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ORIGINAL ARTICLE

Biological activity of antitumoural MGBG: the structural variable

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Abstract The present study aims at determining the structure-activity relationships (SAR's) ruling the biological function of MGBG (methylglyoxal bis(guanylhydrazone)), a competitive inhibitor of *S*-adenosyl-L-methionine decarboxylase displaying anticancer activity, involved in the biosynthesis of the naturally occurring polyamines spermidine and spermine. In order to properly understand its biochemical activity, MGBG's structural preferences at physiological conditions were ascertained, by quantum mechanical (DFT) calculations.

Keywords MGBG · SAMDC · Structure-activity relationships · DFT calculations · Physiological structures · Mitochondrial permeability transition (MPT)

Abbreviations

CsA	Cyclosporin A
$\Delta\Psi$	Electrical membrane potential
DFT	Density functional theory
FCCP	Carbonyl cyanide-*p*-trifluorometoxyphenyl-hydrazone
MGBG	Methylglyoxal bis(guanylhydrazone)
MPT	Mitochondrial permeability transition
PCM	Polarised continuum model
RLM	Rat liver mitochondria
SAMDC	*S*-Adenosyl-L-methionine decarboxylase
SCRF	Self-consistent reaction field
SD	Standard deviation
SSAT	Spermidine–spermine acetyltranferase
TPP^+	Tetraphenylphosphonium cation

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Introduction

Methylglyoxal bis(guanylhydrazone) (MGBG, Fig. 1) is a polycation (at physiological conditions) containing two guanidinium groups, which acts as a competitive inhibitor of *S*-adenosyl-L-methionine decarboxylase (SAMDC) (Wallace and Fraser 2004), being thus involved in the biosynthesis of the biogenic polyamines spermidine and spermine. During the 1960s it garnered a considerable interest for its antineoplastic and strong cytotoxic effects (Mihich 1963), but it was discarded from therapeutical use due to its severe toxicity. However, subsequent clinical investigations have shown that particular administration conditions strongly reduce MGBG's toxicity while preserving its antitumoural action (Von Hoff 1994).

Although the mechanisms underlying the antiproliferative and cytotoxic effects of MGBG are still not well

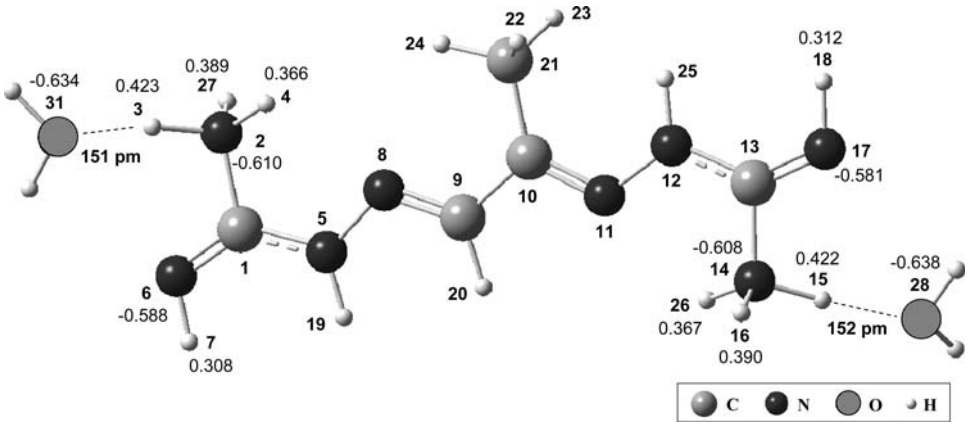

Fig. 1 Schematic representation of the calculated (PCM/B3LYP/6-31G**) lowest energy geometry for diprotonated MGBG, comprising the most relevant atomic charges and the (N)H···O(H) interactions with the solvent (water) molecules (The atom numbering is included)

understood, they are probably linked to the production of reactive oxygen species upon MGBG oxidation (which, however, has not been proved), as well as to its interaction with polyamine metabolism and mitochondrial function. In fact, biogenic polyamine metabolism is one of the main targets of MGBG, which reduces the intracellular polyamine content through induction of spermidine–spermine acetyltransferase (SSAT) (involved in polyamine catabolism) (Regenass et al. 1992) and inhibition of SAMDC (essential for polyamine synthesis) (Williams-Ashman and Schenone 1972). Again polyamine oxidation represents a crucial catabolic pathway in polyamine metabolism by which these polycations can be involved in oxidative stress and apoptosis (Agostinelli et al. 2004). In this regard, it has been reported that MGBG is able to inhibit polyamine oxidase activity in thymus suggesting a positive role for this drug in HIV related diseases (Ferioli and Armanni 2003). Furthermore, some reported studies evidence an increased MGBG antitumour activity in malnourished patients, possibly due to their low intracellular polyamine pool (Von Hoff 1994).

At the mitochondrial level, MGBG exhibits inhibition of protein phosphorylation (Gosalvez et al. 1974; Mailer and Petering 1976; Muhammed et al. 1983; Warrell and Burchenal 1983), impairment of carnitine palmitoyl tranferase and carnitine acetyl transferase (Nikula et al. 1985; Brady et al. 1987), and an overall damaging effect due to selective inhibition of mitochondrial DNA synthesis (Feuerstein et al. 1979). Reported studies on Ehrlich ascites carcinoma cells suggest that the precursor of MGBG, methylglyoxal, induces cytotoxicity by inhibiting the electron flux along Complex I of the mitochondrial respiratory chain (Ray et al. 1994). In isolated rat liver mitochondria (RLM), MGBG was found to display an inhibitory effect towards Ca^{2+}-induced permeability transition in the presence of phosphate and oxalacetate (Toninello et al. 1988), similarly to what is known to occur in the presence of spermine and spermidine (Lapidus and Sokolove 1994; Tassani et al. 1995). In this regard, it should be emphasised that MGBG binding to the mitochondria membrane, at the site responsible for polyamine transport, must be excluded, according to the particular structural characteristics of both the receptors and MGBG (Dalla Via et al. 1999; Toninello et al. 1999). This leads to the conclusion that MGBG's inhibitory effect on polyamine binding to membranes probably arises from conformational changes at the receptor, induced upon binding of the drug to a neighbouring site(s) (Toninello et al. 1999) (in accordance with the induced-fit theory of drug-receptor interaction). The mechanism (or mechanisms) associated to MGBG transport in mitochondria is still not known, nor that associated to its inhibition of mitochondrial permeability transition (MPT) induction.

