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AMINOGUANIDINE: A DRUG PROPOSED FOR PROPHYLAXIS IN DIABETES INHIBITS CATALASE AND GENERATES HYDROGEN PEROXIDE *IN VITRO*

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Abstract—Aminoguanidine (AG) has been proposed as a drug of potential benefit in prophylaxis of the complications of diabetes. We show here that AG irreversibly inhibits catalase with an efficacy similar to aminotriazole. AG also produces hydrogen peroxide, in a transition metal-catalysed process which may be partially dependent upon prior hydrolysis of AG to semicarbazide and hydrazine. These observations may be of importance in proposals for the long term administration of AG in diabetes.

Aminoguanidine (AG†) has been proposed as an agent of potential benefit in prophylaxis of the diabetic complications of kidney, nerve and eye [1–5]. *In vivo*, AG has been shown to lessen the formation of collagen-linked fluorescence associated with the production of advanced glycosylation endproducts (AGE). AG has been postulated to block pathological tissue alterations via inhibition of deleterious long-term changes to proteins associated with hyperglycaemia [6].

AG has been referred to as a "virtually non-toxic nucleophilic hydrazine (LD_{50} = 1800 mg/kg in rodents)" [3]. This would be consistent with an absence of subacute/chronic adverse effects reported from studies in which control animals are administered up to 25 mg/kg AG i.p. daily for periods as long as 6 months. While these observations of low toxicity of a potentially useful compound are encouraging, some caution needs to be observed.

Our interest in this compound was aroused by our (unpublished) observation that AG seemed to increase hydrogen peroxide (H_2O_2) production from glucose, which is prone to a slow metal-catalysed oxidation process of possible relevance to protein modification by glucose in diabetes [7]. We had also become aware of literature showing that AG inhibits copper-containing amine oxidases [8] such as spermine [9] and histidine [10, 11] oxidases. AG had also been suggested to inhibit inducible and constitutive nitric oxide synthases [12] which might have a bearing on the moderate hypotensive effect of AG in animals [13], as commented upon in much earlier studies [14]. Finally, earlier studies indicated that AG could inhibit catalase [15]. In this paper, we re-examine inhibition of catalase by AG and also describe the oxidation of this compound to yield H_2O_2. These observations might be relevant to possible problems of chronic toxicity associated with the compound and merit further study.

MATERIALS AND METHODS

Ammonium ferrous sulphate, hydrazine sulphate and 2-hydroxy-1-naphthaldehyde were obtained from the Aldrich Chemical Co. (Poole, U.K.). Xylenol orange, H_2O_2, sorbitol, catalase (Type C-10), semicarbazide and AG bicarbonate were obtained from the Sigma Chemical Co. (Poole, U.K.). AG bicarbonate salt was dissolved rapidly in 1 N HCl and adjusted to pH 7.4 with sodium hydroxide. All solutions were prepared with chelex-treated double distilled water.

Determination of H_2O_2. The concentration of H_2O_2 in purchased batches was determined using its extinction coefficient of 39.4 M/cm at 240 nm [16]. Standard solutions of H_2O_2 were then used to calibrate the FOX (ferrous oxidation in xylenol orange) assay for hydroperoxide determination as described previously [17]. Briefly, 50 μL of test sample or H_2O_2 standard were mixed with 950 μL of FOX1 reagent (composed of 250 μM ammonium ferrous sulphate, 100 μM xylenol orange, 100 mM sorbitol in 25 mM H_2SO_4) and incubated for 30 min at room temperature before reading absorbance at 560 nm. Where appropriate, flocculated protein was first removed by centrifugation (12,000 $g \times 5$ min). Absorbance data were gathered on a Pye Unicam Series 8700 Spectrophotometer.

Inhibition of catalase. Inhibition of catalase was measured by a discontinuous method using the FOX assay. Catalase (40 Sigma units/mL) was incubated with chemicals of interest at various concentrations and for varying times in 50 mM potassium phosphate buffer at 37° (see legends to figures for further details). Samples were then diluted 8-fold into 200 μM H_2O_2 in potassium phosphate buffer (pH 7.2; 10 mM) and incubated for 10 min at room

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† Abbreviations: AG, aminoguanidine; AMT, aminotriazole; H_2O_2, hydrogen peroxide; FOX, ferrous oxidation in xyenol orange; AGE, advanced glycosylation end-product; PBS, phosphate-buffered saline.

temperature. Residual H_2O_2 was then measured as described above. Where appropriate, blank incubations containing drug but no catalase were also run in order to control for H_2O_2 production by AG and other chemicals of interest.

