MGBG in Combined Anticancer Chemotherapy

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Abstract: The combined activity of MGBG (metylglyoxal-*bis*(guanylhydrazone)) with either cisplatin or carboplatin was investigated, towards the human breast cancer cell line MCF-7. While a dose- and time-dependent cytotoxic activity was already observed for MGBG alone, a synergistic and non-reversible effect was verified upon co-administration with these Pt-based drugs. Furthermore, toxicity against non-neoplastic cells was shown to be quite low for these drug cocktails, as opposed to either Pt-agents alone. These results may hopefully allow to use lower dosages of both MGBG and the Pt-drugs, minimising the drug´s deleterious side effects while keeping antitumour efficacy, in an anticancer strategy that couples interference with polyamine homeostasis with DNA damage.

INTRODUCTION

Methylglyoxal-*bis*(guanylhydrazone) (mitoguazone or MGBG, Fig. **1**) structurally related to the biogenic polyamine spermidine, has been widely studied since the discovery (in 1972) of its selective inhibitory action against S-adenosylmethionine decarboxylase (SAM*dc*) [1, 2]. This direct involvement in biogenic polyamine biosynthesis (spermidine and spermine) [3-6], known to be significantly deregulated in pre-carcinogenic and carcinogenic states [7], prompted the study of this compound as a potential antineoplastic agent, either alone or in combination with conventional drugs.

MGBG´s cytotoxicity was first demonstrated in the 1960´s, towards acute myelogenous leukaemia [8, 9] but it was quickly abandoned as an anticancer agent due to its severe side-effects (*e.g.* mucositis and myelosuppression [10]). Nevertheless, it was later rediscovered, when new clinical trials demonstrated that its scheduled (weekly) administration strongly decreases MGBG´s toxicity while preserving its antitumour properties, particularly in Hodgkin's and non-Hodgkin's lymphoma [4]. Moreover, its use in combined therapy has shown to yield promising results in several types of cancer, namely myeloid leukemia (with difluormethylornithine (α-DFMO) [9], refractory lymphoma (with cisplatin [11]) or breast cancer (with 2'2'-difluorodeoxycytidine (Gemcitabine) [5]).

Breast cancer represents 14% of female cancer deaths worldwide [12], more than one million cases being detected each year. Although extensive preclinical and clinical studies have resulted in modest success in decreasing the morbidity of this type of neoplasia, no successful chemotherapeutic drug is yet available to control the disease. In estrogen-dependent breast cancers such as MCF-7 cell growth is controlled by regulating hormonal factors, namely estrogens.

Therefore, one of the most common chemotherapeutic practices against this kind of neoplasias is administration of antiestrogens, either targeting estrogen synthesis (*e.g.* aromatase inhibitors) or estrogen receptors (selective estrogen receptor modulators, SERM´s). However, most tumours eventually develop resistance to this type of therapy. Additionally, MCF-7 human breast cancer cells are relatively resistant to cisplatin treatment compared to other breast cancer cell lines, and this is further limited by the drug-related adverse effects [13, 14]. Therefore, the development of alternative therapeutic strategies against this type of breast cancer is of paramount importance, namely by enhancing the cells' sensitivity to cisplatin (or other Pt-based conventional drugs) [15], or through combination of antitumour agents with distinct mechanisms of action.

In the present study, the effect of co-administration of MGBG with either cisplatin (*cis*-$(NH_3)_2PtCl_2$, cDDP) or carboplatin ($Pt(NH_3)_2C_6O_4H_6$) (Fig. **1**) was investigated, towards the estrogen-dependent human cancer cell line MCF-7. A sinergistic effect between these two agents is sought, by targetting two distinct mechanisms of action: interference with the polyamine homeostasis (MGBG) and with DNA replication and transcription (Pt-based compounds). Both cell-growth and cell viability were evaluated, as well as the reversibility of the cytotoxic effect and the toxicity towards non-carcinogenic cells (non-immortalised, BJ human foreskin fibroblasts).