The biochemical properties of polyamines and related compounds are recognizedly dependent on their geometrical characteristics, at the molecular level. In order to establish the relationship between the structural preferences of MGBG and its biological action—in particular its transport mechanism(s), binding to macromolecular structures and MPT prevention—a conformational analysis in aqueous solution, as a function of pH, was carried out through theoretical methods. The results obtained agree well with a previous study carried out for the biogenic amine agmatine, which also contains a guanidinium moiety, known to be essential for the activity of this kind of polyamines (Toninello et al. 2006).

Materials and methods

Chemicals

[^{14}C]-MGBG and [^{14}C] spermidine were purchased from Amersham Int. (Buchinghamshire, England), while [^{14}C]-arginine was obtained from NEN (Boston, MA, USA). [^{14}C]-agmatine was prepared by treating [^{14}C]-arginine

with *Escherichia coli* arginine decarboxylase, as reported in (Cabella et al. 2001). Hepes, EGTA and rotenone were purchased from Sigma-Aldrich. Cyclosporin A was kindly provided by Sandoz Pharma, Basel (Switzerland). All other reagents were of the highest quality available.

Quantum mechanical calculations

The quantum mechanical calculations—full geometry optimisation and calculation of the harmonic vibrational frequencies—were performed using the GAUSSIAN 03W program (Frisch et al. 1998) within the density functional theory (DFT) approach, in order to properly describe the electron correlation effects (particularly important in this kind of nitrogen-containing systems). The widely employed hybrid method denoted by B3LYP (Cotton and Feng 1998; Wagener and Frenking 1998), which includes a mixture of Hartree-Foch and DFT exchange terms and the gradient-corrected correlation functional of Lee, Yang and Parr (Lee et al. 1988; Miehlich et al. 1989), as proposed and parametrised by Becke (1988, 1993), was used, along with the double-zeta split valence basis set 6-31G** (Hariharan and Pople 1973; Francl et al. 1982).

Molecular geometries were fully optimised by the Berny algorithm, using redundant internal coordinates (Peng et al. 1996): the bond lengths to within ca. 0.1 pm and the bond angles to within ca. 0.1°. The final root-mean-square (rms) gradients were always less than 3×10^{-4} hartree $bohr^{-1}$ or hartree $radian^{-1}$. No geometrical constraints were imposed on the molecules under study.

The solvent effect (water) was simulated by performing Self-Consistent Reaction Field (SCRF) calculations, using Tomasís Polarised Continuum Model (PCM) (Barone et al. 1998; Cammi and Tomasi 1995; Miertus et al. 1981). This approach defines the molecular cavity as the union of a series of interlocking spheres centered on the distinct atoms of the system. A dielectric constant of 78.39 was used for water. Moreover, two water molecules were explicitly considered in the calculations, in order to account for the (N)H···O(H) interactions between MGBG and the solvent, which are of the utmost importance for an accurate representation of this kind of systems under physiological conditions.

Harmonic frequency calculations were performed for all the lowest energy conformations, at the PCM/B3LYP/6-31G** level, in view of determining if these were real minima in the potential energy surface (conformers). Mulliken atomic charges and total dipole moments were obtained for the different MGBG conformers. The relative populations were determined according to the Boltzmann distribution (at 25 and 37°C), based on the relative conformational energies yielded by the geometry optimisation process.

Biochemical assays

Hepatocytes were prepared from male Wistar rats (weighing 150–200 g) by the collagenase perfusion method (Probst and Unthan-Fechner 1985) and cultured as reported by Cabella et al. (2001), using M199 medium supplemented with 2 mg/ml bovine serum albumin (BSA), 3.6 mg/ml Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 5% horse serum and 1 nmol/ml insulin. After 48 h, the medium was removed, the cells were washed once with 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4 and 0.02% KH_2PO_4 pH 7.2, and suspended in 2 ml of Earl's balanced salt solution with both labeled MGBG, or agmatine. After further incubations at different times, the medium was removed and the cells were washed three times with ice-cold NaCl/phosphate, and frozen at −80°C. MGBG and agmatine uptake were measured after solubilisation of each sample in 1 ml of 0.2 M $HClO_4$ and centrifuging at 50,000*g* for 15 min, followed by total radioactivity counting of the supernatant by liquid scintillation. Cell proteins were determined in the 1 M NaOH solubilised pellet, as described by Bradford (1976).

RLM were isolated in 0.25 M sucrose and 5 mM Hepes (pH 7.4) by conventional differential centrifugation (Schneider and Hogeboom 1950). Mitochondrial protein concentration was assayed by the biuret method, using BSA as a standard (Gornall et al. 1949). Mitochondrial incubations were carried out at 20°C, with 1 mg of mitochondrial protein/ml in the following standard: medium 250 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM Na-succinate and 1.25 μM rotenone. A sucrose-based medium was chosen, in order to compare the results with those obtained in polyamine transport studies (for a review see Toninello et al. 2004). [^{14}C]MGBG, [^{14}C]agmatine and [^{14}C]spermidine uptake was determined by a centrifugal filtration method (Toninello et al. 1985).

Mitochondrial membrane potential ($\Delta\Psi$) values were calculated on the basis of the distribution of the lipid-soluble tetraphenylphosphonium cation (TPP^+) through the inner membrane, measured on a home-made TPP^+-specific electrode (Kamo et al. 1979). Mitochondrial matrix volume was calculated from the distribution of [^{14}C]sucrose and ^3H_2O (Palmieri and Klingenberg 1979).

Mitochondrial swelling was evaluated by the apparent absorbance change of mitochondrial suspensions, measured at 540 nm on a Kontron Uvikon 922 spectrophotometer equipped with thermostatic control.