Determination of hydrazine in AG. The presence of hydrazine in stock solutions of AG was sought by the reaction of hydrazine with 2-hydroxy-1-naphthaldehyde to form a spectrophotometrically-detectable hydrazine [18]. Volumes (10 mL) of hydrazine standard solutions or unknowns were incubated with 1 mL of 1 M sodium acetate/100 mM acetic acid and 1 mL of 120 mM 2-hydroxy-1-naphthaldehyde (in ethanol) for 20 min at 100°. Aldizines were then extracted into 5 mL of chloroform by shaking. Spectral characteristics of the chloroform extract were compared for the hydrazine unknowns and hydrazine standards. Concentrations of hydrazine-like aldizines in AG were estimated by calibration against 412 nm absorbance for hydrazine standards.

Determination of catalase activity in liver slices and erythrocytes. Preparation and incubation of liver slices were performed according to the method of McDanell *et al.* [19]. Male Wistar rats weighing 120–200 g were killed by cervical dislocation under terminal anaesthesia induced by fentanyl citrate (0.01 mg/kg, i.m.) and diazepam (2.5 mg/kg, i.p.) (Janssen, Wantage, U.K.). The livers were rapidly removed and slices of 0.3 mm thickness or less (approximately 100 mg) were cut by hand on a Stadie-Riggs stage with a long razor blade (AH Thomas Co., Philadelphia, PA, U.S.A.). Duplicate slices were put into 25-mL Erlenmeyer flasks containing 5 mL of Ringer solution as described previously [20] and varying concentrations of AG or aminotriazole (AMT). Flasks were incubated at 37°, under oxygen, in a shaking water bath (90 strokes/min). At 2 and 4 hr duplicate slices were removed, washed in phosphate-buffered saline (PBS) and homogenized in 3 mL of cold PBS.

Homogenates were diluted to 200 μg liver (wet weight)/mL with PBS containing 200 μM H_2O_2 and incubated with shaking for 10 min at room temperature under air. Catalase activity was determined by reference to the concentration of H_2O_2 (measured using the FOX assay) remaining at the end of the incubation period. Aliquots (50 μL) of the H_2O_2-exposed homogenate were added to 950 μL of the FOX reagent (which also halted H_2O_2 consumption by catalase) and then incubated for 30 min at room temperature in 1-mL microcentrifuge vials with occasional vortexing. The vials were centrifuged to remove flocculated material (12,000 g × 3 min) and the supernatants were read at 560 nm against H_2O_2 standards. Catalase activity was calculated in terms of H_2O_2 consumed per minute. Under the conditions used, all incubations contained residual H_2O_2. H_2O_2 consumption was approximately linear over the 10 min observed.

Human blood was drawn from healthy volunteers and centrifuged to separate the plasma and buffy layer. Erythrocytes were washed three times in ice-cold PBS. Washed erythrocytes were incubated with various concentrations of AG and with AMT (50 mM) at a final cell volume of 5%. At varying time intervals, 50 μL of the cell suspension were removed and added to 950 μL of lysis buffer (potassium phosphate buffer, pH 7.4; 10 mM) containing 200 μM H_2O_2 and incubated for 10 min at room temperature with shaking. Catalase activity was determined by reference to the concentration of H_2O_2 (determined by the FOX assay) remaining at the end of the incubation period as described above.

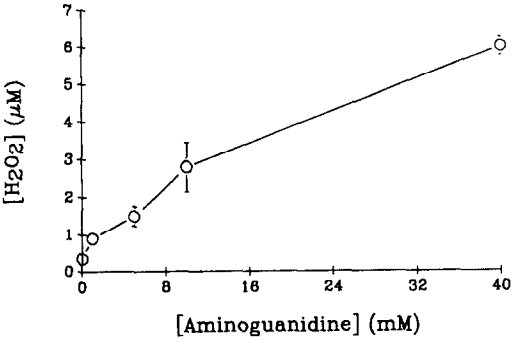


Fig. 1. The accumulation of H_2O_2 by AG. Aminoguanidine at varying concentrations was incubated with potassium phosphate buffer (50 mM, pH 7.4) at 37° for 20 hr. H_2O_2 was determined by the addition of 100-μL samples to 900-μL samples of FOX reagent as described in Materials and Methods. Values given represent the means ± SD of triplicate measurements.

