Anticancer effects of a specific mixture of nutrients in the multidrug-resistant human uterine sarcoma MES-SA/Dx5 and the drug-sensitive MES-SA cell lines

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Abstract. A specific nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract has demonstrated a broad spectrum of antitumor activity against a number of cancer cell lines. In this study, our main objective was to investigate the comparative effects of NM on anticancer parameters, such as cytotoxicity, matrix metalloproteinase (MMP) secretion and Matrigel invasion in the human uterine sarcoma drug-resistant MES-SA/Dx5 and the drug-sensitive MES-SA cell lines. In addition we studied the effects of NM on P-glycoprotein (Pgp) on these cell lines. Cell proliferation was evaluated by MTT assay, MMPs by gelatinase zymography, invasion through Matrigel, morphology by H&E and Pgp expression by Western blot analysis and immunodetection using FITC-conjugated antibody and rhodamine 123 (Rh123) accumulation and efflux assays. NM exhibited antiproliferative effects on MES-SA/Dx5, by 20% at 50 and 100 μg/ml and by 36, 40 and 48% at 250, 500 and 1,000 μg/ml, respectively. By contrast, NM treatment of MES-SA cells resulted in significantly increased cytotoxicity: 40, 46, 65 and 72% at 50, 100, 500 and 1,000 μg/ml, respectively. In both cell lines, zymography demonstrated a band corresponding to MMP-2 in normal cells and MMP-9 with phorbol 12-myristate 13-acetate treatment. The two MMPs showed dose-response inhibition by NM. As shown by Western blot analysis and immunodetection, NM treatment resulted in a dose-dependent decrease in Pgp expression in the MES-SA/Dx5 cell line. The MES-SA cell line does not exhibit Pgp. NM enhanced the accumulation and efflux of the Pgp substrate, Rh123, in the MES-SA/Dx5 uterine sarcoma cell line but not in the drug-sensitive cell line, MES-SA. Therefore, it can be concluded that NM demonstrates potent anticancer effects in both the drug-resistant and sensitive cell lines and modulates Pgp, suggesting its potential therapeutic effects in drug-resistant as well as sensitive cancers.

Introduction

Certain cancer cells develop resistance to therapeutic agents as well as to a broad spectrum of unrelated compounds, and may develop a multidrug-resistant (MDR) phenotype (1-4). MDR is mediated by overexpression of the drug export protein, known as plasma membrane P-glycoprotein (Pgp), which increases transport of the therapeutic agents from the cells, resulting in decreased cellular accumulation of the agents and, thus, reduced therapeutic efficacy (5). Pgp has a molecular mass of approximately 150-170 kDa and is a member of the ATP-binding cassette (ABC) transporters, encoded by the *MDR1* gene in humans (6-8). A number of human tissues normally express Pgp: Cancer cells, lymphocytes, renal, hepatic and testicular cells, intestinal epithelia and the endothelium at the blood-brain barrier. Numerous classes of anticancer drugs are affected by Pgp-mediated resistance. These drugs include anthracyclines, epipodophyllotoxins, inca alkaloids and microtubule drugs (9). In recent decades, significant efforts have been focused on reversing MDR by targeting Pgp. MDR-reversal agents include calcium channel blockers, calmodulin antagonists, steroidal agents, protein kinase C inhibitors, immunosuppressive drugs, antibiotics and surfactants. However, many of these identified MDR-reversing drugs result in side-effects, such as cardiac toxicity, hypotension and congestive heart failure (10,11).

The fluorescent dye rhodamine (Rh)123 has been used extensively as an index of Pgp-mediated transport in rodent and tissue culture models. Rh123 cell exclusion has been employed as a reliable analytical tool to identify potential multidrug resistance reversal agents for use in chemotherapy (12,13). Although arguments have been made that Rh123 is transported by multidrug resistance-associated protein (MRP)1, Perloff *et al* reported that Rh123 transport was unaffected by the MRP1 inhibitors probenecid and indomethacin (14). Furthermore, the National Cancer Institute, in conducting a drug screen in 58 different cell lines, demonstrated a positive correlation between Rh123 transport and the expression of Pgp, but not that of MRP (15).

A specific micronutrient mixture (NM) has exhibited potent anticancer activity *in vivo* and *in vitro* in over 40 cancer cell

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lines (16). The anticancer effects of the NM include: Inhibition of metastasis, tumor growth, matrix metalloproteinase (MMP) secretion, invasion, angiogenesis and cell growth, as well as the induction of apoptosis (16). We are currently investigating whether NM is effective in drug-resistant cancers. We decided to study the effects of NM on the human drug-insensitive uterine sarcoma MES-SA/Dx5 and the drug-sensitive MES-SA cell lines, as the uterine sarcoma human cell line, MES-SA/Dx5, has been reported to overexpress the *MDR1* gene product Pgp (17). The main objective of this study was to evaluate the effects of NM on cell viability, morphology, MMP expression and Matrigel invasion using the human drug-insensitive uterine sarcoma MES-SA/Dx5 and the drug-sensitive MES-SA cell lines in light of the effects of NM on Pgp expression in these cell lines.

Figure 1. Effects of NM on MES-SA/Dx5 and MES-SA cells: MTT 24 h.

Materials and methods

Cancer cell lines and culture. The human drug-insensitive uterine sarcoma MES-SA/Dx5 and the drug-sensitive MES-SA cell lines, obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), were grown in RPMI-1460 medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA, USA). Cells were incubated with 1 ml of medium at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with the NM at 0, 50, 100, 250, 500 and 1,000 μg/ml, in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA), at a dose of 100 ng/ml was added to the cells to induce MMP-9 secretion. The plates were then returned to the incubator. The medium and serum used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL (Long Island, NY, USA). EGCG and other chemicals were obtained from Sigma.

Composition of the NM. The NM was composed of the following, at the indicated doses: Vitamin C (as ascorbic acid and as Mg, Ca, and palmityol ascorbate) 700 mg, L-lysine 1,000 mg, L-proline 750 mg, L-arginine 500 mg, N-acetyl cysteine 200 mg, standardized green tea extract [derived from green tea leaves, obtained from US Pharma Lab; the certificate of analysis indicated the following characteristics: Total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35% and caffeine 1.0%] 1,000 mg, selenium 30 μg, copper 2 mg and manganese 1 mg.