MATERIALS AND METHODS:

Chemicals

Cisplatin, carboplatin, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Dulbecco's modified Eagle's high glucose medium (DMEM-HG), phosphate-buffered saline (PBS) solution, sulforhodamine B (SRB) and Trypan blue (0.4% solution) were purchased from Sigma-Aldrich Chemical Co. (Sintra, Portugal). Antibiotics (penicillin-streptomycin 100x solution) and methylglyoxal-*bis*(guanylhydrazone) (MGBG) were obtained from Janssen (Belgium). Fetal calf serum

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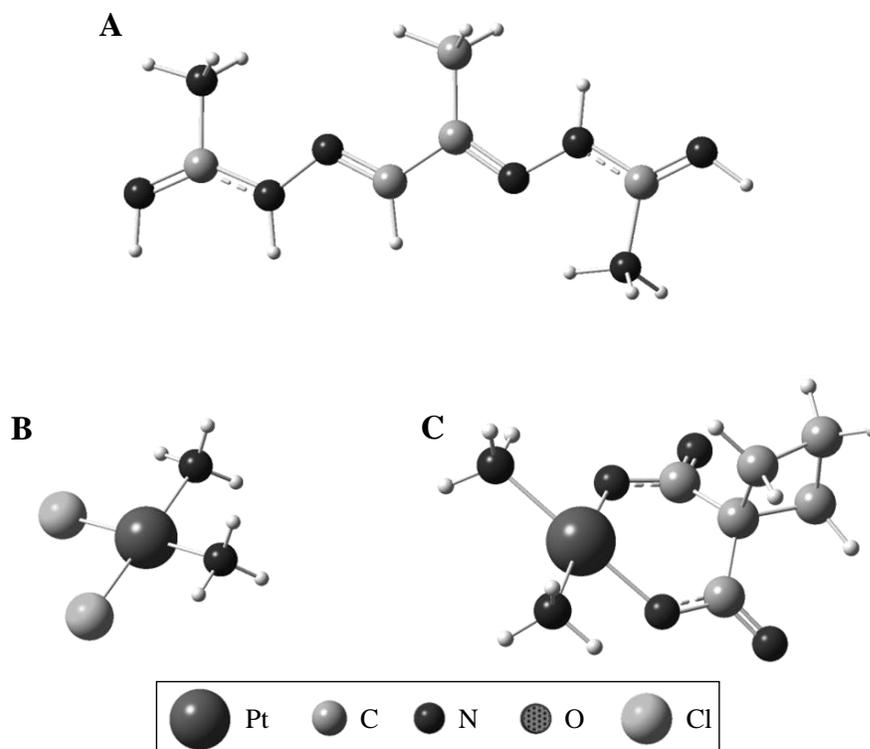
A

B   **C**

⬤ Pt	⬤ C	⬤ N	⬤ O	⬤ Cl

Fig. (1). Structural representation of the compounds under study: (**A**) – MGBG; (**B**) – cisplatin; (**C**) – carboplatin.

(FCS), trypsin-EDTA (0,05%). All the other reagents (*pro analysis* grade) were obtained from Sigma-Aldrich Chemical Co. (Sintra, Portugal).

Cell Lines

The human breast estrogen-receptor positive (ER+) adenocarcinoma cell line MCF-7 was kindly made available by the Centre for Neuroscience and Cell Biology (CNC) of the University of Coimbra, while the non-carcinogenic, non-immortalised, human foreskin fibroblast cells (BJ) were purchased from the American Type Culture Collection (CRL-2522™, ATCC, USA).

Preparation of Solutions

MGBG, cDDP and carboplatin stock solutions were prepared in phosphate-buffered saline (PBS) solution to the desired concentrations (Table **1**) and sterilised by filtration.

MTT was prepared, in a concentration of 0.5 mg/mL, in PBS solution. SRB was used as a 0,5% *(w/v)* solution, in 1% *(v/v)* acetic acid.

Cell Culture

The cells were grown as monolayers, at 37ºC, in a humidified atmosphere of 5% CO_2, in DMEM-HG (4500 mg/mL) medium, supplemented with 10% heat inactivated fetal calf serum, glutamine (13,4 g/L), antibiotics (100 units of penicillin and 100 mg streptomycin) and 1,5 g sodium bicarbonate (pH 7,4). The cell lines were subcultured when 85% confluence was attained, by washing with PBS and trypsinising with 0,05% trypsin-EDTA solution.