Table 1 Calculated relative energies, populations and dipole moments (μ) for the distinct conformers of diprotonated MGBG (in aqueous solution)

Conformer	ΔE/kJ mol^{-1}	Population[a] (%)	μ/D[b]
MGBG 21	0	95.7	1.3
MGBG 22	10.0	1.7	3.5
MGBG 23	10.4	1.5	3.0
MGBG 24	11.7	0.8	9.5
MGBG 25	14.5	0.3	7.1

[a] Calculated at 37°C

[b] 1 D = 1/3 × 10^{-2} Cm

Results and discussion

Quantum mechanical calculations

A complete geometry optimisation was carried out for MGBG in aqueous solution, considering its different protonation states, with particular emphasis on the diprotonated form (H_2MGBG), which is the major species under physiological conditions (pK_a (MGBG) = 7.5 and 9.2; William-Ashman and Seidenfeld 1986).

Five populated conformers (at room temperature) were obtained for dicationic H_2MGBG (Table 1), from which only three have a population (at 37°C) higher than 1%—MGBG 21, MGBG 22 and MGBG 23 (Fig. 2). The most stable geometry MGBG 21, largely predominant at 25°C, displays a coplanar structure and a *trans* orientation of the two (C=)NH groups relative to the NH_3^+ moieties. Also, internal rotation around the C^1–N^5, C^{13}–N^{12} and C^9–C^{10} bonds lead to a distinct orientation of the methyl group (in C^{21}) relative to the main carbon chain of the molecule, the lowest energy geometries being those for which $C^{21}H_3$ and the N^2H_3 terminal are in a *syn* orientation—MGBG 21, MGBG 22 and MGBG 23 species. Actually, the two less stable conformers MGBG 24 and MGBG 25 differ from these only in the relative position of the CH_3 and NH_3^+ groups. MGBG 24, however, displays a quite high dipole moment as compared to the other dicationic species

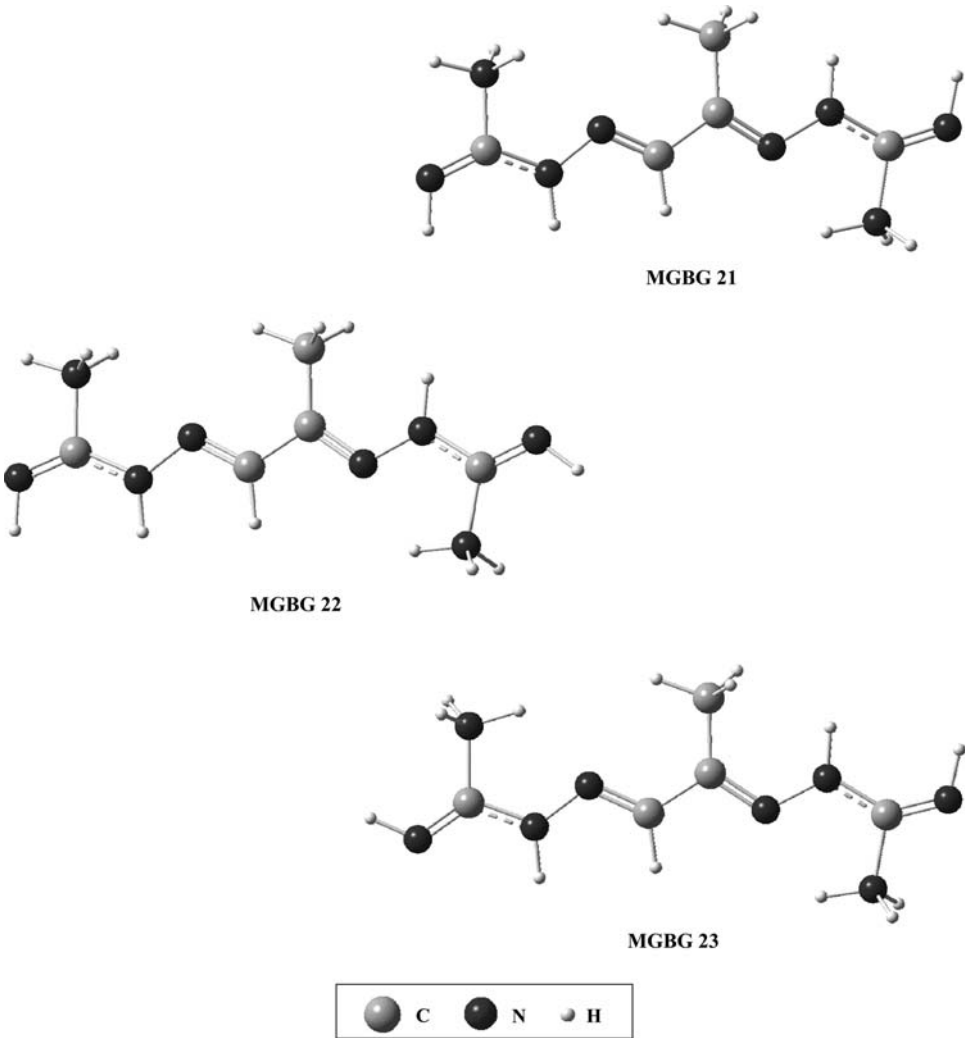

Fig. 2 Schematic representation of the calculated lowest energy conformers for the diprotonated form of MGBG

(Table 1), which may be responsible for an increase of its in vivo population.

Similarly to reported results on the biogenic amine agmatine (Toninello et al. 2006), which contains one NH_3^+ terminal and a guanidinium moiety, a *trans* localisation of the two imino NH's relative to the NH_3^+ groups (MGBG 21) is preferred over a *cis* orientation. In fact, the conformation of the guanidinium group ($(C^1=)N–H$) presently obtained for the most stable MGBG geometry—*trans* relative to the $C^1–N^2$ bond—is identical to the one previously obtained for agmatine (Fig. 3). This is easily understandable for the N-protonated species investigated, in the light of minimisation of the $H^3\cdots H^7$ and $H^{15}\cdots H^{18}$ steric repulsions: conformer MGBG 21 versus MGBG 22 and MGBG 23 (Figs. 1, 2).

