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Preclinical study

Mitoguazone induces apoptosis via a p53-independent mechanism

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Mitoguazone (methylglyoxal bisguanylhydrazone, methyl-GAG or MGBG) is a synthetic polycarbonyl derivative with activity in patients with Hodgkin's and non-Hodgkin's lymphoma, head and neck cancer, prostate cancer, and esophageal cancer. Mitoguazone has also recently been documented to have activity in patients with AIDS-related lymphoma. Among anticancer drugs, mitoguazone has a unique mechanism of action via interference with the polyamine biosynthetic pathway. Polyamines stabilize DNA structure by non-covalent cross-bridging between phosphate groups on opposite strands. In addition, mitoguazone causes uncoupling of oxidative phosphorylation. In this study, the ability of mitoguazone to induce apoptosis by inhibiting the polyamine pathway was assessed in three Burkitt's lymphoma cell lines (Raji, Ramos and Daudi) and one prostate carcinoma cell line (MPC 3). Additional evaluations were performed in two human breast cancer cell lines (MCF7 with wild-type p53 and VM4K with mutated p53) to determine whether the p53 tumor suppressor gene was required for efficient apoptosis induction. The present study demonstrated that mitoguazone induces apoptosis in all the different human cancer cell lines tested in a concentration- and time-dependent way, and triggers a p53-independent programmed cell death in the human breast cancer MCF7 cell line. [© 1998 Lippincott Williams & Wilkins.]

Key words: Mitoguazone, p53-independent apoptosis, polyamines.

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Introduction

Mitoguazone (methylglyoxal bisguanylhydrazone, methyl-GAG or MGBG) is a synthetic polycarbonyl derivative with activity in patients with Hodgkin's and non-Hodgkin's lymphoma, head and neck cancer, prostate cancer, and esophageal cancer.[1] This cytotoxic drug is commonly administered in combination with other chemotherapeutic agents in the MIME protocol (mitoguazone+ifosfamide+methotrexate+etoposide) for patients with refractory lymphomas.[2] More recently, mitoguazone has been documented to have activity in patients with AIDS-related lymphoma even when those patients had progressed on several prior regimens.[3] Mitoguazone has a unique mechanism of action among all the anticancer drugs via its interference with the polyamine biosynthetic pathway.[4] In addition to inhibiting polyamine biosynthesis, mitoguazone also causes disruption of the mitochondrial structure, probably from oxidative phosphorylation uncoupling.[5]

Polyamines (putrescine, spermidine, spermine) are ubiquitous organic cations which play an essential role in cellular growth and differentiation.[6] The biosynthesis of the mammalian polyamines consists of four irreversible steps in sequence from arginine to ornithine, and further conversions to putrescine, spermidine and, lastly, spermine.[5] Ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAM-DC) are the rate-limiting enzymes catalyzing the conversion of ornithine to putrescine and putrescine to spermidine, respectively.[6] With a structure similar to the polyamines, mitoguazone is preferentially taken up by the cell by the same transporter mechanism as spermine[7] and, therefore, has competitive inhibitory activity on SAM-DC.[8] Polyamines stabilize DNA structure by non-covalent cross-bridging between phosphate groups on opposite strands,[9] and polyamine depletion may result in significant changes in chroma-

tin and DNA structure.[10] This destabilization of chromatin structure could increase accessibility to endonucleases and lead to DNA fragmentation.[11] Overall, it would appear that decreases in cellular polyamines pools could be an important trigger for apoptosis.[11]

Programmed cell death pathways are of specific interest as therapeutic targets in cancer cells. In this study, the ability of mitoguazone to induce apoptosis by inhibiting the polyamine pathway was assessed in a variety of human cancer cell lines where this anticancer drug has demonstrated activity, including Burkitt's lymphoma and prostate carcinoma. Additional evaluations were performed in breast cancer cell lines to determine whether the p53 tumor suppressor gene was required for efficient apoptosis induction. Indeed, many anticancer agents such as doxorubicicin, etoposide or 5-fluorouracil induce apoptosis via a p53-dependent pathway.[12] The requirement of the p53 tumor suppressor for efficient activation of apoptosis by these agents provides an attractive explanation for the poor efficacy of these drugs on p53 mutant tumors.[13] Thus, identifying chemotherapeutic agents that act independently of the p53 pathway is of major importance.

