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Effect of Bis(guanylhydrazones) on Growth and Polyamine Uptake in Plant Cells

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Abstract. In the present work the effect of several bis(guanylhydrazones) on the growth of *Helianthus tuberosus* tuber explants was studied. Different aliphatic congeners of glyoxal bis(guanylhydrazone) were tested. Most of the compounds displayed an inhibitory effect on growth, and a correlation between the structure of the molecule and the inhibitory activity was observed. Experiments carried out with glyoxal bis(guanylhydrazone) and its congeners methyl-, ethylmethyl-, and methylpropylglyoxal bis(guanylhydrazones) show that as the total number of side chain carbon atoms in the molecule increases, the inhibitory potency also increases. A depletion of spermidine levels was also found in the explants treated with ethylmethylglyoxal bis(guanylhydrazone), which turned out to be one of the most potent growth inhibitors. The addition of spermidine caused a significant reversion of the antiproliferative action of glyoxal bis(guanylhydrazone). The effect of these compounds on spermidine uptake in protoplasts isolated from carrot phloem parenchyma was also investigated. Only a slight competition was found when antagonists were present at concentrations 20 times higher than the polyamine, thus suggesting that bis(guanylhydrazones) do not share, at least at low concentrations, the polyamine transport system in plant cells.

Key Words. Bis(guanylhydrazones)—Carrot protoplasts—Growth—*Helianthus tuberosus*—Polyamines—Uptake

Abbreviations: AdoMetDC, adenosylmethionine decarboxylase; 2,4-D, 2,4-dichlorophenoxyacetic acid; DMGBG, dimethylglyoxal bis(guanylhydrazone); EGBG, ethylglyoxal bis(guanylhydrazone); EMGBG, ethylmethylglyoxal bis(guanylhydrazone); GBG, glyoxal bis(guanylhydrazone); HF, hormone-free; MGBG, methylglyoxal bis(guanylhydrazone); MPGBG, methylpropylglyoxal bis(guanylhydrazone).

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The bis(amidinohydrazones), known as bis(guanylhydrazones), have been actively studied in recent years (Jänne et al. 1986; Williams-Ashman and Seidenfeld 1986). The interest that these compounds have raised is not exclusively due to their antineoplastic properties. Several bis(guanylhydrazones), of which the methylglyoxal derivative (MGBG) has to date been studied most thoroughly, are known to interfere with the metabolism of natural polyamines, and this property has made them much used tools in the study of the biochemistry and physiological functions of the latter compounds. Considering this purpose, perhaps the most important property of the bis(guanylhydrazones) is their ability to inhibit *S*-adenosylmethionine decarboxylase (AdoMetDC), a key enzyme of polyamine biosynthesis which converts *S*-adenosylmethionine (AdoMet) into the so-called decarboxylated AdoMet that is in turn needed in the conversion of putrescine into spermidine and further in the conversion of spermidine into spermine (Alhonen-Hongisto et al. 1985, Williams-Ashman and Schenone 1972).

A curious feature of the bis(guanylhydrazones) is their structural relationship with two molecules involved in polyamine biosynthesis, namely spermidine and AdoMet; (Jänne et al. 1986). Fig. 1 shows the structural formulas of spermidine free base (*A*), the free base of the bis(guanylhydrazones) of various glyoxals (*B*), and AdoMet (*C*).

The reversal of the antiproliferative activity of MGBG by polyamines, observed in different animal cells and tissues both in vivo and in vitro, was initially believed to be the result of the ability of MGBG to inhibit polyamine biosynthesis. Later, it was found that the mechanism of the antiproliferative action of MGBG almost certainly is not only due to the inhibition of polyamine biosynthesis. Actually, it has been demonstrated that MGBG and polyamines compete for a common cellular transport system in many animal cell types. Excess extracellular polyamines therefore inhibit the entry of MGBG and other bis(guanylhydrazones) into animal cells, thereby