Antiproliferative Activity

The antiproliferative activity of MGBG was evaluated towards the MCF-7 cell line, using the SRB method [16, 17].

Table 1   **Systems Under Study and Concentration Range Used in the Biological Assays**

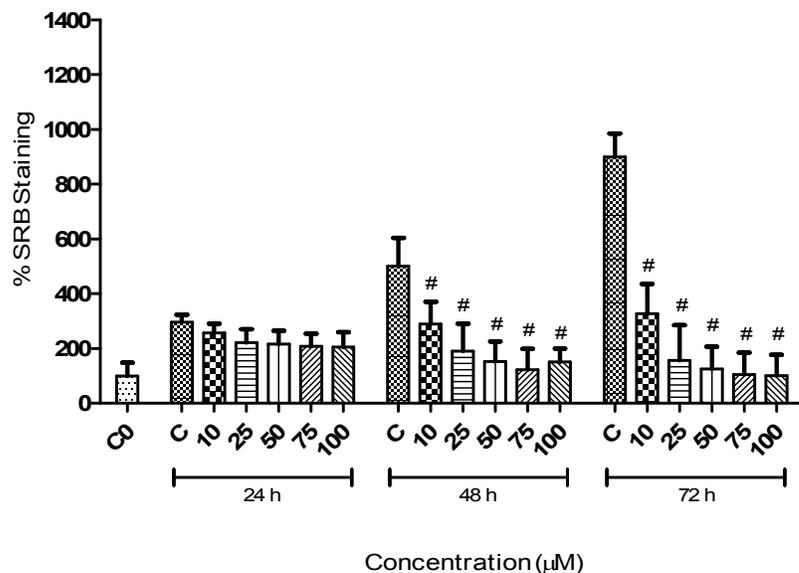|	Concentrations (µM) Cell line	
Drugs	**MCF-7**	**BJ**
MGBG	10, 25, 50, 75, 100	-
cDDP10/MGBG cocktail	5, 10, 20, 50, 100	5, 10, 50
cDDP25/MGBG cocktail	5, 10, 20, 50, 100	5, 10, 50
Carboplatin25/MGBG cocktail	5, 20, 100	-
Carboplatin20/MGBG cocktail	5, 20, 100	-

Fig. (2). Time dependence of the antiproliferative activity of MGBG against the MCF-7 cell line. Cells (3×10^4 cells/cm^2) were incubated with the compounds for periods of 24 to 72 h. Every 24 h the cell density was measured by the SRB colorimetric assay. The drug was removed 72 h after seeding, and the cell growth was assessed following a further 72 h incubation. The data are expressed as a percentage of the control SRB staining, and represents the average±SD from three independent experiments carried out in triplicate ([#]$p < 0.0001$).

The cells were seeded for 24 h, at a density of 3.0×10^4 cells *per* well (in 24-wells plates), prior to adding the compounds. The cells were counted every 24 h, for a total exposure period of 72 h. The compound-containing medium was removed, the cells were fixed (in cold 1% acetic acid:methanol *(v:v)*) and incubated for 2 h at 4°C. Upon air drying, 0.5% *(w/v)* SRB in 1% acetic acid was added. At the end of the staining period (1h at 37°C), the unbound SRB was washed with 1% acetic acid. The plates were air-dried again and the cell-bound dye was solubilized in 10 mM aqueous Tris base. The plates were shaken, and the absorbance mesured at 540 nm, yielding the cell density values.