Material and methods

Cell lines

The Raji, Ramos and Daudi cell lines are human Burkitt's lymphoma cell lines and were obtained from ATTC (Rockville, MD). The MPC3 cell line, a human prostate cancer cell line derived from the passage through a nude mouse model, was a gift from Dr J Ware (Medical College of Virginia, Richmond).[14] The two human breast cancer cell lines used were the MCF7 and the VM4K cell lines. The MCF7, a p53 wild-type, estrogen receptor-positive, progesterone receptor-positive human breast adenocarcinoma cell line, was a gift from Dr CK Osborne (University of Texas, Health Science Center, San Antonio).[15] The VM4K cell line, a gift from Dr R Elledge (University of Texas, Health Science Center, San Antonio), was derived originaly from the MCF7 cell line but contains a mutant p53 suppressor oncogene. The cDNA was derived from a lung cancer cell line with a nucleotide substitution at codon 179, resulting in a mutation from histidine to glutamine. The full-length cDNA was cloned into a 5.5 kb PUC/CMV plasmid with a CMV promoter for p53 expression and a neomycin gene allowing for G418 selection (Geneticin; Gibco/BRL, Gaithersburg, MD).

Tissue culture procedures

Cells from each cell line were divided into 12 75 cm^3 flasks with a concentration of 10^6 cells/flask. A separate flask was prepared for each of the drug concentrations and controls at days 1, 3 and 5. The Raji and Ramos cell lines grew in RPMI 1640 with 10% heat-inactivated fetal bovine serum (HIFBS). The Daudi cell lines were grown in RPMI 1640 with 20% non-heat-inactivated FBS. The growth medium for the MCF7 cell lines was IMEM with 10% HIFBS and bovine insulin 10^{-9} mol/l. The growth medium for MV4K cell lines was MEM with 10% HIFBS, insulin 10^{-9} mol/l, gentamicin and geneticin G418 200 μg ml (Gibco/BRL). Cell growth at days 1, 3 and 5 was determined with a hemocytometer.

Exposure of cells to drug

Three concentrations (0.1, 1 and 10 μg/ml) of mitoguazone were studied. These concentrations were chosen because 0.5 μg/ml of mitoguazone is sufficient to inhibit spermidine synthesis in lymphocytes,[16] and 30 μg/ml or more inhibits protein synthesis and mitochondrial respiration.[17] Moreover, these concentrations are achievable in patients. Indeed, the peak plasma levels of mitoguazone in 12 treated patients with AIDS-related non-Hodgkin's lymphoma ranged from 6.47 to 42.8 μg/ml.[18] Mitoguazone was added on day 0 of culture and was replaced each time the cells were passaged. The cells were harvested, and the degree of apoptosis was quantitated at days 7, 14 and 21.

Evaluation of apoptosis

The degree of apoptosis was quantitated using two methods of terminal deoxynucleotidyl transferase assay (TdT). First, apoptotic cells were identified by direct fluorescence detection of digoxigenin-labeled genomic DNA using the ApopTag kit (S7110 kit; Oncor, Gaithersburg, MD). Residues of digoxigenin-nucleotide were catalytically added to the DNA by the enzyme TdT. The nucleotides formed a heteropolymer of digoxigenin-11-dUTP and dATP. An anti-digoxigenin antibody fragment containing fluorescein was then added and an intense signal was emitted at 523 nm following excitation at 494 nm wavelength. For each evaluation, 1×10^6 cells were pelleted and incubated briefly in 4% formalin. Aliquots of 75 μl were then spread on silanized slides and allowed to dry. The TdT enzyme was added and the slides were incubated for 1 h at 37 C. After incubation, the cells were washed in

a stop/wash buffer for 30 min and antidigoxigenin-fluorescein was added for 30 min. The cells were counterstained with propidium iodide/Vectashield and examined using a fluorescence microscope. A minimum of 500 cells was counted and the percentage of apoptotic cells was calculated.

