Inhibition of glyoxylate conversion to oxalate in cultured human cells by the carbonyl-scavenging drug, aminoguanidine

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Calcium oxalate is the most frequent cause of kidney stones, and is responsible for the damage to kidneys and other organs observed in inherited disorders of oxalate metabolism. Most oxalate produced in the body is derived from its metabolic precursor, glyoxylate. Thus, any means of scavenging glyoxylate to a non-toxic product, thereby diverting it away from oxalate synthesis, has considerable therapeutic implications. Here we show that aminoguanidine, a compound with a proven safety record and used for many years to prevent long-term complications of diabetes, binds glyoxylate covalently and reduces its conversion to oxalate by human liver- and lymphocyte-derived cell lines by >90%. We propose that scavenging glyoxylate with aminoguanidine or its congeners may provide a means of reducing oxalate production *in vivo*, and advocate the tissue culture system described here as a convenient means for testing such agents *in vitro*. A serendipitous finding to emerge from our study was the abiotic and strongly pH-dependent formation of oxalate from ascorbate, which has implications for the contribution of ascorbate to urine oxalate excretion.

Introduction

For some forms of life, oxalate is an essential metabolite. For others, including humans, it is a toxic, non-metabolizable waste product eliminated by the kidney.[1] Toxicity arises from its tendency to form extremely insoluble and virtually indestructible calcium oxalate crystals, which damage cells directly and obstruct the lumen of renal tubules. While some urine oxalate in healthy individuals is derived from intestinal absorption,[2] most is formed endogenously from its chemical precursor, glyoxylate, by the action of the peroxisomal enzyme, glycolate oxidase (GO). The primary role of this enzyme is to oxidize glycolate; its ability to further oxidize glyoxylate to oxalate is a regrettable consequence of its broad substrate specificity. Glyoxylate escaping from peroxisomes into cytosol can also be oxidized to oxalate by the ubiquitous cytosolic enzyme, lactate dehydrogenase (LD).[1]

The major determinant of oxalate production is thus the steady-state level of glyoxylate, which in turn depends on its relative rates of formation and consumption. It is formed from oxidative deamination of glycine catalysed by D-amino acid oxidase or from glycolate by glycolate oxidase, while its removal is effected by transamination back to glycine by alanine glyoxalate aminotransferase (AGT), or egress from the peroxisome and reduction to glycolate by glyoxylate reductase (GR). A defect in the catalytic activity or appropriate subcellular localization of either of these two latter enzymes inevitably leads to an accumulation of glyoxylate, and its conversion to oxalate. Clinical disorders due to defects in either enzyme have been well-characterized, and are referred to as primary hyperoxaluria (PH) types I and II, respectively.[1] The metabolic pathways involved in oxalate production are illustrated in Fig. 1.

Of the two disorders, type I (AGT deficiency) is the more common and clinically severe. It is characterized by progressive renal damage due to deposition of insoluble calcium oxalate within the kidney substance and as kidney stones. As renal function deteriorates, the kidney is no longer able to excrete oxalate, and it deposits throughout the body, a condition termed *systemic oxalosis*; typical sites include bone, heart and blood vessel walls. Figure 2 shows a bone biopsy from a 9-year-old child with primary hyperoxaluria type I. When stained with haematoxylin & eosin (A), calcium oxalate crystals appear as greyish masses with radiating spicules. Multinucleated foreign body giant cells can be seen closely apposed to the crystals, in a (vain) attempt to digest and remove them. When viewed under polarizing light (B), birefringent calcium oxalate crystals stand out as blue and yellow against the red background of normal tissue. The latter method of examination is more sensitive insofar as it clearly shows multiple small crystals not readily apparent by conventional staining. Over 80% of PH type I patients die by the age of 20 years. The only currently effective treatment for this devastating disorder is combined kidney and liver transplantation, the latter being necessary because AGT expression is confined to that organ.

PH type II, due to a defect in the cytosolic enzyme glyoxylate reductase (GR), runs a more benign clinical course, with symptoms usually confined to recurrent kidney stones. Idiopathic calcium oxalate stone disease is another disorder, in which urinary excretion of oxalate is excessive and leads to kidney stone formation.[3,4] Although far commoner than the above two monogenic disorders, the contribution of the genetic and environmental factors implicated in its causation are yet to be ascertained.[5] Acute kidney damage from calcium oxalate deposition can also occur after ethylene glycol ingestion.[6] This compound is the active ingredient of anti-freeze and brake fluid, and is sometimes added illegally to alcoholic beverages. It is oxidized by hepatic alcohol and aldehyde dehydrogenase to glycolate, which undergoes further oxidation to oxalate,[7] as outlined in Fig. 1. Since in all