Cytotoxic Activity and Reversibility of the Compounds' Effect

Cell viability as a function of the compounds' concentration – MGBG and cDDP:MGBG – was determined for both cell lines studied, at a density of 3.0×10^4 cells *per* well (24-wells plates for MCF-7 and 48-wells plates for BJ). The carboplatin:MGBG cocktails were screened only towards the MCF-7 cell line. The cells were seeded for 24 h prior to adding the tested agents. Culture analysis was performed every 24 h, for a total period of 48 and 72 h for the BJ and MCF-7 lines, respectively. A MTT solution (0.5 mg/mL) [18, 19] was then added to each well. After 3 h incubation at 37 °C, the formed formazan crystals were
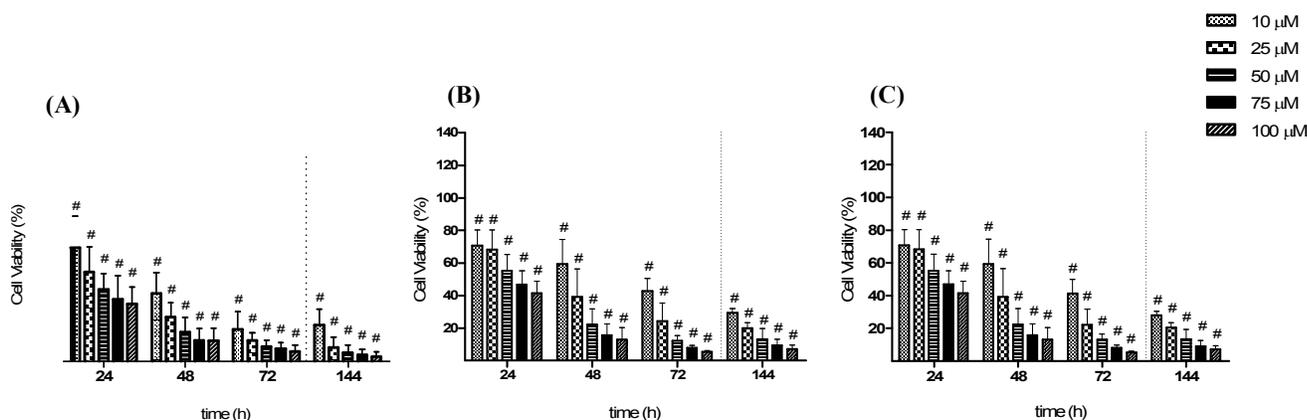

Fig. (3). Time dependence of the cytotoxic effect of the compounds studied against the MCF-7 cell line: (**A**) MGBG, (**B**) cDDP10/MGBG and (**C**) cDDP25/MGBG. Cells (3×10^4 cells/cm^2) were incubated with the compounds for periods of 24-72 h. Cells (3×10^4 cells/cm^2) were incubated with the compounds for periods of 24 to 72 h. Every 24 h the cell viability was evaluated by the MTT colorimetric assay. The drug was removed 72 h after seeding, and the viability was assessed following a further 72 h incubation. The data are expressed as a percentage of the control MTT reduction (100%), and represent the average±SD from three independent experiments carried out in triplicate ([#]$p < 0.0001$).
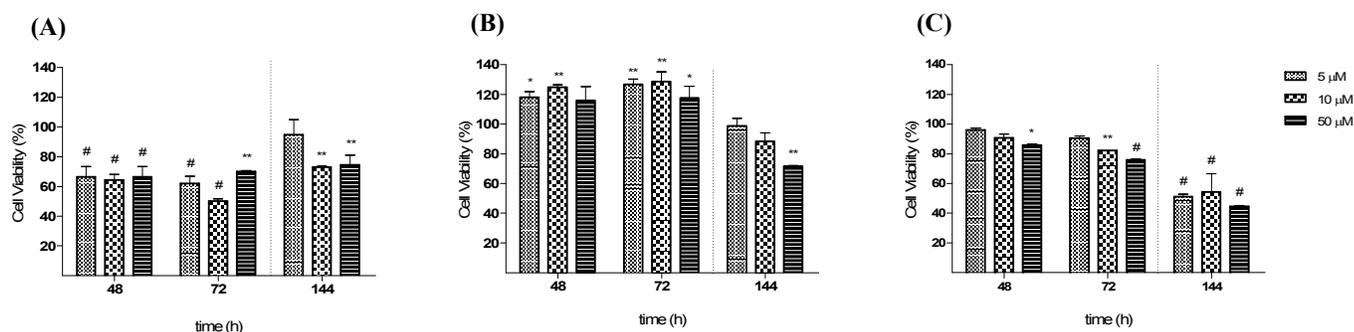
(A)