Cytotoxicity: MTT assay. Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and, hence, of cell viability. After 24-h incubation, the cells were washed with phosphate-buffered saline (PBS) and 500 μl of MTT (Sigma, M-2128) 0.5 mg/ml in medium were added to each well. After MTT addition, the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the
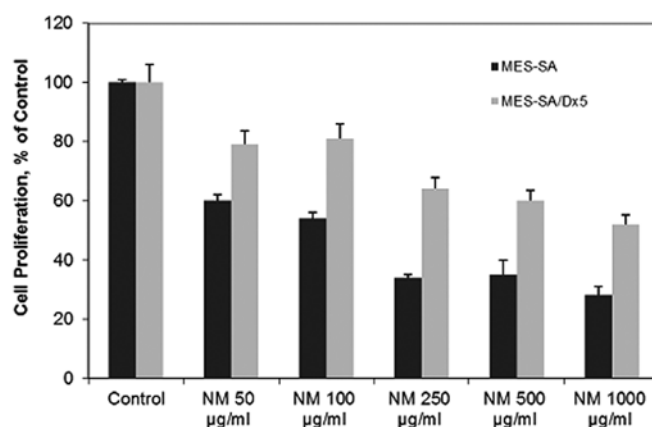
wells, the formazan product was dissolved in 1 ml DMSO and absorbance was measured at 570 nm in a BioSpec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

Gelatinase zymography. MMP expression in conditioned medium was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex Pre-Cast SDS polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture medium (20 μl) was mixed with sample buffer and loaded for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) with tris glycine SDS buffer, according to the manufacturer's instructions (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digest the gelatin in the gel and give clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Matrigel invasion. Invasion studies were conducted using Matrigel (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Therefore, both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 h. Subsequently, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with H&E and visually counted under the microscope.
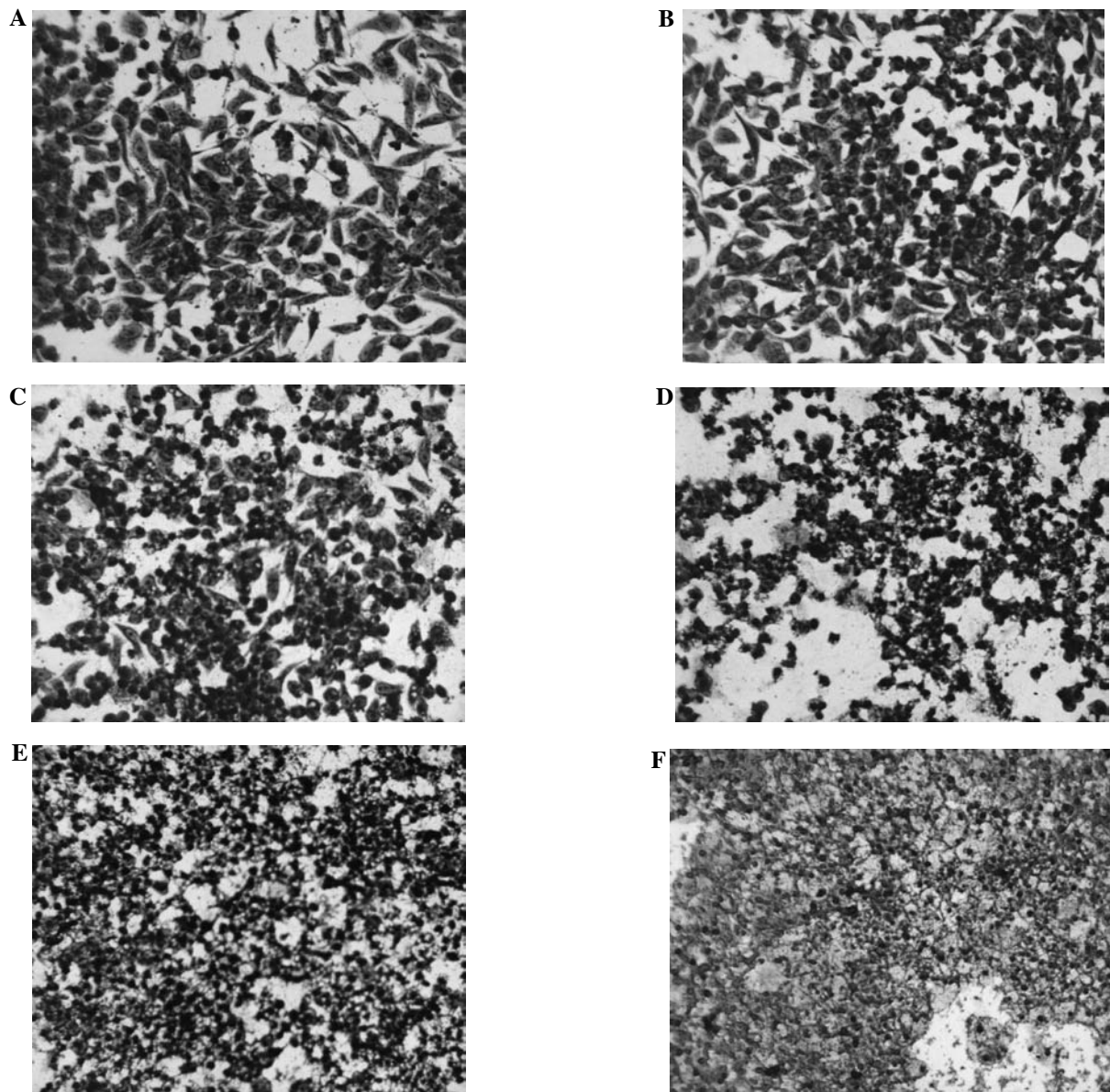
Figure 2. Effects of NM on morphology of MES-SA cells: H&E staining. (A) Control, (B) NM 50 μg/ml, (C) NM 100 μg/ml, (D) NM 250 μg/ml, (E) NM 500 μg/ ml and (F) NM 1000 μg/ml.

Western blot analysis of Pgp and ECL detection. Cells were lysed with 1% Triton X-100 and 0.5% deoxycholate (in phosphate buffer pH 7.4), 1 mM phenylmethylsulfonyl fluoride and centrifuged at 1,000 g. The supernatant was collected and the total protein concentration was determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The cell membrane proteins (10 μg/lane) were separated by 10% SDS-PAGE and electroblotted overnight onto nitrocellulose filters. The filters were incubated sequentially with primary mouse monoclonal anti-Pgp clone F4 at 1:500 and 11RP-conjugated goat anti-mouse IgC at a 1:20,000 dilution. Proteins were visualized by the SuperSignal protein detection kit (enhanced chemiluminescence, ECL), and quantified by scanning densitometry.