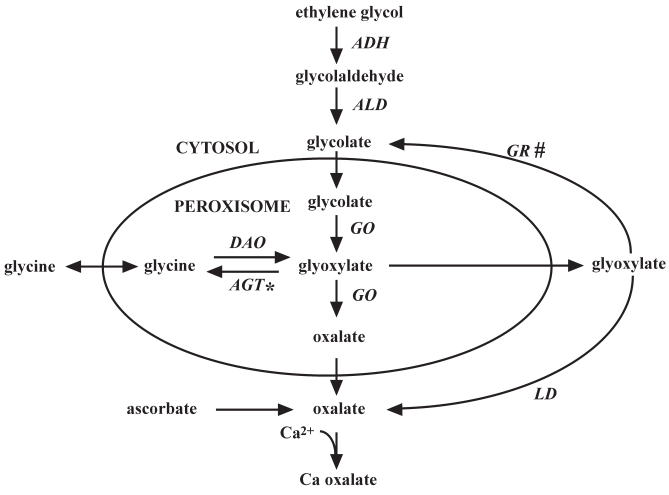

Fig. 1. Metabolic pathways involved in oxalate formation. *DAO* = D-amino acid oxidase; *AGT* = alanine glyoxylate transaminase; *GO* = glycolate oxidase; *GR* = glyoxylate reductase; *LD* = lactate dehydrogenase; *ADH* = alcohol dehydrogenase; *ALD* = aldehyde dehydrogenase; * and # indicate the steps defective in primary hyperoxaluria types I and II, respectively.

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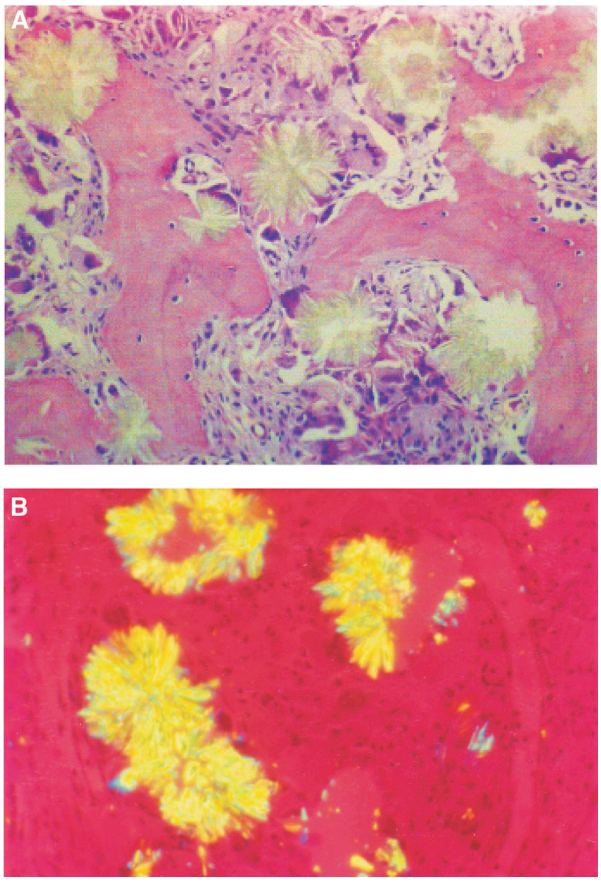


Fig. 2. Histological appearance of oxalate crystals. A bone biopsy from a 9-year-old child with primary hyperoxaluria type I, was stained with haematoxylin & eosin (**A**), or viewed under polarizing light (**B**). When stained with H&E, calcium oxalate crystals appear as discrete spiculated greyish masses with tightly apposed multinucleated foreign body giant cells clustered on their surface, attempting to digest them. Under polarizing light, bone matrix and marrow appears red, whereas birefringent calcium oxalate crystals appear blue and yellow. Magnification ×200.

conditions listed above oxalate is directly derived from glyoxylate, it follows that reduction of the glyoxylate pool by chemical means *in vivo* should decrease endogenous oxalate production and ameliorate its attendant tissue damage. There is experimental evidence that glyoxylate is highly toxic in its own right.[8]

Aminoguanidine has been used for several years to prevent the visual, renal and microvascular degeneration associated with diabetes.[9–11] Its side effects are minor, being confined to nausea, headache and impaired pyridoxine absorption; the last is readily amenable to dietary supplementation with this vitamin. It is believed to act by trapping glucose-derived dicarbonyl compounds such as 3-deoxyglucosone that cross-link long-lived structural proteins, including lens crystallin, collagen and elastin.[12,13] Encouraged by its proven safety record, which would facilitate its use for a novel clinical application, we explored the ability of aminoguanidine to react with the carbonyl group of glyoxylate. We present evidence for covalent binding of aminoguanidine to glyoxylate, and demonstrate the ability of aminoguanidine at low millimolar concentrations to profoundly inhibit conversion of glyoxalate to oxalate by cultured human cells *in vitro*.

Methods

Cell culture

HepG2 cells were grown as adherent monolayers in Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal calf serum at 37°C in the presence of 10% CO_2. For testing their ability to form oxalate, cells were trypsinized, transferred to 3.5-cm wells in multi-well plates and incubated as described above. Glyoxylate and/or aminoguanidine was added from sterile concentrated stock solutions to give the desired final concentration. Lymphoblasts were derived by Epstein-Barr virus transformation of peripheral lymphocytes obtained from donors not known to suffer from disorders of oxalate metabolism. They were grown as a suspension under the same conditions as HepG2 cells.