RESULTS

AG and H_2O_2 production in relation to glycation

We were originally interested in AG in relation to its proposed use as a potential inhibitor of protein modifications associated with enhanced glycation reactions in diabetes. Our preliminary observations led us to postulate that AG could act as a precursor for H_2O_2 since we found that glucose appeared to generate consistently greater amounts of H_2O_2 in the presence of AG (Ou and Wolff, unpublished observations). This result was unexpected. Although

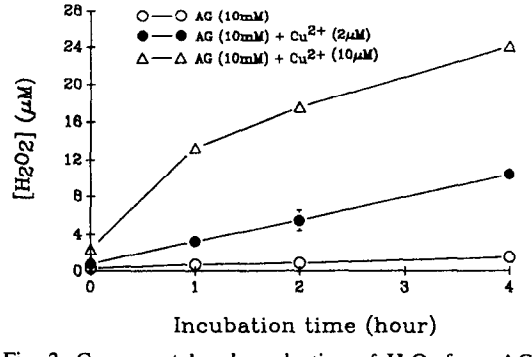


Fig. 2. Copper-catalysed production of H_2O_2 from AG. AG (10 mM) was incubated in the presence or absence of Cu^{2+} (2 or 10 μM) in potassium phosphate buffer (50 mM, pH 7.4) at 37° for up to 4 hr. Samples were taken at various time intervals and analysed for H_2O_2 as described in Fig. 1.

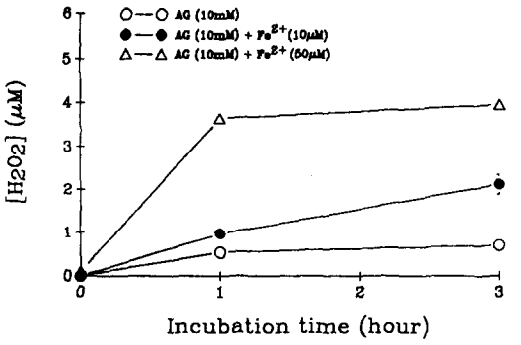


Fig. 3. Iron-catalysed production of H_2O_2 from AG. AG (10 mM) was incubated in the presence or absence of Fe^{2+} (10 or 50 μM) in potassium phosphate buffer (50 mM, pH 7.4) at 37° for up to 4 hr. Samples were taken at various time intervals and analysed for H_2O_2 as described in Fig. 1.

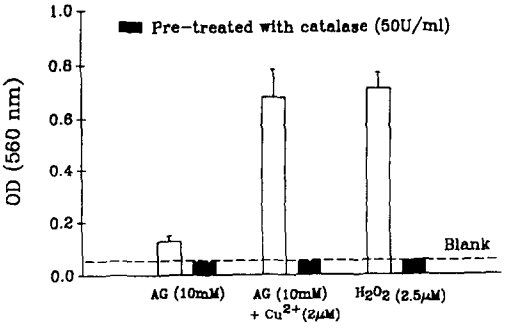


Fig. 4. Effect of catalase on hydroperoxide signal generated by AG. AG (10 mM) was incubated alone or in the presence of Cu^{2+} (2 μM) in potassium phosphate buffer (50 mM, pH 7.4) at 37° for 4 hr. The formation of H_2O_2 (absorbance at 560 nm) in both cases was then determined by the FOX assay. Catalase (final concentration; 50 Sigma units/mL) was also added to samples of incubated AG prior to their measurement by the FOX assay in order to confirm that authentic H_2O_2 was being formed by aminoguanidine. Heat-inactivated catalase produced no reduction in signal (not shown). A positive control of 2.5 μM H_2O_2 measured by the FOX assay is also shown for comparative purposes.