Rh accumulation and efflux assays. Rh accumulation assay was performed in 24-well plates in MES-SA and MES-SA/DX5 cancer cells using fluorescence detection (18,19). Cells were

seeded onto 24-well flat-bottom plates and incubated in 5% CO_2 at 37˚C for 3 days. Rh123 (20 μM) was added to the cells in the absence or presence of NM at doses 100, 250, 500 and 1,000 μg/ml and incubated at 37˚C in 5% CO_2 for 40 min. The culture medium was removed and the cells were washed twice with cold PBS. The green fluorescence of Rh123 was measured at 485/20 nm excitation and 530/25 nm emission by florescence microplate reader (Millipore Cytofluor 2300, Billerica, MA, USA). Each plate included a blank control (no cells), a cell control (which contained cells without NM) and tested wells, which contained cells with NM. In the Rh efflux assay, 20 μM Rh123 were added to the cells for 40 min and the culture medium was removed. The cells were incubated in fresh culture medium with or without NM at 37˚C in 5% CO_2 for an additional hour. Cellular residual Rh123 was measured by a fluorescence microplate reader (Millipore Cytofluor 2300) and images were captured by fluorescence microscopy.
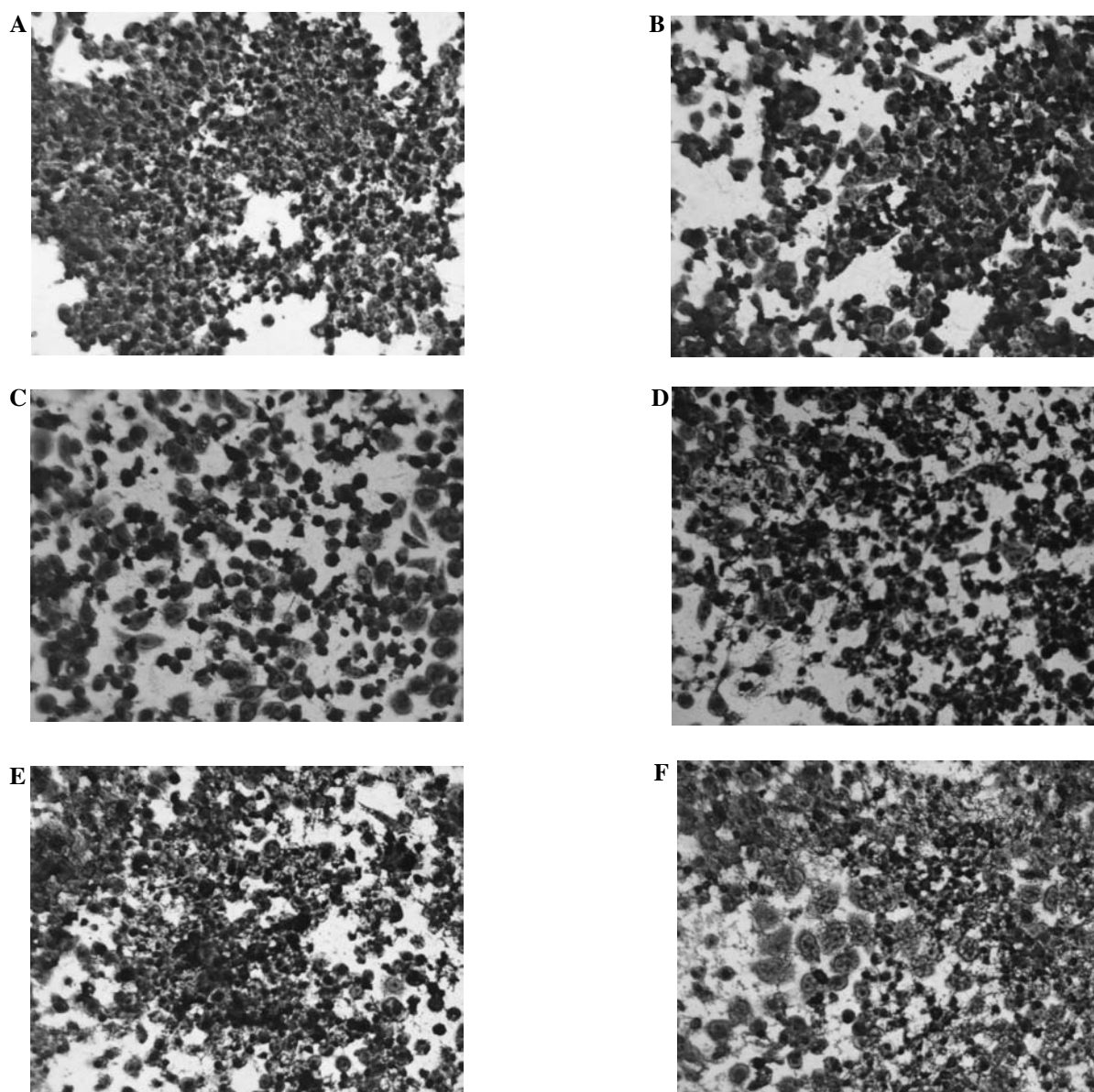
Figure 3. Effects of NM on morphology of MESA/Dx5 cells: H&E staining. (A) Control, (B) NM 50 μg/ml, (C) NM 100 μg/ml, (D) NM 250 μg/ml, (E) NM 500 μg/ml and (F) NM 1000 μg/ml.

Statistical analysis. The results were expressed as the means ± SD. Data were analyzed by independent sample t-test.

Results

Effects of NM on viability of MESA/DX 5 and MES-SA cells. MES-SA/Dx5 cell viability was decreased with NM treatment by 21% at 50 μg/ml and by 36, 40 and 48% at 250, 500 and 1,000 μg/ml, respectively (linear trend R^2, 0.8306; Fig. 1). By contrast, NM treatment of the drug-sensitive cell line, MES-SA, demonstrated significantly higher cytotoxicity to cells, resulting in a 40% decrease in viability compared to the control at 50 μg/ml, 46% at 100 μg/ml, 65% at 500 and 72% at 1,000 μg/ml (linear trend R^2, 0.9271; Fig. 1). The difference in NM cytotoxicity between MES-SA and MES-SA/Dx5 cells reached statistical significance. At NM 100 μg/ml, the cell viability of MES-SA cells was 66.7% (P=0.0005) of that of

MES-SA/Dx5 cells, at NM 500 μg/ml, 58.3% (P=0.001) and at NM 1,000 μg/ml, 53.8% (P=0.001).

Effects of NM on morphology of MESA/DX 5 and MES-SA cells: H&E staining. The morphology of MES-SA (Fig. 2A-F) and MES-SA/Dx5 (Fig. 3A-F) cells was not affected at concentrations of ≤250 μg/ml NM. However, at 500 and 1,000 μg/ml NM both cell lines showed changes, such as apoptosis.