[^{3}H]thymidine uptake

HepG2 cells were incubated for 4 h at 37°C in 1 ml Hank's balanced salt solution (HBSS) containing 1 μCi [methyl-^{3}H]thymidine, 87 Ci/mmol. Cells were then washed 3 times with 1 ml 5% trichloracetic acid, DNA was solubilized in 0.5 ml 0.1 M NaOH, and the radioactivity incorporated was measured in a β-scintillation counter.

Oxalate assay

For most of the oxalate measurements, we used a method developed in-house that is based on precipitation of added radio-labelled calcium. Briefly, 10 μl of a 4 μCi/ml solution of [^{45}Ca]Cl_2 (0.1–1.0 mCi/mmol, Amersham Biosciences) was added to 1 ml of medium, followed by 10 μl of a 0.2 M calcium oxalate (CaOx) suspension to saturate the medium with respect to CaOx and thereby facilitate precipitation of [^{45}Ca]Ox. After 16 h at 4°C, tubes were centrifuged at 7000 × *g* for 10 min, and the supernatant carefully aspirated and discarded. The CaOx pellet was dissolved in 0.1 ml of 10 M HCl, transferred to a counting vial and pooled with 2 × 0.1 ml water washes to ensure quantitative transfer. Radioactivity was measured in a β-scintillation counter, and compared with that obtained for an oxalate standard curve, generated by adding known amounts of oxalate to 1 ml aliquots of fresh medium to give a final concentration ranging from 0 to 0.6 mM. Concerns that exchange of unlabelled calcium in the CaOx suspension with ^{45}Ca would lead to high background counts were dispelled by low blank counts routinely obtained in standard curves (<5% of the counts for the highest oxalate standard), indicating a negligibly slow rate of exchange.

We also used a commercially available oxalate oxidase-based kit designed for measurement of oxalate in urine (Sigma Diagnostics). Briefly, 50 μl aliquots of media or urine were mixed with an equal volume of 10 mM EDTA to free oxalate from complexes with calcium. A spatula tip of activated charcoal (~10 mg) was added, mixed for 5 min, then centrifuged to remove potential inhibitors of subsequent enzymatic steps; 2 μl supernatant was mixed with 10 μl of a combined reagent containing oxalate oxidase (300 U/l), peroxidase (10 000 U/l), 3-(dimethylamino)benzoate (DMAB) (3 mM) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (0.2 mM). Oxalate present was oxidized by oxalate oxidase to CO_2 and hydrogen peroxide, which then oxidized DMAB and MBTH in the presence of peroxidase to yield a coloured indamine dye. Absorbance at 595 nm was measured on a Nanodrop® spectrophotometer, and compared to a similarly treated set of aqueous oxalate standards of 0, 0.2, 0.4 and 0.6 mM. The Nanodrop® was capable of reading absorbance of samples as small as 1 μl, which made the use of the costly Sigma kit reagents economically feasible.

Spectrophotometric assay for residual glyoxylate

Glyoxylate (1 mM) was incubated with aminoguanidine at concentrations varying from 0 to 20 mM in 50 mM phosphate buffer (pH 7.4). After 30 min at 37°C, 40 μl phenylhydrazine (0.5 M) was added, and 30 s later a 40 μl aliquot of reaction mixture was diluted to 1.2 ml with phosphate buffer and the optical density read at 324 nm ($A_{324\ nm}$) against a reagent blank consisting of phosphate buffer alone subjected to the identical procedure.

Results

We initially studied the interaction of pure solutions of glyoxylate and aminoguanidine *in vitro*. Figure 3 shows the effect of aminoguanidine on free glyoxylate concentration. Glyoxylate (1 mM) was incubated with varying concentrations of aminoguanidine for 30 min at 37°C, then residual glyoxalate determined spectrophotometrically by its reaction with phenylhydrazine. Results are expressed as % of absorbance recorded in the absence of aminoguanidine. Failure of $A_{324\ nm}$ to fall to zero

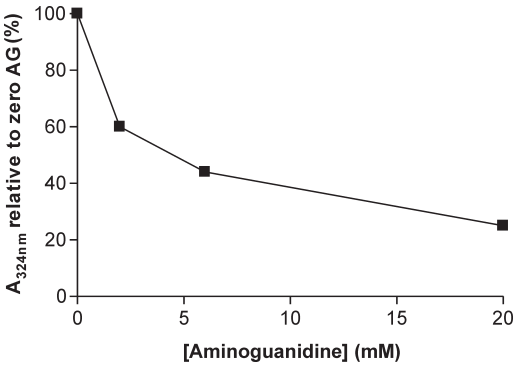


Fig. 3. Decrease in free glyoxylate concentration by aminoguanidine. Glyoxylate (1 mM) was incubated with varying concentrations of aminoguanidine from 0 to 20 mM for 30 min at 37°C, then residual glyoxylate was measured as its phenylhydrazone adduct by absorption at 324 nm.

with increasing aminoguanidine we attribute to reversibility of the glyoxylate–aminoguanidine reaction, in that the phenylhydrazine indicator reagent, present in vast molar excess, competed with aminoguanidine for glyoxylate binding. This method therefore underestimated the extent of binding.

The reaction between glyoxylate and aminoguanidine resulted in characteristic changes in the UV absorption spectrum. Figure 4 shows a time course for the development of an absorption peak at 240 nm observed upon mixing glyoxylate with aminoguanidine, both at an initial concentration of 0.5 mM.