Aquation of MGBG, considering a solvent continuum model *plus* two explicit water molecules, involves formation of specific hydrogen close contacts between the positively charged terminal amino groups of MGBG and H_2O. These are predicted by the calculations to be quite strong: (H)O···H(N) distances of 151 and 152 pm, and ($O^{31}H^3N^2$) and ($O^{28}H^{15}N^{14}$) angles of ca. 170° (Fig. 1).

Apart from diprotonated MGBG, the monoprotonated (HMGBG) and unprotonated (neutral) forms of the molecule were also investigated as to their conformational preferences. Figure 4 comprises the most stable structures calculated for each of these species, in aqueous solution. In fact, the monocationic geometry MGBG 11 is expected to be present at physiological conditions along with the predominant diprotonated species MGBG 21 (Fig. 3), its relative populations (ca. 60:40%) being determined by the amine pK_a values of the molecule. Moreover, the very high dipole moment of the monoprotonated conformer MGBG 11 (μ = 17.2 D) can favour its occurrence at physiological conditions (polar medium).

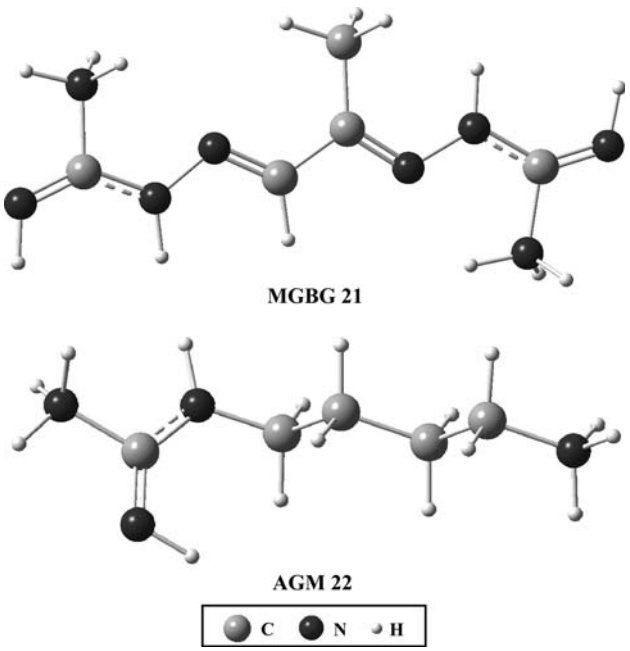

Fig. 3 Schematic representation of the calculated major physiological species of MGBG (MGBG 21) and agmatine (AGM 22 [^{14}C])

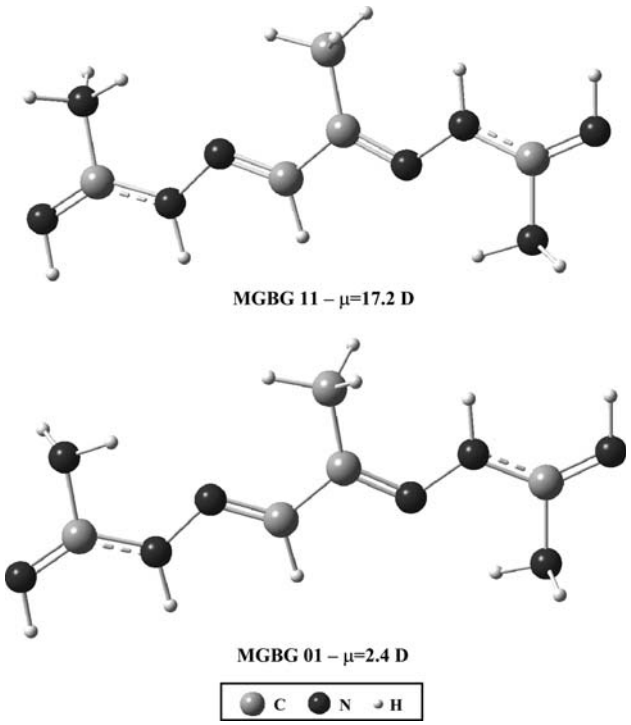

Fig. 4 Schematic representation of the calculated lowest energy conformers for monoprotonated (MGBG 11) and unprotonated (MGBG 01) MGBG, in aqueous solution (The values of the corresponding total dipole moments are included)

Biological activity

The results presently reported refer to particular biological mechanisms associated to MGBG, such as its transport across the plasma and mitochondrial membranes and the prevention of the MPT process. Most probably these events involve different MGBG structures, thus evidencing the importance of their exact knowledge.

As represented in Fig. 5, at the exogenous concentration of 50 μM MGBG is taken up by an hepatocyte culture at about 50 pmol/mg prot., for a 100 min incubation time. This uptake is completely insensitive to the presence of ouabain, while the uncoupler carbonyl cyanide-*p*-trifluorometoxyphenylhydrazone (FCCP) exhibits an inhibition of about 50%. For comparison purposes, Fig. 5 also displays the transport of the biogenic amine agmatine. For a concentration of 50 μM, agmatine is shown to be taken up by hepatocytes at about 40 pmol/mg prot. Also in

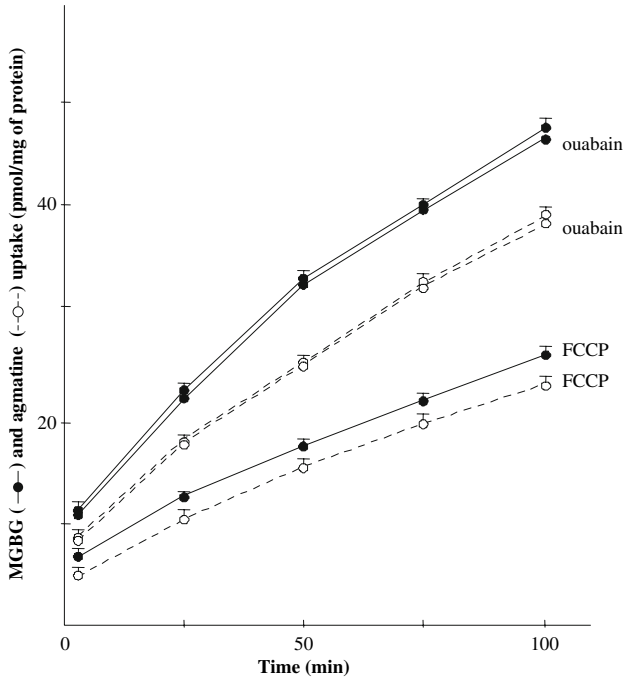

Fig. 5 Time course of MGBG and agmatine uptake by an hepatocyte culture. Hepatocytes were incubated at 37°C (as described in the Experimental Section), in the presence of 50 μM-[^{14}C]MGBG (1 μCi/ml) (*solid lines*) or 50 μM-[^{14}C]agmatine (1 μCi/ml) (*dashed lines*). Where indicated, 5 μM-FCCP or 0.15 mM-ouabain were added (Mean values ± SD of five independent experiments)