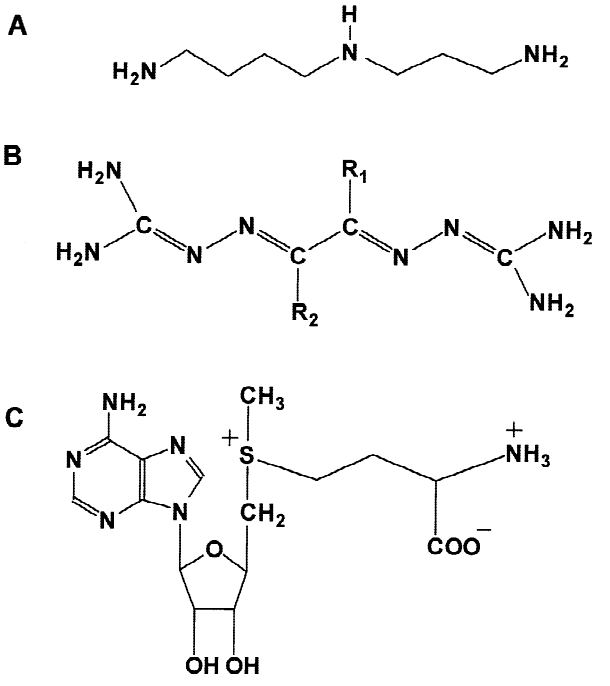


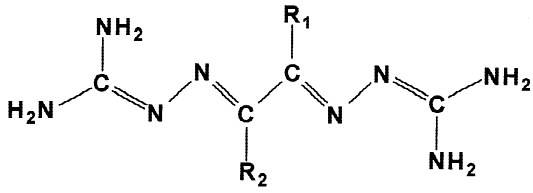
Fig. 1. Comparison of the structural formulas of spermidine free base (*A*), the free base of the bis(guanylhydrazones) of various glyoxals (*B*), and *S*-adenosylmethionine (*C*).

also preventing the antiproliferative action of the latter. The ultimate mechanism of action of MGBG in animal cells is so far obscure (Alhonen-Hongisto et al. 1980, Byers et al. 1987, Elo 1989a, Jänne et al. 1991).

In spite of the biochemical and pharmacological significance of these compounds in animals and, more recently, the systematic analysis of their chemical properties (Elo 1989b, Elo et al. 1989, 1996), nothing is known about the biochemical and physiological action and significance of these compounds in plant cells and tissues, with the exception of the effect of the methylglyoxal analog (MGBG) (Bagni et al. 1981, Lin 1985, Malmberg and McIndoo 1983, Pistocchi et al. 1987).

In the present study the antiproliferative effect and the action on polyamine uptake of GBG and its aliphatic congeners have been elucidated. For this purpose, two well known plant model systems, *Helianthus tuberosus* tuber explants for growth studies and *Daucus carota* protoplasts for uptake competition experiments, were utilized. The use of *Helianthus* tuber explants is justified by the fact that the medullary parenchyma of this tuber contains, during deep dormancy, a low level of polyamines as well as plant hormones, such as auxins and cytokinins, whose concentrations are too low to sustain growth. On the contrary, this tissue reacts positively to an artificial break of dormancy induced by exogenous free polyamines and/or plant hormones (Bagni 1989).

Table 1. Structures of the bis(guanylhydrazones) studied.



Compound	R_1	R_2
GBG	H	H
MGBG	CH_3	H
EGBG	CH_2CH_3	H
DMGBG	CH_3	CH_3
EMGBG	CH_2CH_3	CH_3
MPGBG	CH_3	$(CH_2)_2CH_3$

Materials and Methods

All of the bis(guanylhydrazones) studied were in the free base form and were synthesized according to previously published procedures (Elo et al. 1986, Elo 1989a, Seppänen et al. 1984).

Plants

Tubers of *H. tuberosus* L. cv. OB1, grown in the Botanical Garden of Bologna University and stored in November at 4°C, were utilized for the experiments during the period of dormancy (from December through February) and at the beginning of the natural release from dormancy (beginning of March).

Tubers were sterilized by immersion in a 5% solution of commercial NaOCl and rinsed three times in sterile deionized water. Then, under sterile conditions, homogeneous explants of medullary parenchyma (about 50 mg) were placed on solid medium recommended by Bertossi (1959), containing 5% sucrose and 0.7% Biolife agar. The basal medium was supplemented with 10 μM 2,4-D and bis(guanylhydrazones) at various concentrations (0.2, 1, 5 mM). The hormone-free medium (HF) did not contain either 2,4-D or bis(guanylhydrazones). The compounds tested are shown in Table 1.