To characterize the product(s) formed between glyoxylate and aminoguanidine further, the two compounds, both at 2 mM, were incubated overnight at 37°C, then analysed by positive mode electrospray mass spectroscopy (ESMS). A 131-Da product, consistent with a Schiff base adduct between glyoxylate (74 Da) and aminoguanidine (75 Da) (74 + 75 – 18 = 131) was the principal species observed (Fig. 5A). An aliquot of reaction mixture was analysed by strong cation-exchange HPLC on a PolyLC polysulphoethyl A column, and UV-absorbing material eluting as a single major peak (results not shown) was analysed by ESMS. A small 131-Da peak was observed, alongside a number of

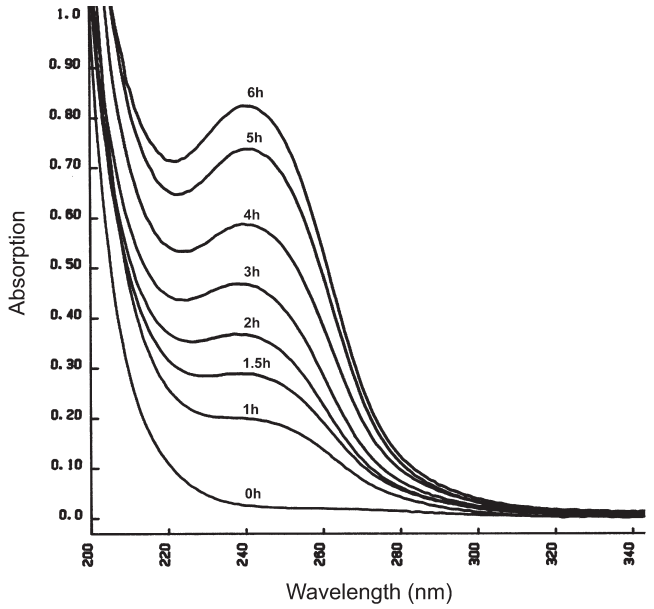


Fig. 4. UV absorption spectrum of the glyoxylate–aminoguanidine complex. Glyoxylate and aminoguanidine were mixed and the UV-absorption spectrum monitored over the next 6 h. Initial concentration of both reactants was 0.5 mM.