Effects of NM on MMP secretion in MESA/DX 5 and MES-SA cells: Gelatinase zymography. In the two cell lines, zymography demonstrated a band corresponding to MMP-2 in normal cells and to MMP-9 with PMA treatment. NM inhibited the secretion of both MMPs in a dose-dependent manner. Zymograms of normal and PMA (100 ng/ml)-treated MES-SA and MES-SA/Dx5 cells are shown in Fig. 4. Densitometry analyses of PMA-treated cells are shown in Fig. 4C and F.
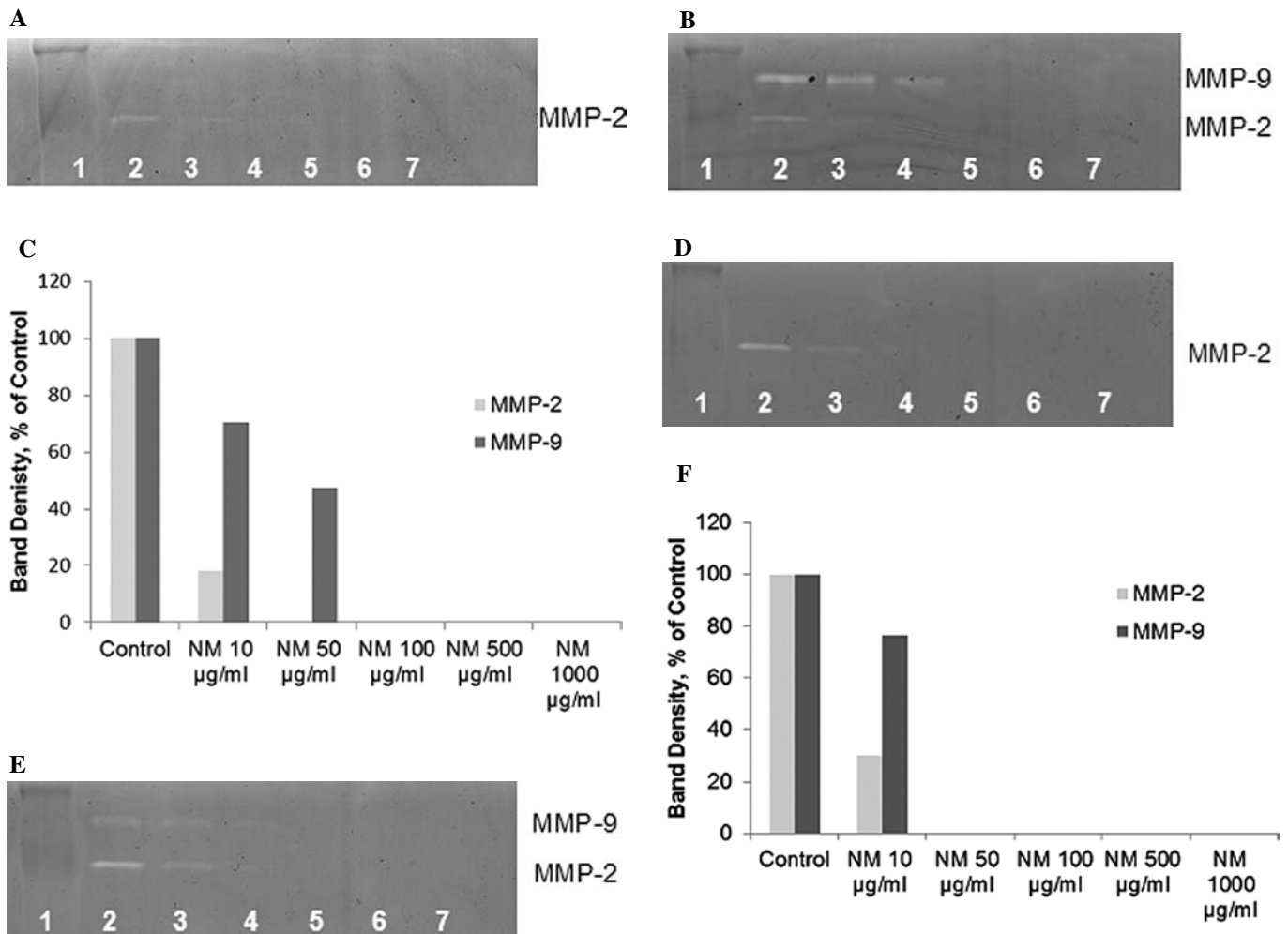
Figure 4. Effects of NM on MMP-2 and -9 secretion in MES-SA and MESA/Dx5 cells. Zymograms of (A) normal MES-SA and (B) PMA-treated MES-SA cells. 1, markers; 2, control; 3-7, NM 50, 100, 250, 500 and 1,000 μg/ml, respectively. (C) Densitometry of PMA-treated MES-SA cells. (D) Zymograms of normal MES-SA/Dx5 and (E) PMA-treated MES-SA/Dx5 cells. 1, markers; 2, control; 3-7, NM 50, 100, 250, 500 and 1,000 μg/ml, respectively. (F) Densitometry of PMA-treated MES-SA/Dx5 cells.

Effects of NM on MESA/DX 5 and MES-SA cell Matrigel invasion. NM inhibited MES-SA and MES-SA/Dx5 cell invasion through Matrigel in a dose-dependent manner with 100% block of MES-SA cell Matrigel invasion at 250 μg/ml NM and MES-SA/Dx5 cell invasion at 50 μg/ml NM, as shown in Fig. 5.

Effects of NM on MESA/DX 5 and MES-SA cell Pgp expression. As shown by Western blot analysis, the MES-SA/Dx5 cell line exhibited dose-dependent Pgp expression, while MES-SA did not exhibit Pgp (Fig. 6A). The NM treatment resulted in a dose-dependent decrease of Pgp expression in the MES-SA/Dx5 cell line, as shown by Western blot analysis (Fig. 6B) and by immunodetection (Fig. 8), whereas MES-SA did not exhibit Pgp by immunostaining (Fig. 7).

Effects of NM on accumulation and efflux of Pgp substrate Rh123. NM enhanced the accumulation and efflux of the Pgp substrate, Rh123, in the MES-SA/Dx5 uterine sarcoma, but not in the drug-sensitive MES-SA cell line. Figs. 9 and 11 show graphical representations of NM effects on Pgp accumulation and efflux, respectively, in both cell lines. Photoluminescent

images of NM effects on MES-SA and MES-SA/Dx5 Rh123 uptake are shown in Fig. 10.