The pH was adjusted to 5.6 with NaOH before autoclaving at 120°C for 20 min. Chemical solutions were sterilized by filtration through 0.22-μm cellulose acetate membranes (Millipore) and added to autoclaved medium. The explants were maintained under a 16-h light/8-h dark photoperiod (Thorn Cool-white, 500 K fluorescent tubes, irradiance 90 μmol m^{-2} s^{-2} at explant level) for 3 weeks at 23 ± 1°C. Explants grown in the absence of 2,4-D (HF) were used as controls to check the dormancy of the tissue. Results are referred to explants treated with 2,4-D.

For protoplast isolation, mature taproots of carrot, *D. carota* L. cv. Ingrid, were grown in fields near Bologna without any addition of growth regulators or antiparasitic agents. They were stored unwashed in sand at 5°C for up to 3 months.

Polyamine Determination

Polyamines were extracted, separated, and detected by the method of direct dansylation described by Smith and Best (1977) using TLC-precoated plates of Silica Gel 60 with concentration zone (Merck).

Ethylacetate:cyclohexane (2:3, v/v) was used as solvent. Spots were scraped from the plates, extracted in acetone on a Vortex mixer, and centrifuged. Fluorescence was measured using a Jasco 550 FP spectrofluorometer (excitation 360 nm, emission 505.5 nm), and results were compared with dansylated standards.

Isolation of Protoplasts

Protoplasts were prepared essentially as described by Keller (1988). Taproots were peeled, and 15 g of the phloem parenchyma was chopped with a single-edge razor blade into cubes of 1 mm^3 and placed in the protoplast medium (0.7 M glycine-betaine, 25 mM MES-Tris, pH 5.5). The tissue was washed three times with the same medium and then incubated for 3 h with 15 mL of the protoplast medium also containing 2% (w/v) Cellulase Y-C and 0.1% (w/v) Pectolyase Y-23 (both from Seishin Pharmaceutical Co. Tokyo, Japan) on an orbital shaker (30 rpm) at 24°C in the light. Protoplasts were collected by filtering through cheesecloth and then washed twice in the protoplast medium by slow centrifugation (25 g for 5 min) and resuspension. The pellet contained 7–10 × 10^6 protoplasts mL^{-1}, as counted in a Burker counting chamber.

Uptake Experiments and Protein Determination

The procedure for polyamine uptake was performed as described by Pistocchi et al. (1988) with slight modifications. Protoplasts were incubated in a medium containing 25 mM MES-Tris (pH 5.5), 0.7 M glycine-betaine, and 7.4 kBq in 4 μL of [^{14}C]spermidine (Amersham, UK, specific activity 4.07 Gbq/mmol). The various compounds used as antagonists of spermidine uptake were also added in the medium, at the concentrations indicated in the Results section. Experiments were performed with 1 mM $CaCl_2$ in the incubation medium. After a 2-min incubation, 150 μL of the mixture was transferred to 400-μL microtubes already containing, from bottom to top: 140 μL of silicon oil AR 200/AR 20 (1/0.5, v/v) (Wacker-Chemie GmbH Germany), 50 μL of 100 mM unlabeled spermidine solution, and 50 μL of silicon oil AR 200/AR 20 (1/0.5, v/v). They were then immediately centrifuged at 13.250 ×*g* for 40 s in a Beckman microcentrifuge B. The excess unlabeled polyamine was inserted between the two silicon oil layers in order to remove most of the surface-bound polyamine (Pistocchi et al. 1988). The microtubes were snapfrozen in liquid nitrogen, and the tips, containing the protoplasts, were cut with a razor blade. The protoplasts were suspended in 500 μL of distilled water, and after a 1-min sonication, 250-μL aliquots were used for the determination of radioactivity in 4 mL of scintillation cocktail (Beckman Ready Gel) with a Beckman LS 1800 scintillation counter. Further aliquots were used for protein determination. Data are the means ± S.E. of three different experiments with duplicate samples.

Protein content was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Results and Discussion

Preliminary experiments were carried out with different bis(guanylhydrazones) at various concentrations (0.02, 0.05, 0.1, 0.15, 0.2, 1, and 5 mM) in order to determine the minimum concentration at which growth of *H. tuberosus* explants was inhibited. Between 0.02 and 0.15 mM none of the compounds tested inhibited growth significantly (data not shown), whereas growth inhibition became evident starting from 0.2 mM.