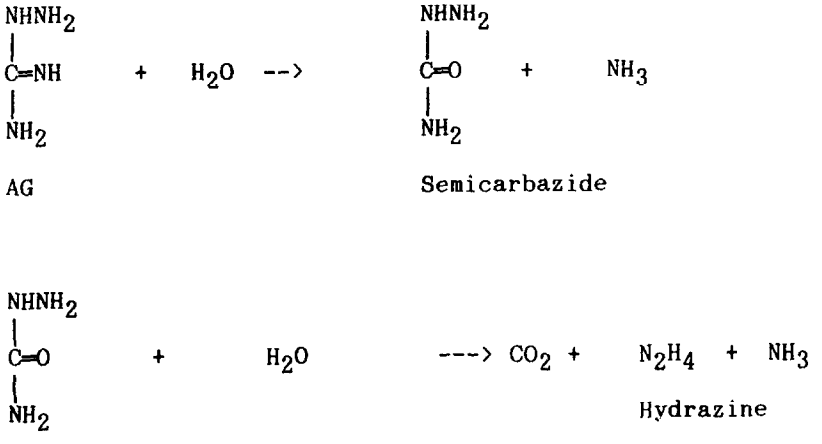
AG is believed to block AGE formation we had previously shown that AGE formation could generally be inhibited by strategies which lowered H_2O_2 formation during glycation [21]. We had thus wrongly postulated that AG would also lower H_2O_2 formation from glucose.

H_2O_2 *formation by AG*

We subsequently observed (Fig. 1) that AG incubated in chelex-treated potassium phosphate buffer slowly accumulated H_2O_2 in a reaction which was catalysed by the addition of copper or iron ions (Figs 2 and 3) and was inhibited (greater than 50%) by the transition metal chelator diethylene-triaminepenta-acetic acid. H_2O_2 production was approximately curvilinear when catalysed by copper ions (Fig. 2) but reached a plateau in the case of iron ions (Fig. 3), probably due to time-dependent insolubilization of catalytic iron. The 560 nm absorbance signal generated by AG in the FOX1 assay was confirmed as H_2O_2 by pre-treatment of the incubated AG solutions with catalase, which abolished colour development (Fig. 4).

Route to H_2O_2 *from AG*

AG decays to semicarbazide via hydrolysis and subsequently to hydrazine [22] (see scheme), which as reducing agents, might be expected to generate H_2O_2 in aqueous solution. Indeed, hydrazine appeared to generate H_2O_2 at a greater rate than AG. After an incubation period of 2 hr, 50 μM hydrazine in the presence of 10 μM Cu^{2+} produced 36 μM H_2O_2 when incubated in 50 mM phosphate buffer at pH 7.4 at 37°. AG (10 mM) generated approximately 19 μM H_2O_2 under the same conditions. The rate of H_2O_2 production from semicarbazide in the absence of added metal was similar to the rate of H_2O_2 production from AG (not shown).



Scheme

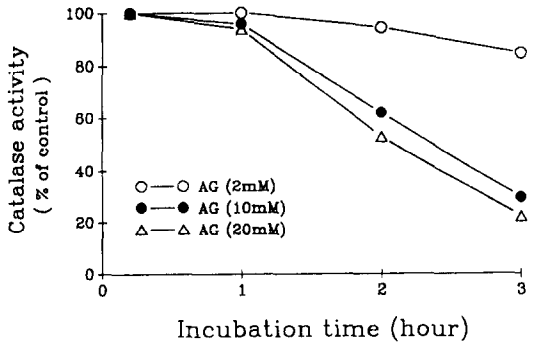


Fig. 5. Time course of inhibition of catalase activity by AG. Catalase (40 units/mL) was incubated with AG (2, 10 and 20 mM) in potassium phosphate buffer (50 mM, pH 7.4) at 37° for 3 hr. At varying time intervals, samples were withdrawn and diluted 8-fold into phosphate buffer (10 mM; pH 7.4) containing 200 μM H_2O_2. After incubation for 10 min at room temperature, residual H_2O_2 was determined by addition of 100-μL samples to 900 μL of FOX reagent, which also stopped H_2O_2 consumption by catalase. Catalase incubated without AG was taken as 100% activity. Data shown are the means ± SD of triplicate measurements.

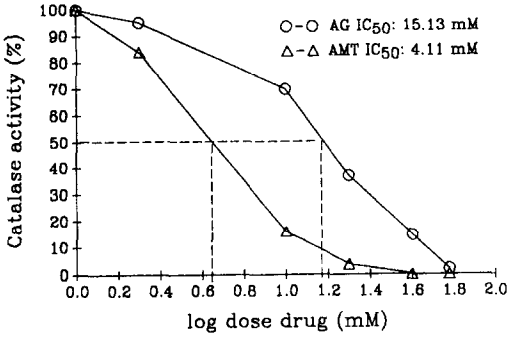


Fig. 6. Dose–response inhibition curves of AMT and AG versus catalase. AG and AMT at varying concentrations were incubated with catalase (40 units/mL) at 37° for 2 hr. Residual catalase activity was measured as described in Fig. 5.

