Anti-Cancer Drugs 1998, 9, pp. 733–738

Preclinical report

Megestrol acetate antagonizes cisplatin cytotoxicity

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Megestrol acetate (MGA) is being widely used for the improvement of appetite and performance status in patients receiving chemotherapy, especially cisplatin-containing therapy. However, little is known about whether MGA has an effect on cisplatin cytotoxicity. We have investigated this using two transitional carcinoma cell lines, i.e. the cisplatin-sensitive parental line NTUB1 and the resistant daughter line NTUB1/P. Combined effects of MGA and cisplatin were assayed with a microculture chemosensitivity method. We explored the level changes of several cisplatin detoxification mechanisms, including metallothionein (MT), glutathione S-transferase-π (GST-π) and glutathione (GSH) levels in cells treated with or without MGA. After treatment with 10 μm MGA for 24 h, the cisplatin IC_{50}s of NTUB1 and NTUB1/P increased 1.4- (p=0.03) and 1.6- (p=0.02) fold, respectively. By median effect analysis, the combinations of MGA and cisplatin in the two cells appeared to produce an antagonistic interaction. By Northern analysis, MT transcript levels in both cells were significantly upregulated after treatment with MGA, as compared to those without treatment. Exposure to MGA in either sensitive or resistant cells did not alter GST-π levels as shown by immunoblotting analysis. Cellular GSH content was increased only in NTUB1/P (p=0.0036) but remained unchanged in NTUB1 cells (p=0.29) after MGA exposure. In conclusion, MGA may antagonize cisplatin cytotoxicity by upregulating cellular MT and GSH levels. Use of MGA in cisplatin-containing chemotherapy may impair tumor response by antagonizing cisplatin antitumor activity. [© 1998 Lippincott Williams & Wilkins.]

Key words: Cisplatin, drug treatment, megestrol acetate, transitional carcinoma.

Introduction

Cisplatin-containing chemotherapy has been widely used in the treatment of a variety of solid tumors, including malignancies of the respiratory, genitourinary and gastrointestinal tracts. Patients receiving cisplatin may become moderately to severely anorexic and emetic, and have poor intake. Megestrol acetate (MGA) has been known for its favorable effects on cancer-related cachexia and anorexia, enhancement of oral intake, and gaining body weight. Numerous studies suggested the liberal use of high-dose MGA in conjunction with systemic chemotherapy, especially cisplatin-containing therapy, to improve patients' nutritional status and hopefully the response rates to cytotoxic therapy.[1,2] However, the drug interaction between cisplatin and MGA has never been explored.

Recently, a randomized clinical trial comparing the response rates of cisplatin-containing chemotherapy with or without additional MGA for small cell lung cancer showed that patients with MGA had a significantly poorer response rate than those without MGA.[3] However, the reason for this has not yet been explored. We were curious about whether there is an antagonistic interaction between MGA effect and cisplatin cytotoxicity in a clinical setting. In this article, we present *in vitro* data showing that MGA-exposed cells are more resistant to cisplatin cytotoxicity than those not exposed to MGA. The possible mechanisms involved have been explored.

Materials and methods

Using cultured transitional carcinoma (TC) cells, combined effects of MGA and cisplatin were evaluated in a microculture chemosensitivity assay with median effect analysis. Since numerous reports have indicated that such cellular mechanisms as metallothionein

This work was supported, in part, by grants NSC85-2331-B-002-317 and NSC86-2314-B-002-118 from National Science Council, Executive Yuan, ROC.

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(MT),[4,5] glutathione S-transferase-π (GST-π),[6,7] and cellular glutathione (γ-glutamylcysteinylglycine, GSH)[8,9] levels are associated with cisplatin chemoresistance, alterations of these cellular factors after MGA exposure were determined.

Cell lines

NTUB1, an immortalized TC cell line raised from a high grade bladder cancer,[10] and its cisplatin-resistant daughter line, NTUB1/P, were used in the study. NTUB1/P can thrive in medium containing 14 μM cisplatin and the cisplatin IC_{50} (drug concentrations inhibiting cell growth by 50%) of this subline is 5.2-fold higher than that of NTUB1. Both lines were maintained in RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL) at 37 °C in humidified air with 5% CO_2.