Rh123 uptake in drug-insensitive MES-SA/Dx5 cells demonstrated increased levels with NM treatment (linear trend R^2, 0.2438), with 180% of control level at 100 μg/ml and 247% at 1,000 μg/ml. By contrast, no significant change in Rh123 was detected in the drug-sensitive MES-SA cells exposed to various concentrations of NM (linear trend R^2, 0.5774). The differences between MES-SA/Dx5 and MES-SA Rh123 levels reached statistical significance. At NM 100 μg/ml, MES-SA Rh123 levels were 57.2% (P=0.009) those of MES-SA/Dx5 cells and at NM 1,000 μg/ml, 38.1% (P=0.003).

In the drug-sensitive MES-SA cells, Rh123 residue demonstrated no significant change with the addition of increasing concentrations of NM (linear trend R^2, 0.5934). Conversely, in the MES-SA/Dx5 cells, RH123 residue increased with NM concentration (linear trend R^2, 0.1699).

Discussion

In the present study, the drug-sensitive human sarcoma cancer cell line was more invasive than the drug-insensitive counter-
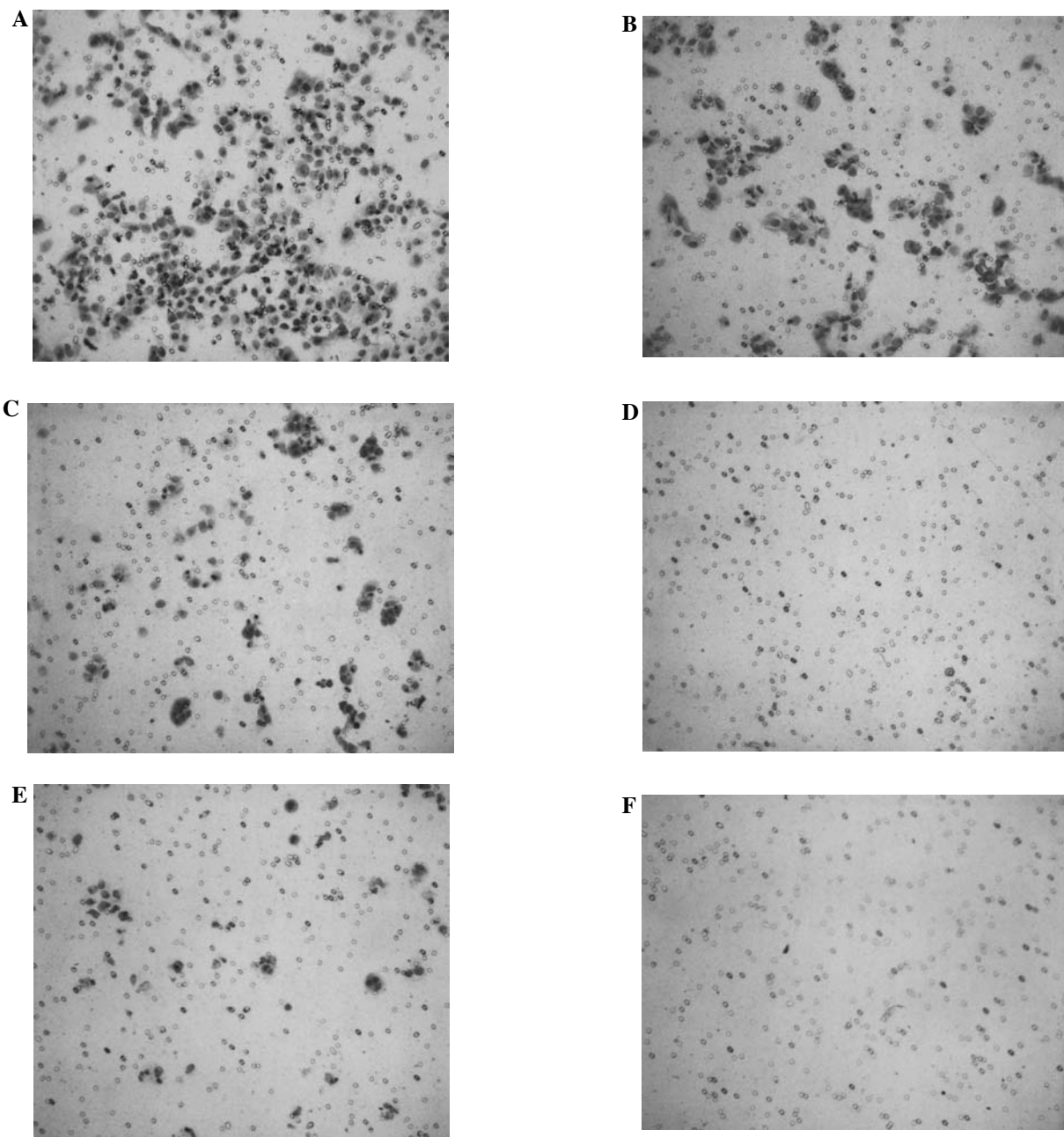
Figure 5. Effects of NM on MESA/Dx5 and MES-SA cell Matrigel invasion. (A) MES-SA control, (B) MES-SA NM 50 μg/ml, (C) MES-SA NM 100 μg/ml, (D) MES-SA NM 250 μg/ml, (E) MES-SA/Dx5 control and (F) MES-SA/Dx5 NM 50 μg/ml.
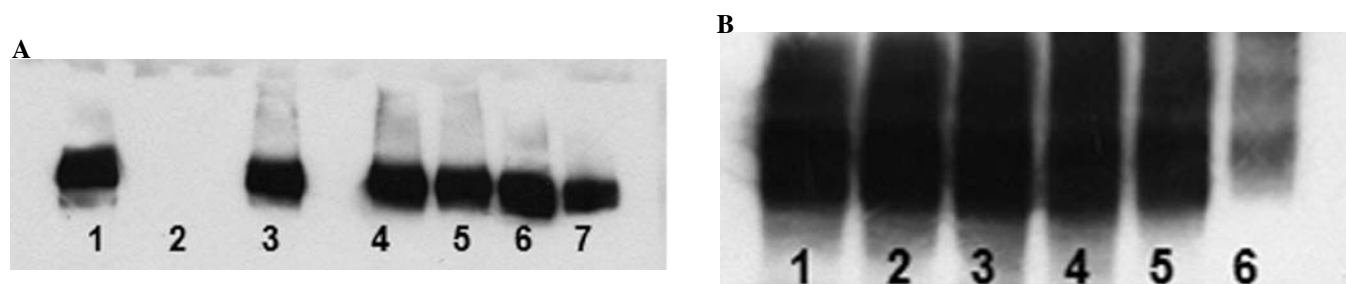