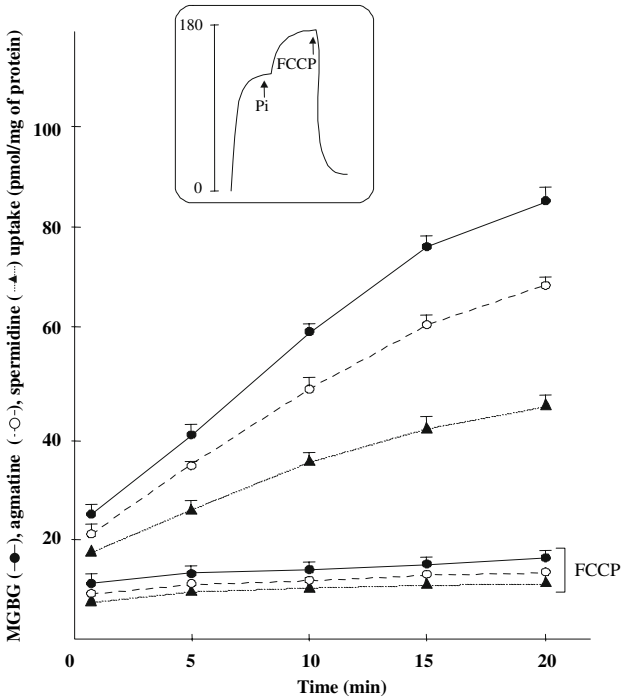

Fig. 6 Time-course of MGBG, agmatine and spermidine uptake by isolated RLM, showing the dependence on the mitochondria energised state. RLM were incubated in standard medium (as described in the Experimental Section), in the presence of 1 mM-phosphate (Pi) with 1 mM-[^{14}C]MGBG (1 μCi/ml) (*solid lines*), 1 mM-[^{14}C]agmatine (1 μCi/ml) (*dashed lines*) or 1 mM-[^{14}C]spermidine (1 μCi/ml) (*dotted lines*). Where indicated, 1 μM-FCCP was added. *Inset* ΔΨ measurement of RLM with and without phosphate. De-energisation by FCCP (Mean values ± SD of five independent experiments)

this case ouabain is ineffective, whereas FCCP induces an inhibition of about 50%. The choice of agmatine for comparison purposes is due to the structural similarity between this amine and MGBG.

Figure 6 depicts the transport of MGBG, at a 1 mM exogenous concentration, in RLM energised by succinate oxidation, in the presence of rotenone (in order to favour substrate oxidation) and phosphate (to obtain a very high ΔΨ, Fig. 6, inset). MGBG transport is compared with that of agmatine, the mechanism of which has recently been characterised (Salvi et al. 2006; Agostinelli et al. 2007), and with that of spermidine, also previously reported (Toninello et al. 1992) (also for a 1 mM concentration). It is verified that MGBG is transported at about 85 nmol/mg prot, for a 20 min incubation period, a higher extent than that measured for either agmatine (≈70 nmol/mg prot.) or spermidine (≈45 nmol/mg prot.). In the presence of FCCP, which collapses ΔΨ (Fig. 6, inset), all these transport processes are almost completely inhibited, demonstrating a clear mechanistic difference relative to the transport mechanism across the plasma membrane (Fig. 5).

The decrease of about 1unit in the apparent absorbance (at 540 nm) of the mitochondrial suspension is plotted in Fig. 7, which is indicative of mitochondrial swelling, induced by 50 μM Ca^{2+} in the presence of phosphate. The total inhibition observed in the presence of the

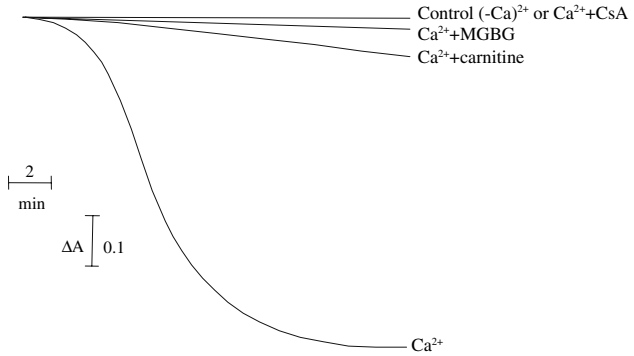

Fig. 7 Inhibition of Ca^{2+}-induced mitochondrial permeability transition by MGBG and carnitine. RLM were incubated in standard medium (as described in the Experimental Section), in the presence of 1 mM-Pi and 50 μM-Ca^{2+}. Where indicated 1 mM-MGBG, 1 mM-carnitine or 1 μM-CsA were added (Five independent experiments yielded almost identical results)

immunosuppressant cyclosporin A (CsA) demonstrates that this absorbance change is due to the opening of the transition pore, related to MPT induction (for a review see Zoratti and Szabò 1995). If RLM are incubated with of 1 mM MGBG, mitochondrial swelling is completely

prevented (Fig. 7), which also occurs for agmatine and spermidine for the same MGBG dosage (results available from the authors upon request). Incubation of RLM with 1 mM carnitine, in turn, results in a strong but not complete inhibition of the swelling.

The results presently obtained lead to the characterisation of the most stable physiological MGBG structures. Furthermore, this work also aims at establishing the MGBG geometry (or geometries) able to cross the plasma and mitochondrial membranes, and to interact with different macromolecular structures in vivo, leading to the known MGBG antitumoural effect.