(B)

(C)

Fig. (4). Time dependence of the cytotoxic effect of the compounds studied against the BJ cell line: (**A**) MGBG, (**B**) cDDP10/MGBG and (**C**) cDDP25/MGBG. Cells (3 x 10^4 cells/cm^2) were incubated with the compounds for periods of 24-72 h. Cells (3 x 10^4 cells/cm^2) were incubated with the compounds for periods of 24 to 72 h. Every 24 h the cell viability was evaluated by the MTT colorimetric assay. The drug was removed 72 h after seeding, and the viability was assessed following a further 72 h incubation. The data are expressed as a percentage of the control MTT reduction (100%), and represent the average±SD from one independent experiments carried out in duplicate (*$p < 0.05$; **$p < 0.001$ and #$p < 0.0001$).

solubilized in dimethylsulfoxide. Finally, the absorbance was measured at 570 nm, and cell viability was determined.

In order to assess the reversibility of this cytotoxic activity, the drug-containing culture medium was removed and fresh medium was added in the last day of incubation, and the cell viability was obtained after 72 h through the MTT test (Figs. **2** to **5**).

Statistical Analysis

The experiments with the MCF-7 cells were performed in triplicate, in three independent assays. Preliminary studies with the BJ cell line were performed in duplicate, in one experiment. The results are expressed as means±SD and compared with the non-treated controls. Statistical analysis were carried out using One-Way ANOVA, followed by Turkey's post-test for the SRB assays, and by Dunnett's post-test for the MTT tests. Statistical comparison between

the data was based on the Pearson correlation coefficient, values with p<0.05 being considered as significant.

RESULTS AND DISCUSSION

The polyamine antimetabolite MGBG was evaluated as to its antiproliferative and cytotoxic properties towards the estrogen-receptor positive (ER+) human breast cancer cell line MCF-7, both alone and co-administered with the conventional Pt-based drugs cisplatin or carboplatin (Table **1**).

The results obtained by the sulforhodamine B method [17] evidence that MGBG exhibits a dose and time-dependent antiproliferative effect towards this cell line, for the concentration range between 10 and 100 µM, after 48 h of incubation (Fig. **2**). This is in agreement with the conclusions attained in a previous study performed in
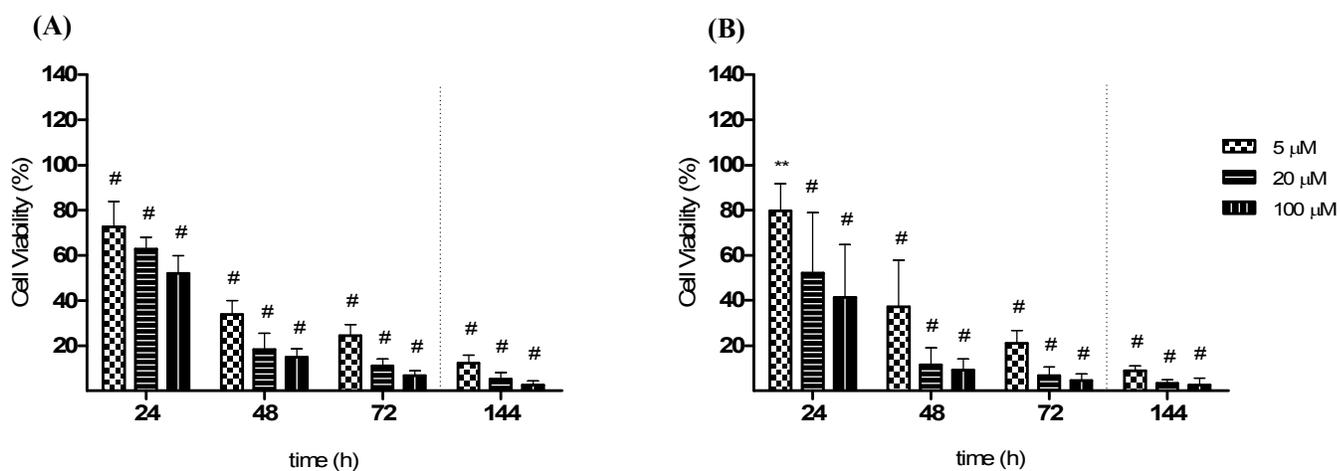
(A)