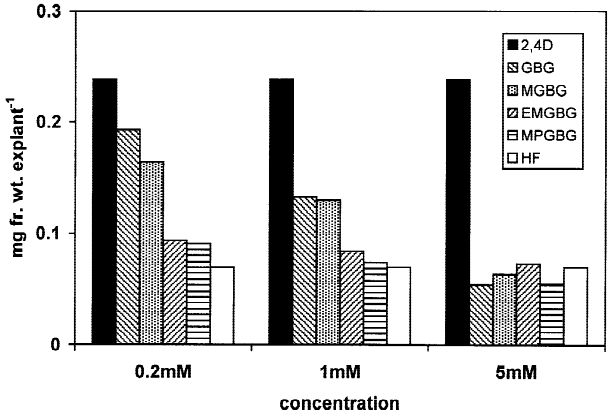


Fig. 2. Effect of different concentrations of bis(guanylhydrazones) on the growth of explants from *H. tuberosus* dormant tubers cultured in vitro. The fresh weights were determined at the 21st day of culture. All of the treatments were significantly different ($p \leq 0.05$) from 2,4-D. Data are the mean of 15 replicates (two experiments).

Figs. 2 and 3 show the effect of different bis(guanylhydrazones) on the growth of tuber explants, on a fresh weight basis, after 21 days in culture. The inhibitory effect was already evident after 5–6 days in culture (data not shown). The explants treated with the highest concentration (5 mM) displayed symptoms of browning, probably because of toxic side effects due to a pronounced synthesis of polyphenols. The set of experiments carried out with GBG, MGBG, EMGBG, and MPGBG (Fig. 2) clearly shows that the growth inhibition increased with an increasing number of side chain carbon atoms in the molecule (Table 1). The effect was clearly dose dependent. At the highest concentration tested, the correlation between inhibitor structure and inhibitory potency vanished, as all inhibitors blocked the growth nearly totally, probably because of toxic side effects.

The results obtained with another set of inhibitors, namely MGBG, EGBG, and DMGBG (Fig. 3) are more difficult to interpret. At the lowest concentration tested, the results obtained with MGBG and DMGBG are in line with the results obtained with the other set of inhibitors, but EGBG appeared to behave differently. At 1 mM concentration, however, the results obtained with EGBG are completely in line with all other inhibitors studied. At the highest concentration tested, EGBG and DMGBG also caused nearly total inhibition. Data expressed on dry weight basis gave a similar pattern (data not shown).

Experiments reported in Fig. 3 were performed at the beginning of the natural release from dormancy of the tuber. It is worth observing that explants grew even in the absence of 2,4-D, although less than in its presence. This suggests that under these conditions, some synthesis of plant growth substances, enough to sustain growth, oc-

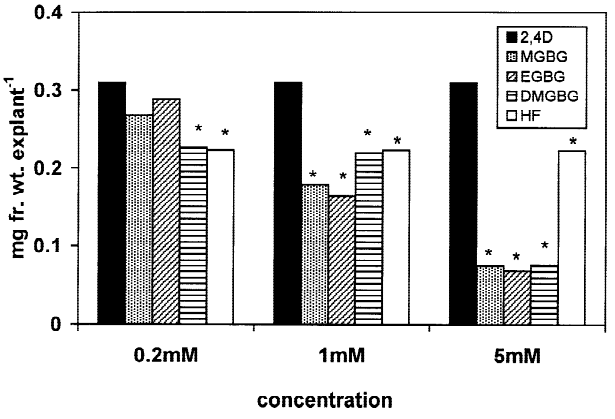


Fig. 3. Effect of different concentrations of bis(guanylhydrazones) on the growth of explants from *H. tuberosus* tubers cultured in vitro. The experiment was performed at the beginning of the natural release of dormancy (beginning of March), and fresh weights were determined at the 21st day of culture. *indicates significant difference ($p \leq 0.05$) from 2,4-D. Data are the mean of 15 replicates (two experiments).

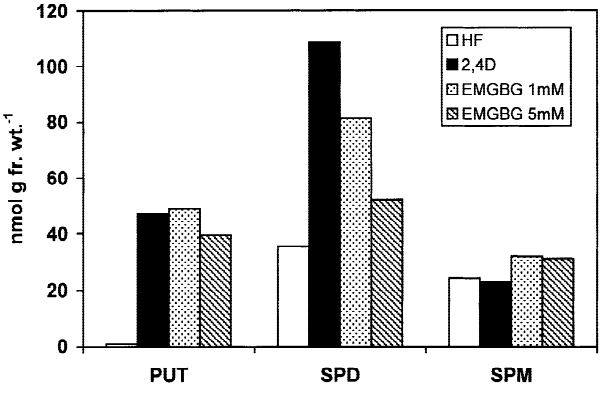


Fig. 4. Free polyamine content in explants from *H. tuberosus* tubers cultured in vitro. The experiment was performed at the beginning of the natural release of dormancy (beginning of March), and fresh weights were determined at the 6th day of culture. PUT, putrescine; SPD, spermidine; SPM, spermine. Data are the mean of four replicates. Error represents less than 10% of the mean values.