Figure 6. (A) Pgp expression of MES-SA and MES-SA/Dx5 cells: Western blot analysis. (B) Effects of NM on Pgp expression of MES-SA/Dx5 cells: Western blot analysis.
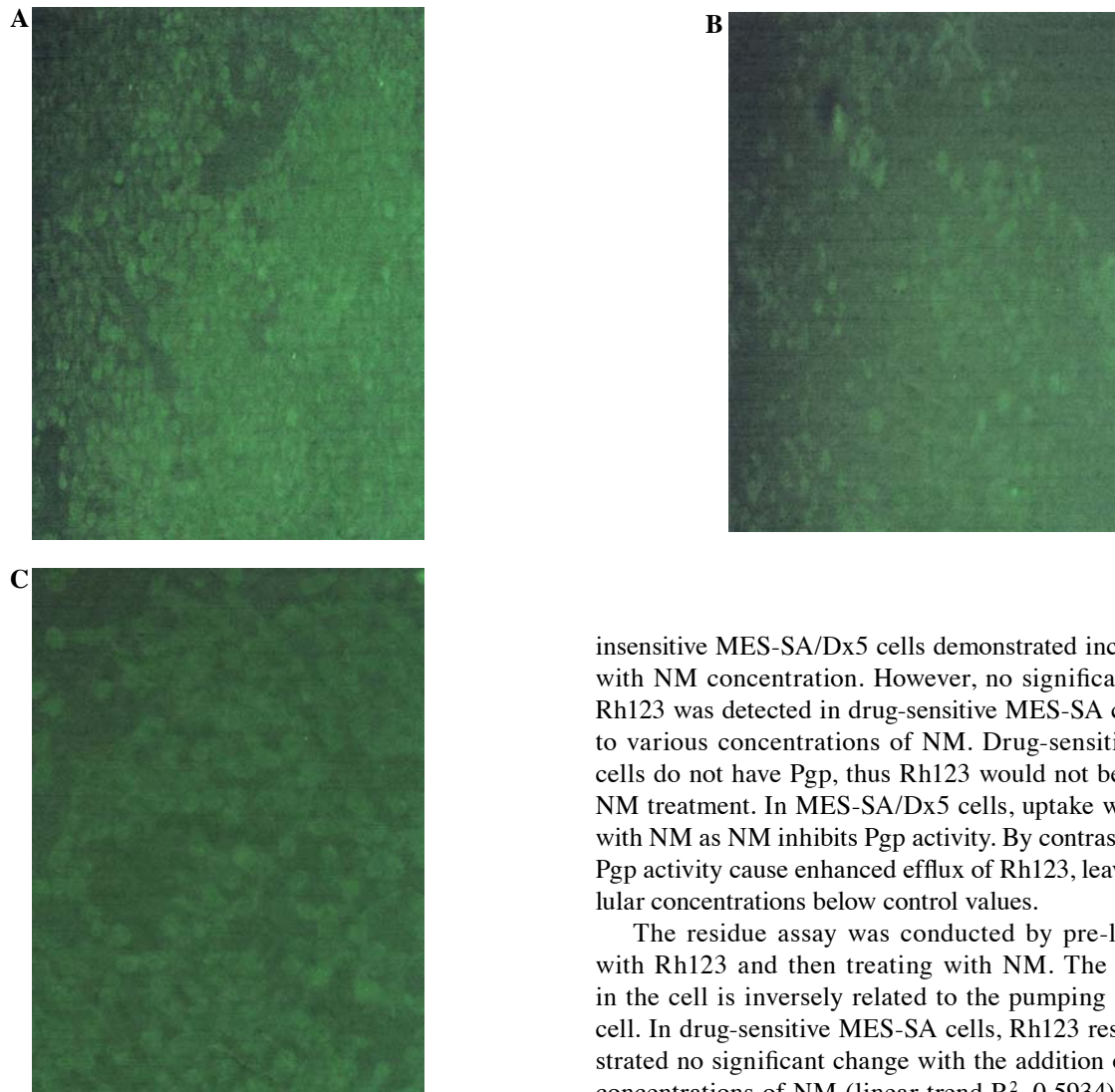
Figure 7. Effects of NM on Pgp expression in MES-SA cells: Immuno-fluorescence. (A) Control, (B) NM 100 μg/ml and (C) NM 250 μg/ml.

part and NM modulated cancer cell MMP activity and invasion of both cell lines, as well as Pgp in MES-SA/Dx5 cells. As regards the Matrigel invasion, drug-insensitive MES-SA/Dx5 cells were less invasive than the drug-sensitive MES-SA cells. At control conditions (no NM present), a substantially larger number of MES-SA cells invaded the Matrigel barrier compared to the MES-SA/Dx5 cells. NM completely blocked Matrigel invasion in drug-insensitive MES-SA/Dx5 cells at a lower concentration compared to the drug-sensitive MES-SA cells. Liang *et al* reported that some tumor cells selected for resistance to drugs are more invasive/metastatic than their non-resistant parental cells, while other tumour cells have not been shown to be correlated with invasion or metastasis (20). Cancer cell invasion has been shown to be correlated with MMP activity, as reported in a previous study (21).

Pgp activity in the cell accumulation assay is measured by the intracellular concentration of the Pgp substrate, Rh123. Thus, inhibition of Pgp activity results in increased intracellular Rh123 concentrations reflected in higher fluorescent signals compared to control cells. Rh123 uptake in drug-

insensitive MES-SA/Dx5 cells demonstrated increased levels with NM concentration. However, no significant change in Rh123 was detected in drug-sensitive MES-SA cells exposed to various concentrations of NM. Drug-sensitive MES-SA cells do not have Pgp, thus Rh123 would not be affected by NM treatment. In MES-SA/Dx5 cells, uptake was increased with NM as NM inhibits Pgp activity. By contrast, inducers of Pgp activity cause enhanced efflux of Rh123, leaving intracellular concentrations below control values.

The residue assay was conducted by pre-loading cells with Rh123 and then treating with NM. The residual dye in the cell is inversely related to the pumping action in the cell. In drug-sensitive MES-SA cells, Rh123 residue demonstrated no significant change with the addition of increasing concentrations of NM (linear trend R^2, 0.5934). Conversely, in the MES-SA/Dx5 cells, Rh123 residue increased with NM concentration. Therefore, the increased residual dye level in MES-SA/Dx5 cells suggests the inhibition of *MDR-1* activity. NM was significantly more cytotoxic to drug-insensitive MES-SA cells than to MES-SA/Dx5 cells, indicating that the overexpression of Pgp in MES-SA/Dx5 cells may play a role in protecting MES-SA/Dx5 cells against NM by transporting NM out of the cell.