Microculture chemosensitivity assay

Cellular chemosensitivities to cisplatin and/or MGA were assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT; Sigma, St Louis, MO) assay to determine cell viability *in vitro*.[11] In brief, 3000 (NTUB1) or 7000 (NTUB1/P) cells per well in 100 μl culture medium were seeded into 96-well microplates and incubated at 37 °C for 24 h before drug exposure. Cisplatin (Pharmacia & Upjohn, Milan, Italy) and MGA (Sigma) reconstituted in culture medium were given either alone or in combination to make the final volume 200 μl per well. Drug exposure was maintained for 72 h at 37 °C before MTT assay. Cell numbers plated were titrated so as to keep control cells growing in the exponential phase throughout the 96 h incubation period before the viability measurement. By the end of drug exposure for 72 h, 50 μl MTT (2 mg/ml in RPMI medium) was added to each well and allowed to react for 2.5 h. Blue formazan crystals that formed were pelleted to the well bottoms by centrifugation, separated from the supernatant and dissolved in 150 μl dimethylsulfoxide (Sigma). The optical density was determined by absorbance spectrometry at 492 nm using a microplate reader (SLT Molecular Device, Untersbergstrabe, Austria). Three separate experiments with triplicate data for each drug combination were performed to obtain the mean effects at given drug concentrations. The drug interaction between cisplatin and MGA was determined by the median effect analysis with the mutually non-exclusive model described by Chou and Talalay.[12] The effects of two-drug combinations (synergism, additivism or antagonism) were transformed into and displayed in the fraction affected (f_a)-combination index (CI) plots. CI values of less than 1 indicate synergism, greater than 1 antagonism and equal to 1 additivism. The IC_{50}s were also calculated by median effect analysis and expressed as mean ± SD.

Metallothionein expression

Northern blotting analysis was performed to evaluate the cellular levels of MT transcript with or without MGA exposure. NTUB1 and NTUB1/P cells were treated with or without 10 μM MGA (non-toxic and serum achievable concentration) for 24 h. Total cellular RNA was obtained by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[13] A digoxigenin (Dig)-labeled hybridization probe (148 bp) for MT was generated by polymerase chain reaction (PCR) to amplify placental cDNA as previously described.[14] The PCR program (25 cycles) used was: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 58.8 °C for 30 s, extension at 72 °C for 60 s and an additional extension time at 72 °C for 10 min. The MT primer pair (sense strand 5′-ATGGACCCCAACTGCTCCTG-3′; antisense strand 5′-ATATGCAGCCCTGGGCACAC-3′) was designed to allow the PCR product to span over a large intron (GenBank Database accession no. K01383).[15] The PCR generated probe was sequenced to confirm the right product. Total RNA (30 μg) was size fractionated in a 1% agarose formamide gel in the presence of DNase. After equilibrating the gel twice in 20 × SSC, the gel was transferred to a positively charged nylon membrane with UV cross-linking. The membrane was prehybridized by incubating with the prehybridization buffer (Boehringer Mannheim, Indianapolis, IN). Hybridization with the Dig-labeled cDNA probe was performed in the hybridization buffer (Boehringer Mannheim) at 50 °C overnight. The membrane was then washed, blocked and developed as suggested by the manufacturer.

GST-π levels

Immunoblotting was performed as previously described[16] with some modifications. Cells (2×10^7) treated with 10 μM MGA for 24 h were harvested, washed twice with phosphate buffered saline (PBS), pelleted and resuspended in 0.1 ml PBS. Cells were frozen at −30 °C for 1 h, thawed on ice and lysed by adding 0.2 ml hypotonic solution (10 mM Tris-HCl,