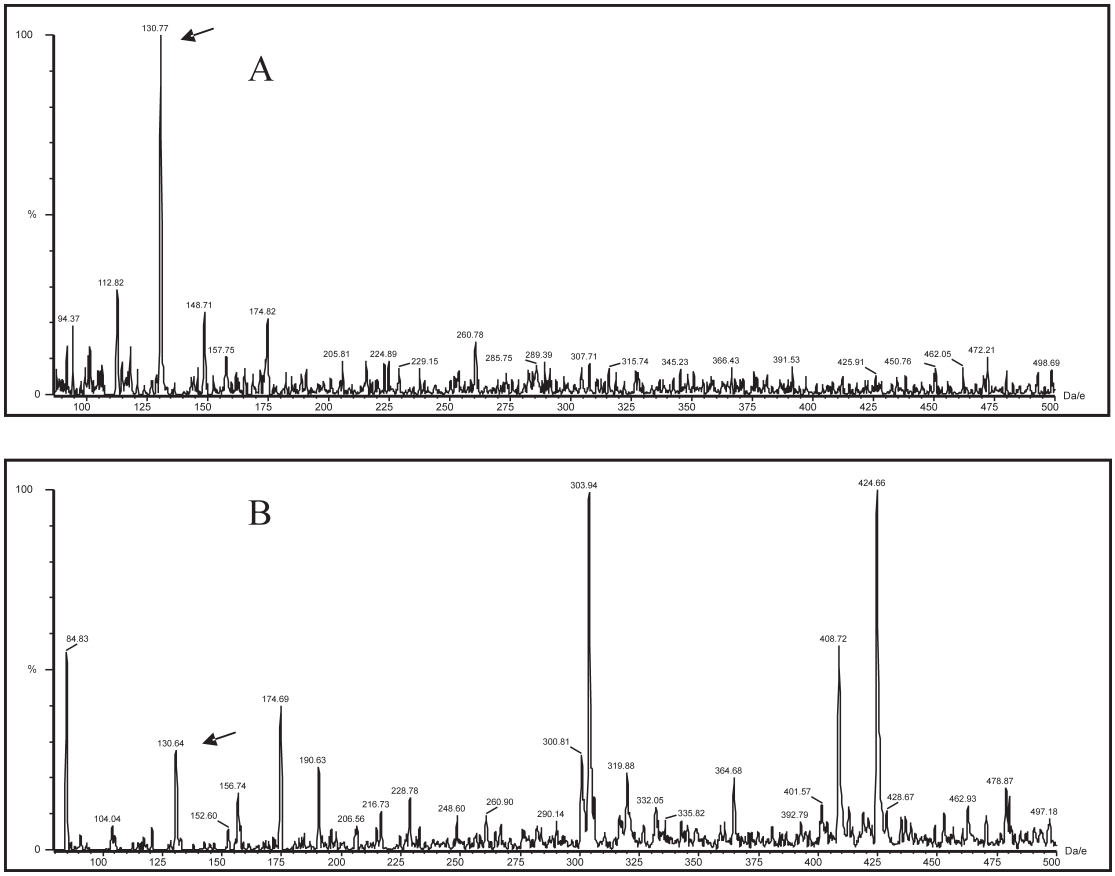


Fig. 5. Characterization of the complex formed between glyoxylate and aminoguanidine by mass spectroscopy. Glyoxylate and aminoguanidine, both at 2 mM, were incubated together for 16 h at 37°C, then analysed directly by electrospray mass spectroscopy, either directly (**A**), or after purification of UV-absorbing material by cation exchange HPLC (**B**). The arrow indicates the 131-Da fragment expected for a Schiff-base adduct between glyoxylate and aminoguanidine.

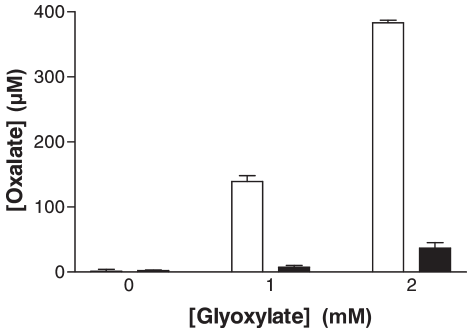


Fig. 6. Glyoxylate conversion to oxalate by HepG2 cells, and its inhibition by aminoguanidine. HepG2 cells were incubated for 48 h with glyoxylate at the concentrations indicated, either in the absence (open bars) or presence (filled bars) of 2 mM aminoguanidine, then oxalate concentration of the medium was measured. Bar height represents the mean of triplicate assays; error bars denote the range.

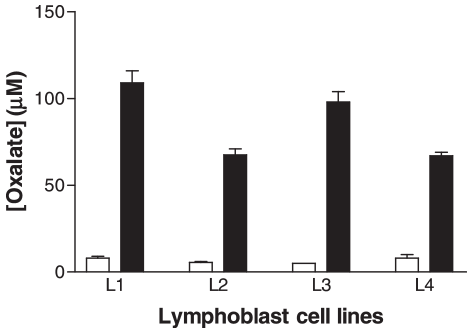


Fig. 8. Oxalate production from glyoxylate by transformed lymphoblasts. Epstein-Barr transformed lymphoblasts prepared from four different human donors (L1–L4) were incubated for 48 h without (closed bars) or with (open bars) 2 mM glyoxylate, then the medium was assayed for oxalate. Bars represent the mean of duplicate measurements, error bars the range.

larger molecular weight species of undetermined structure, presumably representing polymerization products of the initial Schiff base adduct (Fig. 5B). Our interpretation of these data is that glyoxylate and aminoguanidine initially reacted to form a Schiff base, which subsequently polymerized to yield the UV-absorbing material observed spectroscopically.

We next examined whether cultured human cells were able to convert exogenous glyoxylate to oxalate and, if so, whether aminoguanidine inhibited this conversion by sequestering glyoxylate. We initially chose HepG2 cells, since these are derived from liver, the organ primarily responsible for oxalate production *in vivo*. Confluent monolayers of HepG2 cells were incubated in medium containing glyoxylate from 0 to 2 mM, either in the absence or presence of 2 mM aminoguanidine, then the oxalate concentration in the medium was measured as described under Methods. HepG2 cells were indeed able to convert glyoxylate to oxalate; by 48 h, 15–20% of glyoxylate initially added to control cells had been converted to oxalate. Inclusion of aminoguanidine reduced oxalate production by >90% (Fig. 6). In a parallel experiment, uptake of [^{3}H]thymidine by HepG2 cells was determined as a measure of cell viability. Uptake decreased progressively in cells exposed to increasing concentrations of glyoxylate; this decrease was abrogated by inclusion of aminoguanidine (Fig. 7). These results are consistent with the notion that aminoguanidine sequesters glyoxylate, thereby preventing both its conversion to oxalate and any adverse effect it may exert on cell viability. Whether glyoxylate induces cell toxicity directly, as has been proposed,[8] or through its conversion to oxalate, remains to be ascertained.

To check whether ability to convert glyoxylate to oxalate was confined to liver-derived cell lines, we performed comparable

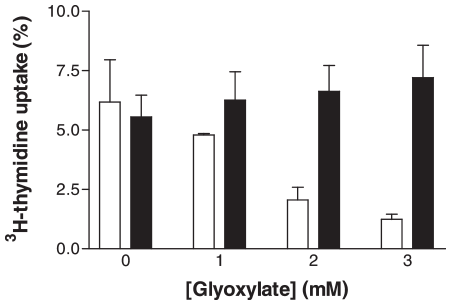
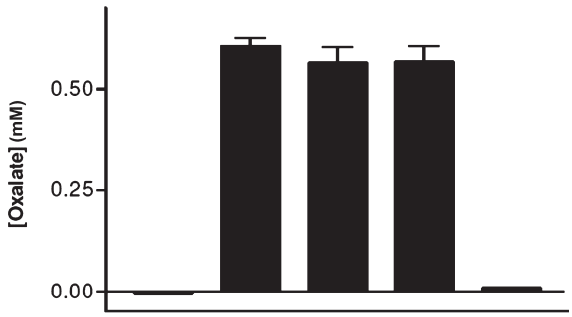