According to the molecule's pK_a values (7.5 and 9.2), both the mono- and diprotonated forms of the molecule are present under physiological pH (ca. 60:40%). Among the five diprotonated structures calculated as conformational minima for MGBG, the most stable and largely predominant one is MGBG 21, with a relative population of 95.7% and a total dipole moment of 1.3 D. However, as mentioned above, at neutral pH a significant fraction of monoprotonated form also occurs. Consequently, in the light of the theoretical data presently gathered, MGBG 21 (μ = 1.3 D) and MGBG 11 (μ = 17.2 D) (Figs. 3, 4) are proposed to be the main MGBG species in biological fluids and inner cell compartments of MGBG-treated patients.

A large number of reports are to be found in the literature on the MGBG transport across the plasma membrane of different cells (for a review see William-Ashman and Seidenfeld 1986), and several authors refer that MGBG shares the same transporter with biogenic polyamines. It is also possible that this drug uses the monoamine transporters EMT and OCT2, which are involved in the uptake of agmatine (Cabella et al. 2001). Actually, since agmatine is a divalent cation at pH 7.4, comprising a guanidinium group, similarly to MGBG (Fig. 3), this hypothesis should not be disregarded. Moreover, MGBG could be transported in the unprotonated, uncharged form, since those transporters exhibit an electroneutral behaviour (Grundemann et al. 2003).

The results represented in Fig. 5 confirm this hypothesis. As previously demonstrated (Cabella et al. 2001), agmatine is transported in hepatocytes by an energy-dependent mechanism, as ouabain, a plasma membrane de-energising agent (Clausen and Flatman 1977) proved to be an ineffective inhibitor of this process. In addition, FCCP, which collapses the electrochemical gradient of "in situ" mitochondria, exhibits a partial inhibition of agmatine transport, as it only excludes the entrance of the amine in the mitochondrial matrix. This data clearly shows that agmatine is transported through an electroneutral mechanism in hepatocyte cultures, supporting the statement that the unprotonated species is involved in this process as observed for EMT and OCT2 transporters (Grundemann et al. 2003). Figure 5 also shows that MGBG is transported in hepatocytes, even to a larger extent than agmatine, suggesting that the presence of two guanidinium groups in its molecule further favours this mechanism. The absence of the ouabain effect and the partial inhibition by FCCP confirm that MGBG is taken up in the uncharged form.

In addition, MGBG is known to cross the mitochondrial membrane: Byczkowski and coworkers (Byczkowski et al. 1981; Byczkowski and Porter 1983) reported that MGBG is taken up by RLM when incubated in energised media, while Diwan et al. (1988) observed an enhancement of MGBG transport in RLM submitted to outer membrane lysis. These observations are confirmed by the results shown in Fig. 6, in different media, in order to establish high $\Delta\Psi$ values (of about 180 mV, Fig. 6, inset) and induce MGBG transport to a higher extent (Byczkowski et al. 1981; Byczkowski and Porter 1983; Diwan et al. 1988). Comparison with agmatine and spermidine transport processes clearly evidences the significance of the presence of two guanidinium groups in the MGBG molecule, which leads to a larger transport extent in mitochondria relative to both agmatine (one guanidinium) and spermidine (one amino-butyl and one amino-propyl group). The decrease in spermidine transport as compared to agmatine and MGBG is due to its lower dipole moment at physiological pH (μ = 2.76 D), (M. P. M. Marques, unpublished results) despite its higher charge (3+) (Toninello et al. 1992a, b), emphasising the importance of this parameter for polycation transport in mitochondria. Recently, it was demonstrated that agmatine is carried through the mitochondrial membrane using a channel or a single-binding centre-gated pore exhibiting an electrophoretic behaviour (Salvi et al. 2006; Agostinelli et al. 2007). Agmatine is proposed to cross the mitochondrial membrane in the monoprotonated form, due its high polarity and the significant $\Delta\Psi$ at the mitochondrial surface. On the basis of these previous results for a similar polyamine, and considering the very high dipole moment of monoprotonated MGBG 11 (μ = 17.2 D, even higher than that of monovalent agmatine, μ = 15.8 D), it is suggested that MGBG is transported in mitochondria in the MGBG 11 conformation. Moreover, a slight amount of MGBG may also be transported as a divalent cation, in the MGBG 24 form, since this geometry has a dipole moment of 9.5 D, much higher than that of the predominant dipositive species MGBG 21 (Table 1).

Interaction of MGBG with the macromolecules within the cell, in order to exert its biological functions, is strongly dependent on its conformational behaviour. The drug is expected to interact with the biomolecular structures by means of weak interactions—hydrogen bonds, Van der Walls forces, cation/π interactions, electrostatic close-contacts between hydrated charged groups, etc.—as

verified for biogenic polyamines (Dalla Via et al. 1985, 1999). Due to the π-resonance occurring in the guanidinium groups, MGBG has a quite rigid structure, surely not as flexible as that of spermine or even spermidine (Toninello et al. 1999). This implies that MGBG will only bind to particular biological sites. A very important observation arose from a study on MGBG inhibition of carnitine palmitoyl transferase (Brady et al. 1987): the guanidinium moiety was found to occupy the quaternary ammonium site of carnitine, thus suggesting that an anionic group should be located in the active site for an effective interaction with the amine to occur. Indeed, the same work reports that the MGBG chain extends with that of acylcarnitine, allowing an immino group from MGBG to occupy a position similar to the acyl carbonyl, enabling the formation of a thiolaminal with a thiol at the active site, by substituting the normal interaction of the acyl carbonyl with the SH group. This type of observations reveals a tight conformational accordance between MGBG and its biological receptors, the sites of interaction being required to display a particular architecture for the binding to be efficient. This is very surprisingly confirmed by the observation that carnitine, although with a lesser efficacy as compared to MGBG, is also able to inhibit MPT (Fig. 7) (see also Di Lisa et al. 1985), suggesting that the critical binding site responsible for MPT inhibition by both MGBG and carnitine has an identical geometry to that of the active site of carnitine palmitoyl tranferase. MGBG does not easily bind to an anionic group and needs a particular receptor, with a well-defined geometry. This fact was previously discussed by William-Ashman and collaborators (1986), who, however, ascribed it to the low electrical charge of the molecule. The present work denies this hypothesis, since it proves that MGBG occurs predominantly in a positively charged form in the physiological medium.