curred in the tissue. Thus, the different behavior of the inhibitors may be related to differences in the endogenous level of plant growth substances, in particular polyamines, as reported previously (Bagni and Serafini-Fracassini 1985) and to their interactions with exogenously supplied hormones. In spite of this, the inhibitors were able to partly (1 mM) or completely (5 mM) block the growth to the level of explants from tubers during deep dormancy grown without 2,4-D (see Fig. 2, HF). Fig. 4 shows the effect of EMGBG, which turned out to be one of the most potent inhibitors of growth (Fig. 2), on polyamine content in the explants.

Spermidine was the most abundant polyamine found in the tuber during dormancy, followed by spermine, whereas putrescine was present only in trace amounts (Fig. 4, HF). Polyamines were present in this tissue only in the free form, and no covalently bound polyamines were detected in the perchloric acid-soluble fraction (mostly as cinnamic acid derivatives), as reported previously (Bagni et al. 1994). Already after 6 days of culture, coinciding with the first macroscopic signs of growth inhibition, a sharp decrease in the levels of spermidine, clearly dose-dependent, was evident in explants treated with EMGBG. This depletion of spermidine levels might account for the growth inhibition. EMGBG has been shown to be one of the two most potent bis(guanylhydrazone)-type inhibitors of the AdoMetDC of *Saccharomyces cerevisiae* (Elo et al. 1986, 1988) and would be expected to inhibit also the AdoMetDC of *H. tuberosus*, as found for MGBG in the same tissue (Barbieri et al. 1983). Thus, it is reasonable to assume that spermidine in explants treated with 2,4-D plus EMGBG was markedly and in a dose-dependent manner decreased compared with explants treated with 2,4-D alone. Yet, EMGBG, at the early stages of growth, did not totally revert the increase in spermidine concentration caused by 2,4-D.

The difference in polyamine content, in particular putrescine, in the tissue treated with 2,4-D plus inhibitors compared with that without 2,4-D could reflect a differential uptake rate of 2,4-D compared with that of the inhibitor. A more rapid absorption of 2,4-D compared with the inhibitor may activate the biosynthesis of polyamines in the early phases following the break of dormancy. In fact polyamine synthesis has been reported to be one of the first events occurring after the break of dormancy, as it begins within 15 min after excision of the tissue, in the early G_1 phase of the first cell cycle (Bagni and Serafini-Fracassini 1985).

Because the K_i values of the inhibitors for the AdoMetDC of *H. tuberosus* are not known, the correlation between K_i (AdoMetDC) and growth inhibition cannot be investigated. Yet, it appears that the growth inhibition of *H. tuberosus* might be correlated with AdoMetDC inhibition because (when going from the unsubstituted congener GBG up to the three-carbon-substituted level, i.e. EMGBG) the increase in the number of side chain carbon atoms also increased the inhibitory potency of the compounds toward the AdoMetDC of *S. cerevisiae* (Elo et al 1986). Most of the bis(guanylhydrazones) have been tested for their ability to compete with spermidine for uptake into cells. For this purpose, protoplasts isolated from the phloem parenchyma of *D. carota* taproots were utilized because as demonstrated previously (Bagni et al. 1994), they represent a good model system for transport studies.

As far as the effect of inhibitors on polyamine uptake is concerned, none of the compounds tested inhibited spermidine uptake into carrot protoplasts up to concentrations 20 times higher than the polyamine. At these

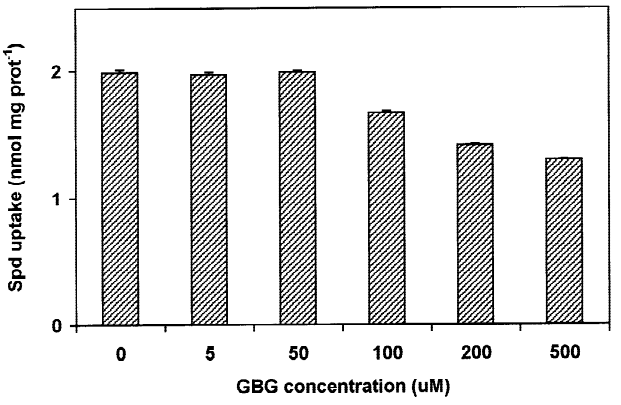


Fig. 5. Effect of GBG on spermidine uptake in carrot protoplasts. Uptake was initiated by adding simultaneously GBG and [^{14}C]spermidine to medium containing protoplasts, and it was followed for 2 min. Data are the means ± S.E. of three different experiments with samples in duplicate.