(B)

Fig. (5). Time dependence of the cytotoxic effect of the compounds studied against the MCF-7 cell line: (**A**) carboplatin25/MGBG and (**B**) carboplatin50/MGBG. Cells (3 x 10^4 cells/cm^2) were incubated with the compounds for periods of 24-72 h. Cells (3 x 10^4 cells/cm^2) were incubated with the compounds for periods of 24 to 72 h. Every 24 h the cell viability was evaluated by the MTT colorimetric assay. The drug was removed 72 h after seeding, and the viability was assessed following a further 72 h incubation. The data are expressed as a percentage of the control MTT reduction (100%), and represent the average±SD from three independent experiments carried out in triplicate (**$p < 0.001$ and #$p < 0.0001$).

cultures of rat hepatocytes [20] as well as on a reported study on human histiocytic lymphoma that evidenced a decrease in protein synthesis after 20 to 25 h of exposure to the compound [21]. MGBG´s antiproliferative activity is suggested to be delayed due to conformational rearrangements undergone by the molecule at physiological conditions (since several species, with distinct activities, may coexist in the plasma).

MGBG's cytotoxic effect was also evaluated (Fig. **3**), through the MTT assay for mitochondrial dehydrogenase activity [16, 18, 19]. Exposure to MGBG led to a cell viability decrease that progresses over incubation time (from 24 to 72 hours). This may be related to changes in mitochondrial permeability due to reduction of the intracellular concentration of spermidine and spermine, since these are known to play a key role in the inhibition of mitochondrial permeability transition [22, 23]. This cytotoxic effect was found to be irreversible, as the cells could not recover and viability was kept at low values upon MGBG removal from the cell culture medium (after 72 h of exposure). Also, this compound provided a larger cytotoxicity than cisplatin, for the same cancer line and concentration range [personal data, unpublished].

MGBG´s cellular uptake is proposed to occur through the transport system of biogenic polyamines [20]. By inhibiting S-adenosylmethionine decarboxylase, it leads to a decrease of the spermidine and spermine intracellular concentration, consequently reducing *in vitro* cell growth and viability. This diminished polyamine pool affects DNA synthesis [1] by destabilization of the DNA molecule, as previously demonstrated in human leukemia cells (HL-60) [24].

Knowing that MGBG displays significant antiproliferative and cytotoxic activities against the breast cancer line MCF-7 (Figs. **2** and **3**), its effect when combined with the chemotherapeutic drugs cisplatin (cDDP) or carboplatin was assessed. These experiments were performed by using a mixture of the two compounds, with varying concentrations of MGBG and a constant cDDP or carboplatin dosage. The results for cDDP:MGBG, comprised in Figs. (**3B** and **3C**) for a cisplatin concentration of 10 and 25 µM, respectively, evidence a clear cytotoxicity towards the MCF-7 cells, already for 25 µM MGBG after 48 hours of incubation, attaining a maximum (*ca.* 7% cell viability) at 72 hours of drug exposure, for MGBG´s highest concentration (100 µM). Irreversibility of the drug effect was also observed for these

cocktails, probably as a result of a twofold mechanism: formation of cDDP–DNA adducts responsible for an irreversible damage (*e.g.* through inefficiency of the cell repair mechanisms [25, 26], coupled to the MGBG inhibitory action at the polyamine level (interference with polyamine biosynthesis). These results reflect a significant sinergistic effect between MGBG and cisplatin: viability decrease of *ca.* 17% upon a 72 h incubation with cDDP-10 µM:MGBG-50 µM, as compared to a viability loss of 40% for the same cDDP dosage and identical experimental conditions [27, 28].