Drug resistance to chemotherapeutic agents accounts for treatment failure in more than 90% of patients with metastatic cancer (22). A major mechanism of this resistance is the enhanced efflux of chemotherapeutic agents due to overexpression of Pgp. Studies have shown that compounds in plants, including vegetables and fruits, not only have anticancer activities but may also modulate Pgp activity, rendering them more effective in treating cancers deemed resistant due to production of Pgp. For example, polyphenols have been reported to exhibit anticancer activity and to modulate Pgp activity in various cancer cell lines (23-26).

The NM is a mixture of nutrients that act on critical physiological targets in cancer progression and metastasis. The anticancer effects of the individual constituents of the NM have been reported in both clinical and experimental studies (27-29). Green tea (23), vitamin C (30) and selenium (31) have been shown to reverse multidrug resistance. Adequate supplies
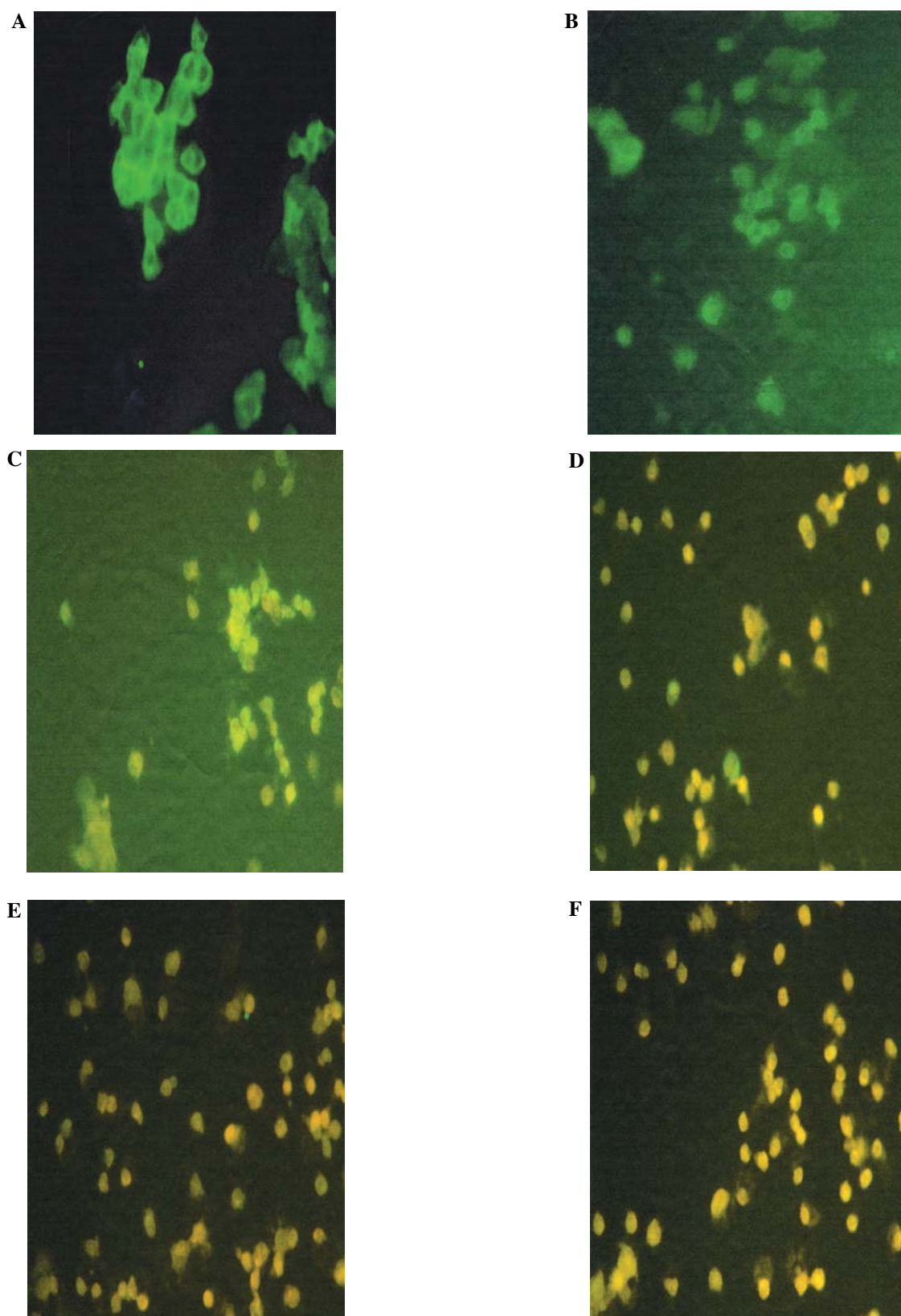
Figure 8. Effects of NM on Pgp expression in MES-SA/Dx5 cells: Immunofluorescence. (A) Control, (B) NM 50 μg/ml, (C) NM 100 μg/ml, (D) NM 250 μg/ ml, (E) NM 500 μg/ml and (F) NM 1,000 μg/ml.

of ascorbic acid and the amino acids, lysine and proline, are essential for optimal extracellular matrix (ECM) formation and structure as these nutrients insure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine contributes to ECM stability as a natural inhibitor of plasmin-induced prote-olysis (32,33). Green tea extract has been shown to control cancer cell growth, metastasis, angiogenesis, as well as other
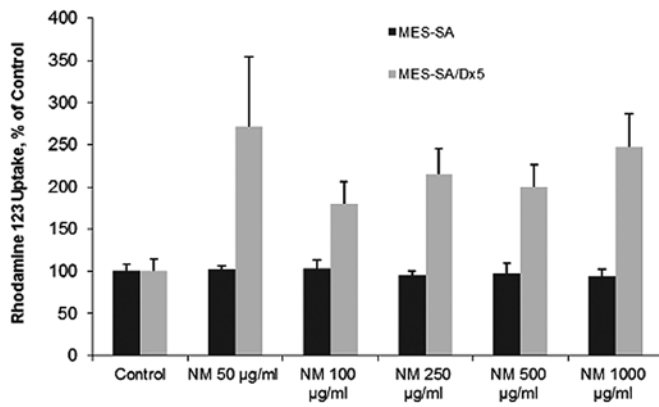
Figure 9. Effects of NM on MES-SA and MES-SA/Dx5 Rh123 uptake.

aspects of cancer progression (34-40). N-acetyl cysteine and selenium have been observed to inhibit MMP-9 and invasive activities of tumor cells, as well as migration of endothelial cells through ECM (41-43). Ascorbic acid is known to exert cytotoxic and antimetastatic actions on malignant cell lines (44-48). Arginine is a precursor of nitric oxide (NO); any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells (49).