From the presently obtained MGBG structural data, the mono- and diprotonated species of the molecule are the ones proposed to be involved in the drug's interaction with its biological targets. Although both H_2MGBG (MGBG 21) and HMGBG (MGBG 11) are present at physiological conditions, the requirement for charged guanidinium groups for the drug-receptor interplay to occur renders the diprotonated form the most effective.

The structural study now performed for MGBG in aqueous solution, as a function of its protonation degree, allows a thorough characterisation of the most stable geometries of the molecule at physiological conditions. Since the activity of this kind of systems (e.g. biogenic polyamines) is known to be ruled by their structural preferences, the knowledge of the conformational behaviour of MGBG is essential for the understanding of its biological role, namely its effect on enzyme activity, polyamine transport and MPT induction. As to the latter, the opening of the transition pore is proposed to be strictly related to programmed cell death and, consequently to all pathological processes linked to apoptosis.

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References

Agostinelli E, Arancia G, Dalla Vedova L, Belli F, Marra M, Salvi M, Toninello A (2004) The biological functions of polyamine oxidation products by amine oxidases: perspectives of clinical application. Amino Acids 27:347–358

Agostinelli E, Tempera G, Molinari A, Salvi M, Battaglia V, Toninello A, Arancia G (2007) The physiological role of biogenic amines redox reactions in mitochondria. New perspectives in cancer therapy. Amino Acids 33:175–187

Barone V, Cossi M, Tomasi J (1998) Geometry optimization of molecular structures in solution by the polarizable continuum model. J Comp Chem 19:404–417

Becke AD (1993) Density-functional thermochemistry. III. The role of exact exchange. J Chem Phys 98:5648–5652

Becke AD (1988) Density-functional exchange-energy approximation with correct asymptotic behavior. Phys Rev A 38:3098–3100

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brady LJ, Brady PS, Gandour RD (1987) Effect of methylglyoxal bis(guanylhydrazone) on hepatic, heart and skeletal muscle mitochondrial carnitine palmitoyltransferase and beta-oxidation of fatty acids. Biochem Pharmacol 36:447–452

Byczkowski JZ, Porter CW (1983) Interactions between bis(guanylhydrazones) and polyamines in isolated mitochondria. Gen Pharmacol 14:615–621

Byczkowski JZ, Salomon W, Harlos JP, Porter CW (1981) Actions of bis(guanylhydrazones) on isolated rat liver mitochondria. Biochem Pharmacol 30:2851–2860

Cabella C, Gardini G, Corpillo D, Testore G, Bedino S, Solinas SP, Cravanzola C, Vargiu C, Grillo MA, Colombatto S (2001) Transport and metabolism of agmatine in rat hepatocyte cultures. Eur J Biochem 268:940–947

Cammi R, Tomasi J (1995) Remarks on the use of the apparent surface charges (ASC) methods in solvation problems: iterative versus matrix-inversion procedures and the renormalization of the apparent charges. J Comp Chem 16:1449–1458

Clausen T, Flatman JA (1977) The effect of catecholamines on Na-K transport and membrane potential in rat soleus muscle. J Physiol 270:383–414

Cotton FA, Feng X (1998) Density functional theory study of transition-metal compounds containing metal-metal bonds. 2. Molecular structures and vibrational spectra of dinuclear tetracarboxylate compounds of molybdenum and rhodium. J Am Chem Soc 120:3387–3397

Dalla Via L, Di Noto V, Toninello A (1999) Binding of spermidine and putrescine to energized liver mitochondria. Arch Biochem Biophys 365:231–238

Dalla Via L, Di Noto V, Siliprandi D, Toninello A (1985) Uptake of spermine by rat liver mitochondria and its influence on the transport of phosphate. Biochim Biophys Acta 815:399–404

Di Lisa F, Bobileva-Guarriero V, Jocelyn P, Toninello A, Siliprandi N (1985) Stabilising action of carnitine on energy linked processes in rat liver mitochondria. Biochem Biophys Res Commun 131:968–973

Diwan JJ, Yune HH, Bawa R, Haley T, Mannella CA (1988) Enhanced uptake of spermidine and methylglyoxal-bis(guanylhydrazone) by rat liver mitochondria following outer membrane lysis. Biochem Pharmacol 37:957–961

Ferioli ME, Armanni A (2003) Polyamine oxidase activity in rats treated with mitoguazone: specific and permanent decrease in thymus. Amino Acids 24:187–194

Feuerstein B, Porter CW, Dave C (1979) A selective effect of methylglyoxal-bis(guanylhydrazone) on the synthesis of mitochondrial DNA of cultured L1210 leukemia cells. Cancer Res 39:4130–4137

Francl MM, Pietro WJ, Hehre WJ, Binkley JS, Gordon M, DeFrees D, Pople JA (1982) Self-consistent molecular orbital methods. XXIII. A polarization-type basis set for second-row elements. J Chem Phys 77:3654–3665

Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Zakrzewski VG, Montgomery JA Jr, Stratmann RE, Burant JC, Dapprich S, Millam JM, Daniels AD, Kudin KN, Strain MC, Farkas O, Tomasi J, Barone V, Cossi M, Cammi R, Mennucci B, Pomelli C, Adamo C, Clifford S, Ochterski J, Petersson GA,Ayala PY, CuiQ, MorokumaK,Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Cioslowski J, Ortiz JV, Baboul AG, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Gomperts R, Martin RL, Fox DJ,Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Andres JL, Gonzalez C, Head-Gordon M, Replogle ES, Pople JA (1998) Gaussian 98, Revision A.9. Gaussian Inc., Pittsburgh, PA, USA

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766

Gosalvez M, Blanco M, Hunter J, Miko M, Chance B (1974) Effects of anticancer agents on the respiration of isolated mitochondria and tumor cells. Eur J Cancer 10:567–574