Similarly, co-administration of MGBG and carboplatin caused a more pronounced cytotoxicity relative to each of the two isolated compounds (Fig. **5**, Table **2**). Also, this synergistic effect was more pronounced than for the cDDP:MGBG cocktails: the same drug dosage (25 µM) led to a much lower LD_{50} in the case of carboplatin – 1.268 *vs* 7.825 (Table **2**). Nevertheless, maximum cytotoxicity was verified for longer incubation times than for the cDDP cocktails, as a consequence of the slower hydrolysis kinetics of carboplatin [29], which leads to a longer time for drug activation within the cell. Actually, cisplatin's chloride atoms are expected to undergo an easier substitution than carboplatin's leaving groups [30].

The LD_{50} values (lethal dosis of drug yielding a 50% of cell viability decrease) were determined for all the compounds tested (Table **2**). Both cDDP:MGBG and carboplatin:MGBG cocktails were responsible for lower LD_{50} dosages as compared to cDDP alone, for 48 and 72 h incubation times. Relative to MGBG, however, a sinergism was only verified for 48 h of drug exposure.

In order to assess the degree of toxicity of the agents under study towards non-neoplastic cells, experiments were carried out against non-immortalised human foreskin fibroblasts (BJ cell line), through the MTT colorimetric assay. Both MGBG and the cDDP:MGBG cocktails were found to induce a relatively low toxicity towards the BJ line – no more than 60-65 % viability loss, even for a 72 h exposure and a cDDP-25 mM:MGBG-50 µM dosage (Fig. (**4C**). These observations can be interpreted in the light of physiological differences between neoplastic and non-neoplastic cells, which respond differently to the drug action. In the case of the MCF-7 cell line, the tested agents probably undergo a quick and extensive internalisation as a consequence of the rapid cancer cell growth thus causing a severe and irreversible effect. In turn, human foreskin

Table 2. LD_{50} **Values (µM) Towards the MCF-7 Cell Line, for the Compounds**

Drugs	LD50 (µM)		
	24 h	48 h	72 h
MGBG	44.640	8.284	1.737
cDDP	56.090	30.550	[a]13.010
cDDP10/MGBG cocktail	49.450	6.285	3.793
cDDP25/MGBG cocktail	49.450	7.825	3.429
Carboplatin25/MGBG cocktail	N.A.	1.268	0.845
Carboplatin50/MGBG cocktail	106.400	2.719	1.066

N.A. – experimental data did not yield convergent results [a][28].

fibroblasts appear to recover from the MBGB´s cytotoxic effect, as the slower dividing cells internalise smaller amounts of compound [2, 31]. This, however, does not discard contribution from other mechanisms, that may differ between cancer and healthy cells.

Co-administration of MGBG and the conventional drugs cisplatin or carboplatin towards the MCF-7 cell line clearly led to a significant synergistic effect relative to the Pt-agent when given separately. This constitutes a promising result regarding the use of MGBG in newer chemotherapeutic anticancer regimes, since it allows to use lower dosages of both this compound and the Pt-based agents, thus minimising drug´s deleterious side effects and acquired resistance.

Furthermore, evaluation of the drug's reversibililty and of its effect on non-neoplastic cell lines (for both MGBG alone and the cocktails) are of paramount importance for assessing effectiveness, administration conditions/patient compliance and toxicity. These are essential parameters to measure and interpret, in view of developing efficient anticancer strategies that couple interference with polyamine homeostasis with DNA damage.

CONCLUSIONS

MGBG has been shown to affect cell growth and viability through modulation of polyamine intracellular pool, for an estrogen-dependent human breast cancer cell line. Co-administration of this SAMDC inhibitor and the convencional Pt-based drugs cisplatin or carboplatin, in turn, led to a synergetic effect.

This constitutes a promising result regarding the use of MGBG as a part of an anticancer strategy, since allows to use lower dosages, when combined to either cisplatin or carboplatin, thus minimising or even avoiding MGBG's deleterious side effects.

Furthermore, evaluation of the drug's reversibililty (both for MGBG alone and the cocktails) and of its effect on non-neoplastic cell lines are of paramount importance for assessing effectiveness, administration conditions and toxicity. These are essential parameters to measure and interpret, in view of developing efficient anticancer strategies that couple with polyamine homeostasis (*via* the polyamine biosynthetic pathway).

ACKNOWLEDGEMENTS

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