pH 7.4, 10 mM NaF, 5 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin) at 4°C for 20 min with gentle mixing. An aliquot of hypertonic solution (1 M NaCl, 0.3 ml) was added to further lyse the nuclei. The lysate was then incubated at 4°C for 20 min and centrifuged at 4°C for 20 min to collect the supernatant. An aliquot of 100 μl of the protein extract and the same volume of sample buffer (0.5 ml 0.5 M Tris-HCl, pH 6.8, 0.4 ml glycerol, 0.8 ml 10% sodium dodesylsulfate, 0.2 ml 2-mercaptoethanol, 0.1 ml 0.05% bromophenol blue and 2 ml distilled water) were mixed and boiled for 5 min to disrupt the disulfide linkage. An aliquot of the mixture containing 60 μg protein was resolved in 12% SDS-polyacrylamide gel. The gel was then treated with the transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) ethanol) for 30 min and was electrotransferred to a polyvinylidene difluoride membrane. The membrane was washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked with skim milk (5% in TBS-T) for 1 h. The membrane was incubated with rabbit anti-human GST-π polyclonal antibody (Signet, Dedham, MA; 1:200 dilution in 5% skim milk) at 4°C overnight. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Caltag, San Francisco, CA; 1:400 in 5% skim milk). Band signals were developed with 3,3'-diaminobezidine tetrahydrochloride (1 mg/ml in 0.1 M Tris buffer, pH 7.2 and 0.02% H_2O_2) for at least 30 min.

GSH contents

Cellular GSH levels were assayed with the following biochemical method. NTUB1 and NTUB1/P cells were treated with or without 10 μM MGA for 24 h. An aliquot of 5×10^6 cells was resuspended in 0.5 ml 5% metaphosphoric acid (Sigma) at −30°C for 20 min. Cells were lysed by three cycles of freeze-and-thaw. After centrifugation at 4°C for 20 min, the cell lysate in supernatant was collected. Cellular GSH level was determined with the GSH-400 kit (Oxis International, Portland, OR) according to the instructions from the manufacturer. In brief, 50 μl of the supernatant was mixed with 400 μl buffer (200 mM potassium phosphate, 0.2 mM diethylene-triamine pentaacetic acid and 0.025% lubrol, pH 7.8). Then 25 μl chromogenic reagent in 0.2 N HCl and 25 μl 30% NaOH were sequentially added and thoroughly mixed. The mixture was incubated in the dark at room temperature for at least 10 min. The optical density of the target chromogen was determined by a spectrophotometer (DU640i; Beckman, Fullerton, CA) at 400 nm. A series of controls of known GSH concentrations prepared from pure GSH powder (Sigma) was used to generate a standard curve. Three separate experiments with triplicate data in each were done.

Statistical analysis

Two-tailed Student's *t*-test was carried out to analyze the differences of IC_{50}s and cellular GSH contents between cells treated with and without MGA. A difference was considered significant with $p < 0.05$.

Results

Microculture chemosensitivity assay

After exposure to 10 μM MGA for 24 h, the cisplatin IC_{50}s of NTUB1 and NTUB1/P increased 1.4-(1.60 ± 0.14 to 2.16 ± 0.28 μM, $p=0.03$) and 1.6-(8.37 ± 1.17 to 14.00 ± 2.37 μM, $p=0.02$) fold, respectively, as compared to those without MGA exposure. The effects of two-drug combinations of MGA and cisplatin were shown in f_a-CI plots (Figure 1). The results indicate an antagonistic interaction between the two agents in both NTUB1 and NTUB1/P cells.

Metallothionein expression

After treatment with 10 μM MGA for 24 h, both NTUB1 and NTUB1/P cells were shown to express

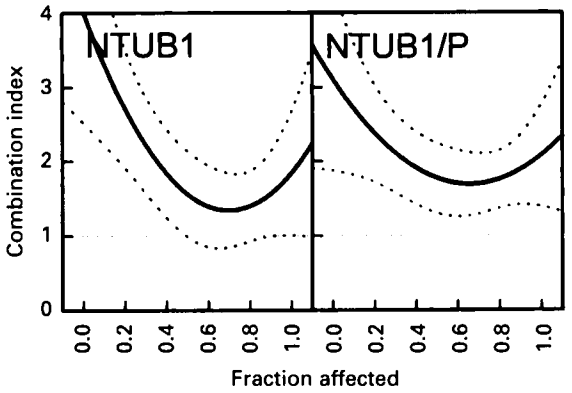

Figure 1. Fraction affected (f_a)–combination index (CI) plots of the drug combination of cisplatin and megestrol acetate. The regression lines (solid lines) with respective 95% confidence ranges (dotted lines) are shown in both NTUB1 and NTUB1/P cells. Both interaction curves traverse through regions where CI is greater than 1, indicating the two drugs are antagonistic to each other.