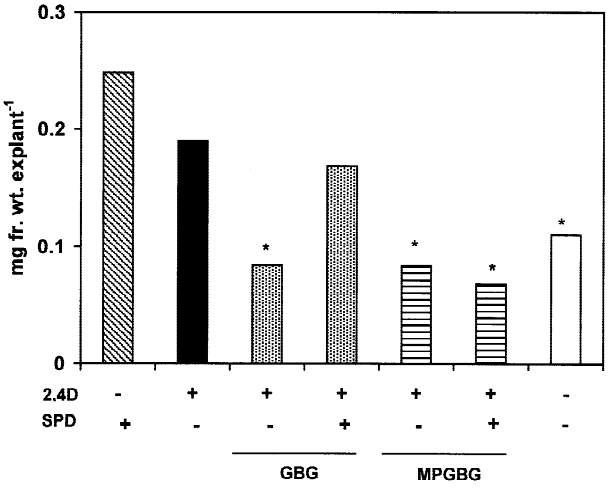


Fig. 6. Prevention of antiproliferative effects of GBG and MPGBG by coincubation with spermidine (SPD). *H. tuberosus* explant cells were coincubated with 1 mM GBG or MPGBG and 0.1 mM spermidine. Fresh weights were determined at the 21st day of culture. *indicates significant difference ($p \leq 0.05$) from 2,4-D (*black bar*).

concentrations, a slight inhibitory effect was observed, and it increased by increasing the concentration of the inhibitor further. Fig. 5 shows the effect of GBG on spermidine uptake, and it reflects the pattern found with all other inhibitors (data not shown). This suggests that, in spite of the structural similarity, the bis(guanylhydrazones) do not compete with spermidine for entry into carrot protoplasts up to high concentrations.

Contrary to L1210 mouse leukemia cells, in which bis(guanylhydrazones) and particularly the glyoxal, methylglyoxal, and ethylglyoxal ones equally inhibited the uptake of spermidine, thus indicating that these compounds had roughly equal affinity for the polyamine carrier (Alhonen-Hongisto et al. 1984), in carrot protoplasts bis(guanylhydrazones) seem to have a low affinity for the spermidine carrier, probably because of their steric hindrance. In fact they compete for spermidine influx only at concentrations 20- to 40-fold higher than the polyamine, even though a slight competition between MGBG and spermidine was reported previously (Antognoni et al. 1993). Nevertheless, in other systems, EGBG and DMGBG are poorly taken up also in mammalian cells (Jänne and Morris 1984). These results are in agreement with previous studies performed using carrot cells, where a specific spermidine transport system was not shared by MGBG (Pistocchi et al. 1987). Moreover, recent studies carried out utilizing unusual aliphatic and branched polyamines as putative substrates of the polyamine transport system in carrot protoplasts have confirmed its specificity toward the common polyamines because no competition was found (Antognoni and Bagni 1997).

When cells were treated with 1 mM GBG, which inhibited growth by about 50% (Fig. 2), spermidine, when given simultaneously, was able to abolish the antiproliferative effect almost completely (Fig. 6). Because our results on uptake competition seem to suggest that GBG does not share the spermidine transport system, it is likely that the reversion of growth inhibition by spermidine was caused by the increase of intracellular pools by the exogenous polyamine rather than by a block of inhibitor uptake by spermidine. Conversely, the antiproliferative effect exerted by MPGBG was not prevented by the addition of spermidine (Fig. 6).

In conclusion, the different bis(guanylhydrazones) tested affect the growth of *H. tuberosus* tuber explants, and this inhibition is probably caused by a decrease in spermidine content, as this polyamine represents the most abundant polyamine in the tissue. With regard to the effect exerted by these compounds on polyamine uptake, the scarce competition observed suggests a strict specificity of the polyamine transport system, although a different uptake rate of these inhibitors compared with polyamines cannot be excluded.

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