 ± 0.7	6.5 ± 0.3	6.2 ± 0.3
	(C)	6.3 ± 0.6	6.6 ± 0.3	6.0 ± 0.7
	(D)	6.2 ± 0.5	7.3 ± 1.2	6.7 ± 0.6

*Values are significantly different from those of the control group by Student's t-test, ($p < 0.05$).

DISCUSSION

The antimalarial properties of azomethine H are discussed in concert with those of others drugs such as chloroquine and quinine, because they share important structural features. Although many thousands of thiosemicarbazone analogue drugs have been synthesised and tested for antimalarial activity, side biological effects of these drugs are, in some time, more deleterious and important than the antimalarial activity. Chloroquine induced inhibition of a lysosomal enzyme phospholipase A_2, has been also proposed to result in some toxic effects, such as the accumulation of phospholipids in various tissues.[15] Pyrimethamine dapsone (Maloprin) is associated with agranulocytosis. Mefloquine may provoke severe neuropsychiatric reactions.[16]

The dose-response relationships of saline-azomethine H involved increased serum amylase activity (Table I). We observed that amylase was increased at a dose of 160 mg/kg. Kuile et al[17] observed that side effects of mefloquine were dose related and induced dizziness, anorexia, nausea, vomiting and fatigue. The dose used was 15 to 25 mg/kg/day at during 7 days course (175 mg/kg). In this paper we used a single i.p. dose 1g/kg of ascorbic acid-azomethine H and HCl-azomethine H, and a dose of 80 to 640 mg/kg of saline-azomethine H (Table I).

The levels of ALT does not fully explain the dose dependent toxicity of azomethine H, but it is clear that there is a dose dependent side effect on amylase activity and that, hepatic damage was not observed until a dose of 640 mg/Kg of saline—azomethine H (Table I).

On the other hand, the dose-response relationships reflects the chemical nature of azomethine-H, since it may be decomposed itself in salicylaldehyde and H-acid, decreasing the half-life, and its activity. Ascorbic acid has been used as antioxidant, and for maintenance of pH to inhibit azomethine-H hydrolysis.[18] Azomethine obtained with ascorbic acid is more stable since the Schiff's base formation, imine group azomethine H presence, from a salicylaldehyde and H-acid is catalyzed by an acid, and the condensation has been shown to be a first order reaction, in presence of ascorbic acid[19] Ascorbic acid contributes to stabilization of the chemical structure of azomethine-H, and with the biological activity, since certain molecular features are essential for efficacy.[20]

The biological side effects included pancreatic damage, since ascorbic acid-azomethine H increased amylase activity from its basal level at 3 and 7 days (Table II). Hilmy *et al.*[21] showed that serum amylase is a marker for pancreatic lesion. This observation also indicates that development of the pancreatic damage was time related since no significant difference was observed from day 60 onwards (Table II). Alterations in serum levels of ALT are liver specific and have been considered as a tool to study varying cell viability and changes in cell membrane permeability.[22] Based on this observation, increased serum ALT activity (Table II) reflects hepatic damage. This toxic effect of azomethine H was clear with the LD_{50} determination and necropsy of the rats. For mortality index LD_{50} determination, azomethine H solutions were injected i.p. to 4 groups of 5 rats (200g) as 1.0 mL of a single dose of 0.5, 1.0, 2.0 and 4.0 g/kg. We observed that: all rats increased the respiratory rate, at 15 minutes after injections; all rats that received a dose of 4.0 g/kg died immediately after injection; some rats that received doses of 2.0 and 1.0 g/kg died at 24–48 hours and others within 7 to 10 days. Rats that received a dose of 0.5 g/kg survive at day 10 onwards, and were sacrificed 14 days after treatment. The necropsy of the rats that died within 10 days after injection revealed hepatic and pancreatic hemorragic necrosis, with disruption of cytosol in some cells. Based on these observations only liver and pancreas biochemical markers were determined in serum.

Enhanced generation of ROS or reduced levels of natural scavengers have been implicated in a wide range of pathological conditions.[23] Reactive oxygen species such as ($O_2^{\bullet -}$) and (H_2O_2) are known to be cytotoxic to cells damaging lipids. Lipid peroxidation is an important toxic event of ($O_2^{\bullet -}$) causing final cell death. Cell and tissue destruction can often lead to more lipoperoxide formation because antioxidants are diluted out and transition metal ions, that can stimulate the peroxidation process are release from disrupted cells. So, the peroxidation process proceeds as a free radical chain reaction. The length of the propagation chain depends upon superoxide dismutase activity.[24] Catalase and glutathione peroxidase catalyse the conversion of hydrogen peroxide to water, thus catalase and GSH-PX could reduce the tissue injury by removing the (H_2O_2). SOD catalyses the destruction of superoxide radical by dismutation and (H_2O_2) formation. Since no alterations in catalase and GSH-PX activities were observed by azomethine H treatment, while SOD activities were increased, we can suppose that ($O_2^{\bullet -}$) generated by azomethine H is of primary importance in the pathogenesis of the tissues damage. Superoxide anion-induced cell damage may occur before being converted to (H_2O_2) by SOD. GSH-PX and catalase activities were considered as a means of differentiating between alternative cytotoxic mechanism. This study allows conclusions to be drawn regarding which reactive oxygen metabolite plays a role in azomethine H activity. These observations suggested the role of superoxide radical with respect to azomethine H tissue injury.

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