higher MT transcript levels than those without by Northern blotting analysis (Figure 2). By densitometric analysis, the intensities of MT transcript signals of NTUB1 and NTUB1/P after MGA treatment were 2.0- and 2.3-fold stronger, respectively, than those without. It indicates that MGA treatment upregulated MT transcript expression in TC cells, which was the case for both cisplatin-sensitive and -resistant cells. There was no significant difference in MT expression between NTUB1 and NTUB1/P, suggesting the absence of MT involvement in cisplatin resistance in the TC cell model.

GST-π levels

Figure 3 shows that treatment with 10 μM MGA for 24 h did not change cellular GST-π levels of either NTUB1 or NTUB1/P as determined by immunoblotting analysis. We have also performed a semi-quantitative reverse transcription (RT)-PCR assay which also failed to show any change in GST-π levels in either of the two cells after MGA treatment (data not shown). No significant differences in band intensity were detected in basal expressions of the two cells, suggesting GST-π is not involved in secondary cisplatin chemoresistance in the cell model used.

Glutathione content

Cellular GSH level was determined by a biochemical method. The GSH levels of NTUB1 cells treated with and without MGA were 0.125 ± 0.003 and 0.121 ± 0.005 μmol per 5×10^6 cells (mean ± SD), respectively. There was no significant difference by Student's *t*-test (p=0.29). For NTUB1/P cells, however, MGA-exposed cells showed a significantly higher GSH level than those without (0.173 ± 0.006 versus 0.141 ± 0.006 μmol per 5×10^6 cells, p=0.0036). NTUB1/P had higher GSH levels than NTUB1 (p=0.012), suggesting that elevation of GSH contents may be responsible for secondary cisplatin resistance in TC cells.

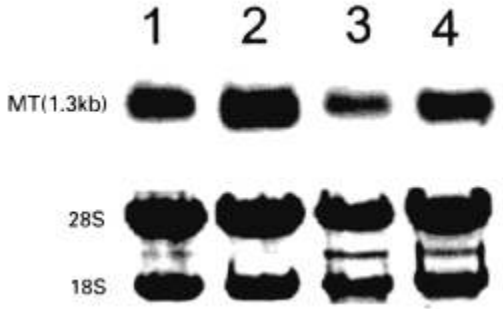

Figure 2. Northern blotting analysis of metallothionein expression in cells treated with or without 10 μM MGA for 24 h. Lanes 1 and 2, NTUB1. Lanes 3 and 4, NTUB1/P. Cells treated with MGA (lanes 2 and 4) expressed higher MT levels than those without (lanes 1 and 3).

Discussion

In the present study, we demonstrated that MGA antagonizes cisplatin cytotoxicity in a TC cell model.

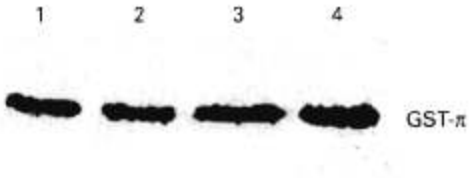

Figure 3. Immunoblotting of GST-π from cells treated with or without 10 μM MGA for 24 h. Lanes 1 and 2, NTUB1. Lanes 3 and 4, NTUB1/P. Lanes 2 and 4, MGA treated. There is no difference in GST-π levels between cells treated with and without MGA.