Formation of hydrazine and semicarbazide by AG hydrolysis coupled with H_2O_2 production by these hydrolysis products makes it difficult to determine whether hydrazine, semicarbazide or their precursor, is immediately responsible for the presence of H_2O_2 in incubated solutions of AG. 2-Hydroxy-1-naphthaldehyde-mediated determination of hydrazine in fresh and incubated solutions of AG showed, however, that hydrazine was present at a steady-state level of less than 0.02% of the AG concentration. Thus a 10 mM solution of AG contained an upper limit of 5 μM hydrazine (not shown). This quantity of hydrazine would contribute less than 20% of total H_2O_2 derived from AG. It can thus be tentatively concluded that AG forms H_2O_2 directly in addition to producing the oxidant via its hydrolysis products.

Inhibition of catalase by AG and aminotriazole

We also observed that AG inhibited catalase activity in a time- (Fig. 5) and dose-dependent manner (Fig. 6). Under the conditions used, the IC_{50} for AG was found to be 15.33 mM compared with 4.11 mM for AMT, used here as a reference catalase inhibitor. The IC_{50} for semicarbazide was considerably higher (in excess of 65 mM) under these conditions. Hydrazine was found not to inhibit catalase. Since hydrazine failed to inhibit catalase and semicarbazide was an inefficient inhibitor, it can reasonably be concluded that the effects of AG on catalase are unlikely to be caused by solution contamination by its hydrolysis products.

Inhibition of catalase by AG in human erythrocytes and rat liver

The irreversible inhibition of catalase by AMT is known to require the continuous and simultaneous presence of H_2O_2 [23, 24]. In the absence of a continuous supply of H_2O_2, the inhibition of catalase by AMT is of a largely ionic type, which is reversible [23, 24]. The inhibition of catalase by AMT shown in Figs 5 and 6 is of this reversible type. Since both AG and its hydrolysis products generate H_2O_2, it is difficult to determine whether the inhibition of catalase by AG is of the reversible or irreversible type under these conditions. The mode of inhibition was thus investigated by examining the inhibition of catalase in liver slices as well as in erythrocytes

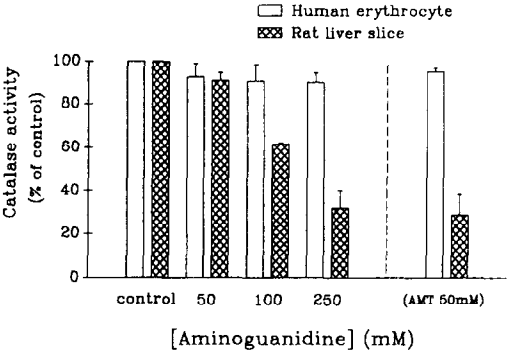


Fig. 7. AG inhibits catalase in liver slices but not in erythrocytes in the absence of an H_2O_2-generating system. (1) Well-washed human erythrocytes were suspended in phosphate-buffered saline (at 5% v/v) containing varying concentrations of AG or 50 mM AMT in a final volume of 10 mL. After incubation with constant shaking at 37° for 2 hr, 50-μL aliquots were removed and added to 950 μL of phosphate buffer (10 mM, pH 7.4) containing 200 μM H_2O_2. The lysates were further incubated for 10 min at room temperature. Aliquots (100 μL) were then added to 900 μL FOX reagent, incubated at room temperature for 30 min, centrifuged at 12,000 g × 5 min before reading absorbance of the supernatant at 560 nm. (2) Liver slices prepared as described in Materials and Methods were homogenized in PBS after incubation with varying concentrations of AG or 50 mM AMT at 37° for 2 hr. Diluted homogenate (200 μg wet weight liver/mL) was then incubated with 200 μM H_2O_2 in phosphate buffer (10 mM, pH 7.4) for 10 min prior to determination of residual H_2O_2. In both cases, data were obtained from duplicate incubations.