Fig. 7. Glyoxylate toxicity to HepG2 cells reversed by aminoguanidine. HepG2 cells were incubated for 48 h with glyoxylate at the concentrations indicated, either in the absence (open bars) or presence (filled bars) of 3 mM aminoguanidine. Medium was replaced with fresh medium containing [^{3}H]thymidine, then incubated for a further 4 h. Incorporation of [^{3}H]thymidine into acid-insoluble material (DNA) was determined and expressed as a percentage of the [^{3}H]thymidine added. Bars represent the mean of triplicate measurements, error bars the range.

experiments on lymphoblasts transformed by Epstein-Barr virus. As shown in Fig. 8, four independent lymphoblast cell lines were able to convert approximately 5% of the glyoxylate initially present to oxalate in 48 h. Inclusion of an equimolar amount of aminoguanidine (2 mM) reduced oxalate production by 90%, similar to our findings with HepG2 cells (results not shown).

Encouraged by these *in vitro* results, we undertook a pilot experiment to ascertain whether aminoguanidine affected oxalate excretion in healthy human subjects. One of us (P.B.) ingested aminoguanidine, 100 mg 3-hourly for 24 h, and compared urinary oxalate excretion during that period with the 24 h immediately before and after. No untoward side effects were experienced, but, rather disappointingly, neither was any significant change in oxalate excretion observed (results not shown).

We next tested whether HepG2 cells were able to produce oxalate from substrates other than glyoxylate. Neither glycine, glycolate nor ethylene glycol, tested at concentrations up to 50 mM, served as oxalate precursors (results not shown). Ascorbate, however, was converted to oxalate even more efficiently than was glyoxylate (>20% conversion within 24 h). Rather unexpectedly, oxalate production did not require the presence of cells, nor was it affected by aminoguanidine, iron chelation, or exclusion of foetal calf serum from the medium (Fig. 9), indicating that it was non-enzymatic, non-iron-dependent and did not proceed via a reactive aldehyde intermediate such as glyoxylate.



Incubation time (h)	24	24	24	24	0
[Ascorbate] (mM)	0	2	2	2	2
[Desferrioxamine] (mM)	0	0	1	0	0
[Aminoguanidine] (mM)	0	0	0	5	0

Fig. 9. Ascorbate to oxalate conversion unaffected by iron chelation, aminoguanidine, or serum. DMEM, devoid of cells or foetal calf serum, was supplemented with ascorbate, desferrioxamine and/or aminoguanidine as detailed above, and incubated at 37°C in the absence of cells for the times indicated. Oxalate concentration was then measured. Samples were processed in duplicate; bar height reflects the mean, error bars the range.

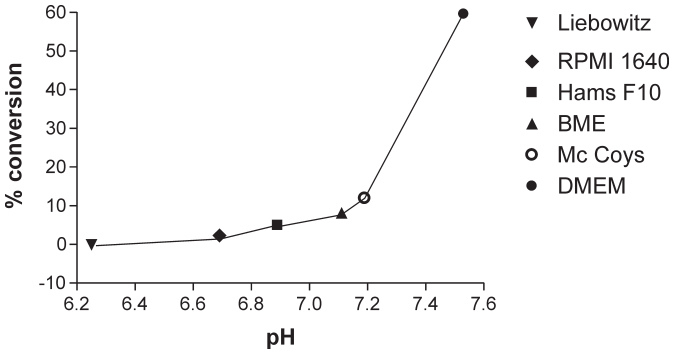


Fig. 10. Dependence of conversion of ascorbate to oxalate on nature of medium. Six commercially available tissue culture media were supplemented with 2 mM ascorbate and incubated for 24 h at 37°C in an atmosphere of 10% CO_2. Oxalate concentration and pH of the media were measured at the end of 24 h by the in-house method, and % ascorbate converted to oxalate plotted as a function of pH.

No oxalate was detected when ascorbate-supplemented DMEM was analysed immediately, excluding the possibility of oxalate contamination of the ascorbate used (Fig. 9). Oxalate formation was, however, critically dependent on the nature of the tissue culture medium. It proceeded rapidly in DMEM, but not at all in HBSS. In a search for the crucial abiotic factor promoting non-enzymatic conversion of ascorbate to oxalate, we tested six commonly used tissue culture media, and found that the only component of media that correlated with conversion efficiency was [HCO_3^-]. Figure 10 shows the exponential relationship between conversion efficiency and operating pH of the medium when equilibrated with 10% CO_2.