Furthermore, in contrast to the toxic side-effects of current chemotherapy, the NM is a safe therapeutic agent. In a previous *in vivo* study addressing safety issues, we found that gavaging adult female ODS rats (weighing 250-300 gm) with the NM (at 30, 90 or 150 mg per day for seven days), had neither adverse effects on vital organs (heart, liver and kidney), nor on the associated functional serum enzymes, indicating that this mixture is safe to use even at these high doses, which far exceed the normal equivalent dosage of the nutrient (50).

In conclusion, the drug-resistant cancer cell line was more responsive to NM compared to the drug-sensitive cell line, which suggests therapeutic potential for all cancers. As NM modulates Pgp, it has significant potential in treating resistant cancers that secrete Pgp. Therefore, in contrast to chemotherapeutic drugs that not only have potent toxic effects but are ineffective in treating drug-resistant cancers, the NM not only has low toxicity but has also shown potential efficacy in the treatment of drug-resistant cancer by the modulation of
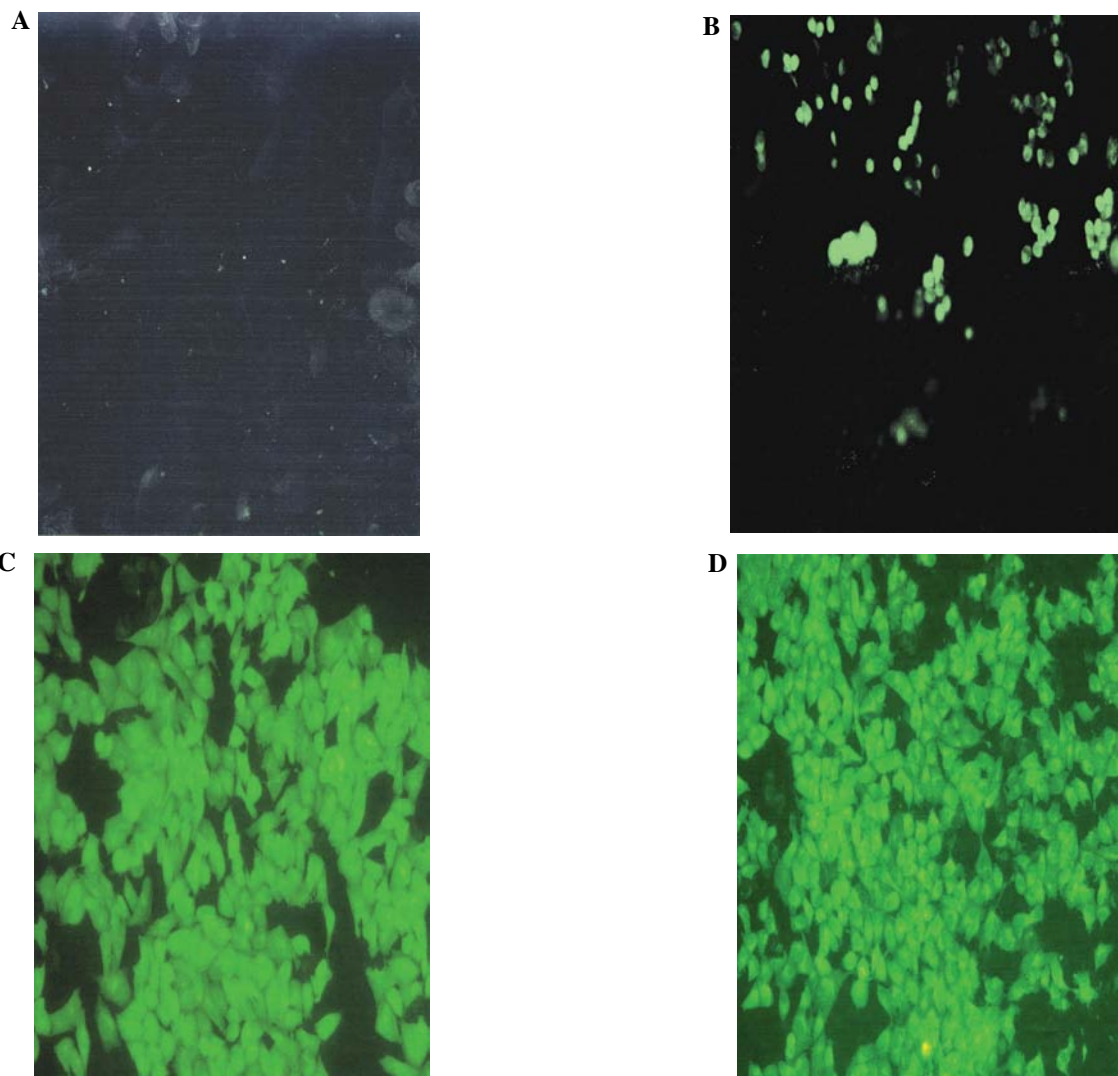

Figure 10. Effects of NM on MES-SA and MES-SA/Dx5 Rh123 uptake. (A) MES-SA/Dx5 control, (B) MES-SA/Dx5 NM 250 μg/ml. (C) MES-SA control and (D) MES-SA NM 250 μg/ml.
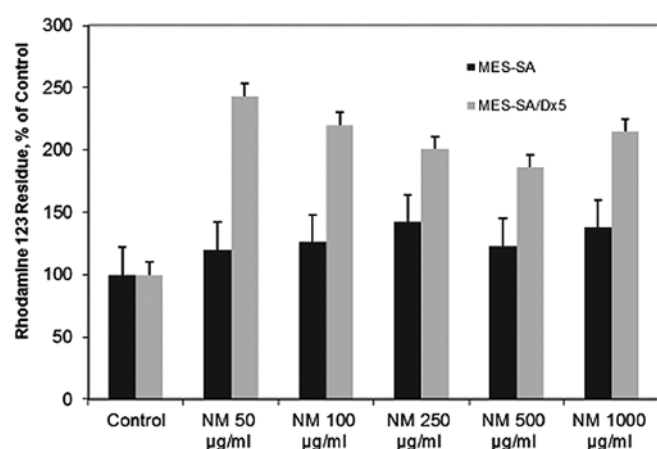
Figure 11. Effects of NM on MES-SA and MES-SA/Dx5 Rh123 residue.

Pgp, rendering increased cell concentration of NM and, thus, increasing anticancer activity.

Acknowledgements

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