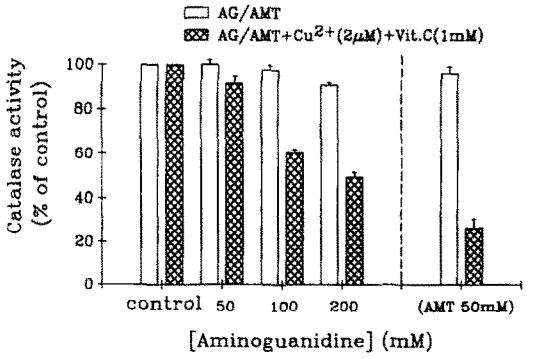


Fig. 8. AG inhibits erythrocyte catalase in the presence of Cu^{2+}/ascorbate. Human erythrocytes were suspended (5% v/v) in PBS in the presence and absence of a H_2O_2-generating system composed of Cu^{2+} (2 μM) and ascorbate (1 mM) and varying concentrations of AG and 50 mM AMT. After incubation at 37° for 2 hr with shaking, erythrocytes were lysed by the addition of a 50-μL sample to 950 μL phosphate buffer (5 mM, pH 7.4) containing 200 μM H_2O_2 and incubated for 10 min prior to determination of residual H_2O_2. The steady-state level of H_2O_2 in the copper/ascorbate incubations was 81.5 ± 3.5 μM.

challenged, and unchallenged, by a high flux of H_2O_2 produced by a combination of Cu^{2+} and ascorbate and by comparing with the effects of AMT under these conditions.

Figure 7 shows the relative effects of varying concentrations of AG, and one concentration of AMT (50 mM) on rat liver slices and human erythrocytes in the absence of other additions. Both AG and AMT inhibited catalase in rat liver slices, which generate endogenous H_2O_2. AMT was approximately five times as efficient an inhibitor as AG as also seen for inhibition of pure catalase in the experiments outlined above. By contrast, neither compound inhibited human erythrocyte catalase at the concentrations and under the conditions employed in the absence of the H_2O_2-generating system (Fig. 7). In the presence of Cu^{2+} (2 μM) and ascorbate (1 mM), however, human erythrocyte catalase was inhibited by both AG and AMT (Fig. 8). These results with liver slices and with erythrocytes exposed to a steady flux of H_2O_2 suggest that AG shares the characteristic of AMT of being capable of inactivating catalase irreversibly in a manner which is dependent upon the continued supply of H_2O_2. AG, like AMT, can thus act as a "suicide substrate" for catalase.

DISCUSSION

AG is shown to generate H_2O_2 at a low rate *in vitro* directly, or via its hydrolysis products semicarbazide and hydrazine. This may be of relevance to model studies of AGE formation in which proteins are incubated with glucose, in the presence of AG, for extended periods *in vitro*. H_2O_2 produced by AG oxidation could conceivably be involved in chromophore bleaching. Semicarbazide can be postulated to react with protein amino groups via its free carbonyl group thus competitively inhibiting glycation.

AG also appears to inhibit catalase in a manner reminiscent of AMT. This inhibition, like AMT, is probably reversible in the absence of a constant source of H_2O_2 and irreversible in its presence. It should be noted that the concentrations of AG required to inhibit catalase here *in vitro* are considerably higher than those which are expected to obtain *in vivo* (approximately 250 μM in an animal treated i.p. with 25 mg/kg AG). Inhibition of catalase by AG, like AMT, seems, however, to be both time-dependent and dependent upon endogenous H_2O_2 production. Extent of inhibition *in vivo* would be dependent upon rates of endogenous H_2O_2 production, rates of catalase turnover and tissue levels of AG. Small chronic doses of AG could thus have a considerable impact upon catalase levels. By way of comparison, a single, 100 mg/kg dose of AMT (giving approximately 1 mM in plasma) leads to a 50% decrease in renal catalase in the normal rat within 1 hr [25].

Our results suggest that chronic administration of AG may be associated with toxicity reminiscent of acatalasaemia, or exposure to AMT. If the effects are solely reminiscent of hereditary acatalasaemia then few adverse effects would be expected, with the possible exception of oral ulceration [26]. If, however, AG acts like AMT then more severe adverse effects might be anticipated. Thus, although the acute toxicity of AMT in rodents is very low (oral $LD_{50} = 14.7$ g/kg), much smaller degrees of chronic AMT exposure (5 mg/kg i.p. giving 50 μM in plasma) result in inhibition of iodine uptake by the thyroid, abnormal thyroid growth and thyroid tumours in animals [27]. Effects relating to inhibition of catalase need to be investigated as part of the formal chronic toxicity testing of AG as a pharmacological entity.

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