To confirm that enhanced conversion of ascorbate to oxalate in DMEM could be ascribed to its high operating pH, we examined conversion efficiency in three different media (DMEM, RPMI and Hams-F10) at pH values ranging from 6 to 8. For this experiment, the commercial oxalate oxidase method was used for measuring oxalate. As is apparent from Fig. 11, there was a marked and equivalent pH dependence of oxalate formation in all three media, with approximately 10-fold greater oxalate formation at pH 8 than at pH 6. Interestingly, negligible oxalate accumulated when water was substituted for medium, pointing to a requirement for some unknown component present in all three media. This component must also be present in urine, since urine exhibited the same pH dependence of oxalate formation. The appreciable amount of oxalate measured in urine incubated at pH 6, compared with the culture media, we attribute to oxalate present in urine *ab initio*, rather than that formed during incubation. This experiment also indirectly served to validate the in-house oxalate method, insofar as it yielded the same pH dependence and comparable absolute values for oxalate formation from ascorbate as did the experiment depicted in Fig. 10, when the in-house method was employed.

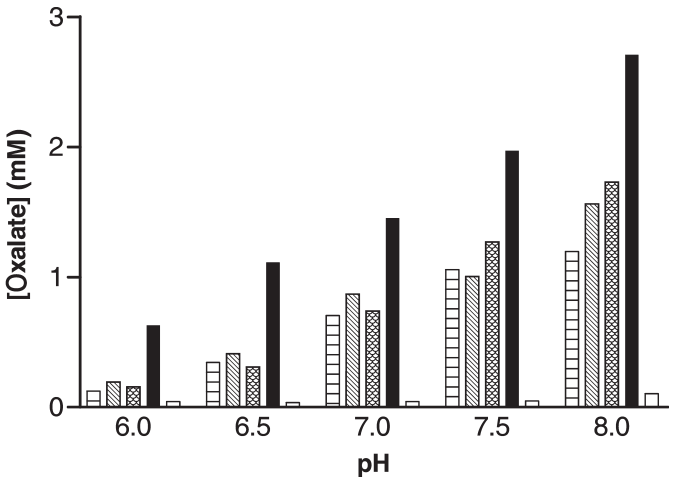


Fig. 11. pH-dependence of conversion of ascorbate to oxalate in different tissue culture media and in urine. Three different media, water, or urine were supplemented with 5 mM ascorbate and 25 mM HEPES, and pH adjusted by addition of NaOH. After incubation for 24 h at 37°C, oxalate was measured by the commercial oxalate oxidase method. DMEM is depicted by horizontal striped bars, RPMI by hatched bars, Hams-F10 by checked bars, urine by solid bars, and water by open bars.

Discussion

Various treatment modalities have been tried in primary hyperoxaluria in an attempt to minimize calcium oxalate deposition in the urinary tract. Reducing dietary intake by excluding oxalate-rich foods such as spinach and rhubarb has limited value, since over 50% of urine oxalate is derived from endogenous production rather than from intestinal absorption.[2] Attempts to reduce urinary calcium by dietary calcium restriction are counterproductive, as dietary calcium binds oxalate in the bowel lumen, thereby preventing its absorption.[2,14] Reducing the whole body oxalate pool with oxalate degrading enzymes, such as oxalate oxidase or oxalate decarboxylase entrapped in dialysis membranes or red cell ghosts, has been tried, but remains largely experimental.[15]

The recognition that metabolically generated glyoxylate is the obligate substrate for endogenous oxalate production has led to a number of novel approaches to treatment. These include decreasing glyoxylate synthesis, inhibiting its conversion to oxalate, or diverting it along alternative chemical pathways. A number of glycolate oxidase inhibitors, including tris-hydroxymethyl-aminomethane (TRIS), reduce glyoxylate formation from glycolate, and carry the additional advantage of simultaneously inhibiting glyoxylate conversion to oxalate.[1] Glycolate formation from glycolaldehyde can be reduced by the aldehyde dehydrogenase inhibitor, disulfiram, an agent used for alcohol aversion therapy. Blocking glyoxylate formation from its other major precursor, glycine, has been attempted by a variety of means, including dietary glycine restriction, benzoate administration, or use of D-amino acids as inhibitors of D-amino acid oxidase.[1]

Diverting glyoxylate into alternative chemical pathways has been explored. Transamination to glycine can be enhanced by stimulating AGT with pharmacological doses of its vitamin co-factor, pyridoxine.[1] Pyridoxine supplementation has indeed led to improvement in certain cases of PH type I. Given that AGT is a peroxisomal enzyme, non-specific stimulation of peroxisomal proliferation with drugs such as fibrates has been attempted, but in humans unfortunately boosts mitochondrial rather than peroxisomal levels of AGT.[1] Non-enzymatic trapping of glyoxylate as a thiol adduct with cysteine[16] or penicillamine[17] to form a thiazolidine derivative has also been demonstrated *in vitro*. In our study, we showed that aminoguanidine in the low millimolar concentration range also trapped glyoxylate, thereby effectively reducing both its inherent toxicity and its conversion to oxalate by cells in culture. Glyoxylate toxicity has been demonstrated before.[8,18] Poldelski *et al.* showed a decline in cellular ATP and release of lactate dehydrogenase from isolated renal tubular cells exposed to glyoxylate, although concentrations of glyoxylate used in that study,[18] namely 13–65 mM, far exceed the 1–3 mM used here. Either HepG2 cells are more sensitive than renal tubular cells to glyoxylate toxicity, or thymidine incorporation is a more sensitive reflection of cellular well-being.