Grundemann D, Hahne C, Berkels R, Schomig E (2003) Agmatine is efficiently transported by non-neuronal monoamine transporters extraneuronal monoamine transporter (EMT) and organic cation transporter 2 (OCT2). J Pharmacol Exp Ther 304:810–817

Hariharan PC, Pople JA (1973) The influence of polarization functions on molecular orbital hydrogenation energies. Theor Chim Acta 28:213–222

Kamo N, Muratsugu M, Hongoh R, Kobatake Y (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 49:105–121

Lapidus RG, Sokolove PM (1994) The mitochondrial permeability transition. Interactions of spermine, ADP, and inorganic phosphate. J Biol Chem 269:18931–18936

Lee C, Yang W, Parr RG (1988) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. Phys Rev B 37:785–789

Mailer K, Petering DH (1976) Inhibition of oxidative phosphorylation in tumor cells and mitochondria by daunomycin and adriamycin. Biochem Pharmacol 25:2085–2089

Miehlich B, Savin A, Stoll H, Preuss H (1989) Results obtained with the correlation energy density functionals of becke and Lee, Yang and Parr. Chem Phys Lett 157:200–206

Miertus S, Scrocco E, Tomasi J (1981) Electrostatic interaction of a solute with a continuum. A direct utilizaion of AB initio molecular potentials for the prevision of solvent effects. J Chem Phys 55: 117–129

Mihich E (1963) Current studies with methylglyoxal-bis(guanylhydrazone). Cancer Res 23:1375–1389

Muhammed H, Ramasarma T, Kurup CK (1983) Inhibition of mitochondrial oxidative phosphorylation by adriamycin. Biochim Biophys Acta 722:43–50

Nikula P, Ruohola H, Alhonen-Hongisto L, Janne J (1985) Carnitine prevents the early mitochondrial damage induced by methylglyoxal bis(guanylhydrazone) in L1210 leukaemia cells. Biochem J 228:513–516

Palmieri F, Klingenberg M (1979) Direct methods for measuring metabolite transport and distribution in mitochondria. Methods Enzymol 56:279–301

Peng C, Ayala PY, Schlegel HB, Frisch MJ (1996) Using redundant internal coordinates to optimize equilibrium geometries and transition states. J Comp Chem 17:49–56

Probst I, Unthan-Fechner K (1985) Activation of glycolysis by insulin with a sequential increase of the 6-phosphofructo-2-kinase activity, fructose-2,6-bisphosphate level and pyruvate kinase activity in cultured rat hepatocytes. Eur J Biochem 153:347–353

Ray S, Dutta S, Halder J, Ray M (1994) Inhibition of electron flow through complex I of the mitochondrial respiratory chain of Ehrlich ascites carcinoma cells by methylglyoxal. Biochem J 303:69–72

Regenass U, Caravatti G, Mett H, Stanek J, Schneider P, Muller M (1992) New *S*-adenosylmethionine decarboxylase inhibitors with potent antitumor activity. Cancer Res 52:4712–4718

Salvi M, Battaglia V, Mancon M, Colombatto S, Cravanzola C, Calheiros R, Marques MPM, Grillo MA, Toninello A (2006) Agmatine is transported into liver mitochondria by a specific electrophoretic mechanism. Biochem J 396:337–345

Schneider WC, Hogeboom GH (1950) Intracellular distribution of enzymes. V. further studies on the distribution of cytochrome *c* in rat liver homogenates. J Biol Chem 183:123–128

Tassani V, Biban C, Toninello A, Siliprandi D (1995) Inhibition of mitochondrial permeability transition by polyamines and magnesium: importance of the number and distribution of electric charges. Biochem Biophys Res Commun 207:661–667

Toninello A, Battaglia V, Salvi M, Calheiros R, Marques MPM (2006) Structural characterization of agmatine at physiological conditions. Struct Chem 17:163–175

Toninello A, Dalla Via L, Di Noto V, Mancon M (1999) The effects of methylglyoxal-bis(guanylhydrazone) on spermine binding and transport in liver mitochondria. Biochem Pharmacol 58:1899–1906

Toninello A, Dalla Via L, Testa S, Siliprandi D (1992a) Electrophoretic polyamine transport in rat liver mitochondria. Amino Acids 2:69–76

Toninello A, Dalla Via L, Siliprandi D, Garlid KD (1992b) Evidence that spermine, spermidine, and putrescine are transported electrophoretically in mitochondria by a specific polyamine uniporter. J Biol Chem 267:18393–18397

Toninello A, Di Lisa F, Siliprandi D, Siliprandi N (1985) Uptake of spermine by rat liver mitochondria and its influence on the transport of phosphate. Biochim Biophys Acta 815:399–404

Toninello A, Salvi M, Mondovi B (2004) Interaction of biologically active amines with mitochondria and their role in the mitochondrial-mediated pathway of apoptosis. Curr Med Chem 11:2349–2374

Toninello A, Siliprandi D, Castagnini P, Novello MC, Siliprandi N (1988) Protective action of methylglyoxal bis (guanylhydrazone) on the mitochondrial membrane. Biochem Pharmacol 37:3395–3399

Von Hoff DD (1994) MGBG: teaching an old drug new tricks. Ann Oncol 5:487–493

Wagener T, Frenking G (1998) Theoretical study of transition metal compounds with molybdenum–andtungsten–phosphorus triple bonds. Inorg Chem 37:1805–1811

Wallace HM, Fraser AW (2004) Inhibitors of polyamine metabolism. Amino Acids 26:353–365

Warrell RP, Burchenal JH (1983) Methylglyoxal-bis(guanylhydrazone) (Methyl-GAG): current status and future prospects. J Clin Oncol 1:52–65

Williams-AshmanHG, Schenone A (1972) Methyl glyoxal bis(guanylhydrazone) as a potent inhibitor of mammalian and yeast S-adenosylmethionine decarboxylases. Biochem Biophys Res Commun 46:288–295

Williams-Ashman HG, Seidenfeld J (1986) Aspects of the biochemical pharmacology of methyl glyoxal bis(guanylhydrazone). Biochem Pharmacol 35:1217–1225

ZorattiM, Szabò I (1995) The mitochondrial permeabilitytransition. Biochem Biophys Acta 1241:139–176