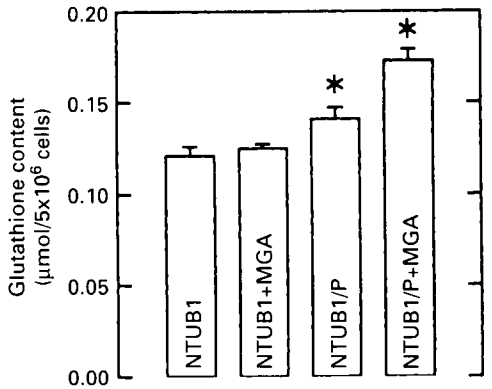

Figure 4. GSH contents in cells treated with or without 10 μM MGA for 24 h. Data are presented as mean ± SD. A significantly higher GSH level was observed in NTUB1/P cells treated with MGA than those without (*p=0.0036). There is no difference in GSH contents in NTUB1 cells (p=0.29). NTUB1/P has a significantly higher basal level of GSH than NTUB1 (p=0.012).

The MGA may act through several possible pathways, including upregulation of MT expression in both cisplatin-sensitive and -resistant cells, and increase of cellular GSH content in resistant cells. MGA treatment did not cause significant differences in GST-π protein levels in either cisplatin-sensitive or -resistant cells. From a practical point of view, this antagonistic phenomenon should be recognized by oncologists who prescribe MGA liberally in daily practice to patients receiving cisplatin-containing chemotherapy. Concurrent use of MGA and cisplatin may lower cisplatin antitumor effects and therefore impair tumor response rates in cisplatin-containing chemotherapy.

Earlier reports from numerous phase II studies testing the potential MGA beneficial effects on the appetite, performance status and hence tumor response rates in patients receiving cisplatin-containing chemotherapy showed that MGA increased patients' appetite and body weight during the study period.[1,2] Some authors claimed that response rates were improved as well by the addition of MGA. Several reports also showed that MGA may abrogate the chemoresistance of a variety of chemotherapeutic agents in some cancer models,[2,17] which resulted in MGA being prescribed more liberally by clinical oncologists in an attempt to enhance chemotherapeutic effects. In 1996, the North Central Cancer Treatment Group reported the results of a randomized clinical trial which was designed to evaluate the effect of additional MGA on chemotherapy with cisplatin plus etoposide in 243 patients with advanced-stage small cell lung cancer.[3] Patients were randomized into chemotherapy with or without oral MGA. Although patients with MGA had increased body weight (p=0.004) and significantly less nausea (p=0.002) and vomiting (p=0.02), they had a significantly poorer tumor response rate than those without MGA (68 versus 80%, p=0.03). Accordingly, patients with MGA, as expected, did not show a survival advantage to those without (median 8.2 versus 10.0 months, p=0.49). The authors stated that a poorer quality of life of the MGA group at study entry may have influenced the outcome. Nevertheless, the possible drug interaction between MGA and cisplatin has never been explored or even considered since then. Recently, we noticed the antagonism between MGA and cisplatin *in vitro*, as shown in the microculture chemosensitivity assay. Further laboratory work shown in this study indicated that MGA upregulated the MT detoxification pathway and cellular GSH content. Our *in vitro* data may explain, in part, why patients with MGA had a poorer response rate than those without.

In our model, basal GSH contents of NTUB1 cells were upregulated as they gained secondary resistance to cisplatin in NTUB1/P cells, suggesting two mechanism may be responsible for cisplatin resistance in TC cells. Similar findings have been reported by others in TC cell models.[6] Although others did, we failed to demonstrate the involvement of MT machinery in secondary cisplatin chemoresistance in our cell model.[5,18] It is not uncommon to see a discrepancy in the chemoresistance mechanisms in different reports. Distinct cell models used and different ways of inducing secondary chemoresistance may result in the discrepancy between those reports.

We would like to emphasize that *in vitro* findings as presented here do not necessarily reflect the *in vivo* situation in a clinical setting. To confirm the biological effects of MGA on cisplatin resistance, the levels of MT, GST-π, GSH and other related factors should be examined in human tissues or cells from patients exposed to MGA. The cellular responses to MGA in different tissue types may vary significantly. What we used in this study were TC cell lines instead of lung cancer cell lines, which might be more relevant to the lung cancer clinical trial mentioned above. However, the data presented here suggested that we should be cautious about the liberal use of MGA in cisplatin-containing chemotherapy, unless further clinical evidence indicates otherwise.

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(Received 26 May 1998; revised form accepted 26 June 1998)