The question as to whether glyoxylate serves any useful function in a cell needs to be addressed. Glyoxylate is a key compo-

nent of the glyoxylate cycle, a modified form of the citric acid cycle whereby isocitrate is converted via glyoxylate to oxaloacetate. This cycle, which allows net carbohydrate synthesis from fat, was originally believed to be confined to microorganisms and germinating seeds of higher plants. More recently, however, convincing evidence has been presented of its existence in mammalian tissues; specifically, neonatal rat liver and starved adult rat liver, where it assists in maintaining blood glucose, as well as in rat epiphyseal cartilage after exposure to vitamin D.[19–21] Not surprisingly, enzymes of the cycle are confined to peroxisomes, where glyoxylate is formed. The high K_m of malate synthase for glyoxylate (3 mM)[20] is indirect evidence that glyoxylate may reach millimolar concentrations, at least within the peroxisome, although levels of glyoxylate in normal tissues and tissues from patients with primary hyperoxaluria and ethylene glycol intoxication have, to our knowledge, never been measured directly.

These encouraging *in vitro* findings need to be validated *in vivo*. Rather disappointingly, ingestion of 100 mg aminoguanidine 3 hourly over 24 h by one of us (P.A.B.) had little effect on oxalate excretion. This dose is substantially greater than the 300 mg/day ingested over 15 months as part of a large clinical trial conducted to assess the efficacy of aminoguanidine on retarding onset of diabetic nephropathy.[22] Plasma levels of aminoguanidine after the first few doses can be estimated by assuming a pseudo-steady state, where aminoguanidine entering the circulation from the gut equals that eliminated by the kidney. Rate of entry = 100/3 mg/h, whereas rate of elimination (assuming clearance by glomerular filtration and a glomerular filtration rate of 100 ml/min) = [A] × 0.1 × 60 = [A] × 6 mg/h, where [A] = plasma level of aminoguanidine in mg/l. Solving for [A] yields 5.5 mg/l, or 0.075 mM, which is appreciably lower than the concentration required, according to Fig. 3, to reduce glyoxylate substantially. Thus, higher plasma levels of aminoguanidine (or a more potent aminoguanidine analogue – see below) will be required to scavenge glyoxylate effectively *in vivo*. Another possible reason for failure of aminoguanidine to lower glyoxylate in healthy subjects might be its inability to access the peroxisome readily, where, under physiological conditions, glyoxylate oxidation to oxalate occurs. By contrast, in hyperoxaluric syndromes accumulated glyoxylate leaks from the peroxisome into the cytosol, where it is oxidized to oxalate by cytosolic lactate dehydrogenase,[23] and where it might be more accessible to scavenging by aminoguanidine.

In terms of the fate of the aminoguanidine–glyoxylate complex and its polymerization products, we predict that, by virtue of their water solubility, they will not accumulate or dissociate, but will rapidly be cleared by glomerular filtration, thereby depleting the whole body glyoxylate pool. Moreover, aminoguanidine might not be the most effective glyoxylate scavenging agent; a recent study indicated that the drug, hydralazine, scavenges acrolein (CH_2=CH-CHO), a toxic aldehyde formed during lipid peroxidation, more than three times faster than an equimolar concentration of aminoguanidine.[24] Preliminary results in our laboratory confirm its superior efficacy with respect to glyoxylate (results not shown).

While screening other potential substrates for oxalate production by HepG2 cells, we observed that ascorbate was readily converted to oxalate in certain media, and that conversion was, rather unexpectedly, not dependent on the presence of cells or serum. On testing media of diverse composition for ability to support oxalate generation from ascorbate, the only component that consistently correlated with conversion efficiency was bicarbonate, and thus medium pH. This finding may have a bearing on the contribution of dietary ascorbate to urinary oxalate excretion *in vivo*, or even *ex vivo* as an analytical artefact.[25] Although the literature abounds with conflicting reports regarding the importance of ascorbate as an oxalate precursor[26] *in vivo*, the impact of variations of pH within the physiological range on the non-enzymatic conversion of ascorbate to oxalate has, to our knowledge, not been explored. A recent study examined the non-enzymatic conversion of radiolabelled ascorbate to oxalate and L-threonate by *Rosa* cells, and identified several novel intermediates, including 4-O-oxalyl-L-threonate, but not glyoxylate, in the process.[7] That study implicated heat-stable but otherwise unidentified components of the medium as enhancers of oxalate formation, but did not explore the effect of pH. Our finding that conversion of ascorbate to oxalate is unaffected by aminoguanidine supports the contention that it proceeds via chemical intermediates not including glyoxylate, thus making it unlikely that aminoguanidine will affect oxalate production from ascorbate *in vivo*.

Conclusions

We show here that glyoxylate is readily converted to oxalate by both hepatic and non-hepatic cell lines, and that such conversion is strongly inhibited by aminoguanidine. As rationale, we provide biochemical evidence for covalent binding between glyoxylate and aminoguanidine, and propose that aminoguanidine, or a more potent derivative thereof, holds promise as a drug for reducing oxalate production in various forms of oxalate overproduction and calcium oxalate stone disease. We furthermore demonstrate the critical importance of small pH change within the physiological range for non-enzymatic oxidation of ascorbate to oxalate, and note the implications this might have for the contribution of ascorbate to oxalate excretion *in vivo*.

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