The second method used to quantitate apoptosis was flow cytometry. Cells were removed from the flask and centrifuged for 6 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 2 ml of PBS without Ca^{2+} and Mg^{2+} and recentrifuged as above. The supernatant was removed, and the cells were resuspended in EM grade paraformaldehyde 0.5% (Electron Microscopy Sciences, Fort Washington, MD) PBS for 10 min at room temperature. The cells were centrifuged, washed once with PBS and then resuspended in 1 ml of PBS. The cell suspension was added to 2.5 ml of cold (−20°C) absolute ethanol for a 70% final concentration of ethanol. The cells were aliquoted at 10^6 cells for each test. Then 1 ml of PBS was added and centrifuged for 6 min at 4°C. The supernatant was removed and the pellet was washed twice in 1 ml of PBS. After the final wash, the supernatant was removed and 50 μl of TdT Reaction Mixture (Boehringer Mannheim kit 220-582) was added to each tube, except the control tube with Reaction Mixture without TdT, and incubated at 37°C for 30 min. After incubation, the cells were washed with PBS 2 ml, the supernatant was removed, and 100 μl of FITC-avidin Reaction Mixture (Vector, Burlingame, CA) was added to each tube and incubated for 30 min at room temperature in the dark. Following the incubation, the cells were washed once in PBS 2 ml and resuspended in 1 ml of propidium iodide (PI) 5 μg/ml. Flow cytometric measurements were collectd on a Coulter Epics Elite flow cytometer using a 488 nm laser line for fluorochrome excitation. The FITC and PI emissions were split using a 550 nm dichroic filter, with FITC emissions collected through a 525/30 nm filter and the PI emissions collected through a 675 nm long pass filter.

Statistical analysis

The Fisher's exact test at a significance level of 0.05 was used to compare the degree of apoptosis in mitoguazone-treated cells and in untreated cells on days 1, 3 and 5.

Results

In Raji cell lines, mitoguazone induced a significant amount of apoptosis compared to the untreated cells from the concentrations of 0.1 μg/ml and higher on day 3 (p=0.038 and p=0.037 compared to untreated cell lines on day 3 with apoptosis measured both by flow cytometry and fluorescence detection) (Figures 1A and 2A). In the Ramos and Daudi cell lines, the 0.1 μg/ml concentration did not induce significant apoptosis. Significant apoptosis was detected from day 3 in these cell lines with the 1 μg/ml mitoguazone concentration ($p<0.01$ and $p<0.01$, respectively, on day 3 with apoptosis quantitated by flow cytometry) (Figures 2 and 3). In the MPC3 cell lines, mitoguazone induced significant apoptosis from day 3 at 1 μg/ml concentrations ($p<0.01$ on day 3 with apoptosis quantitated by flow cytometry) (Figure 4A). In the two human breast cancer cell lines, MCF7 and VM4K, mitoguazone at a concentration of 10 μg/ml induced

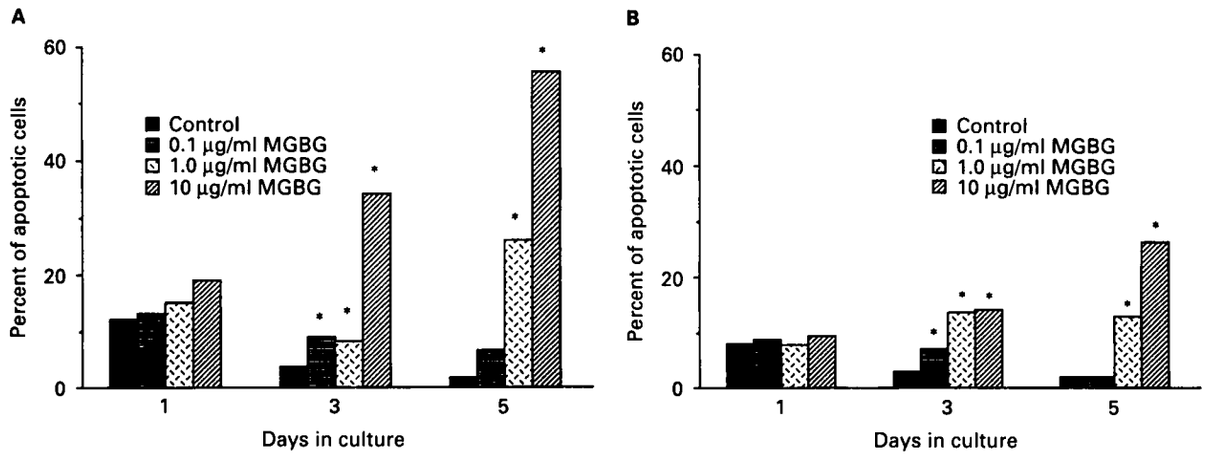

Figure 1. Quantitation of apoptosis in Raji cells by (A) TdT fluorescence and (B) flow cytometry. *p<0.5.

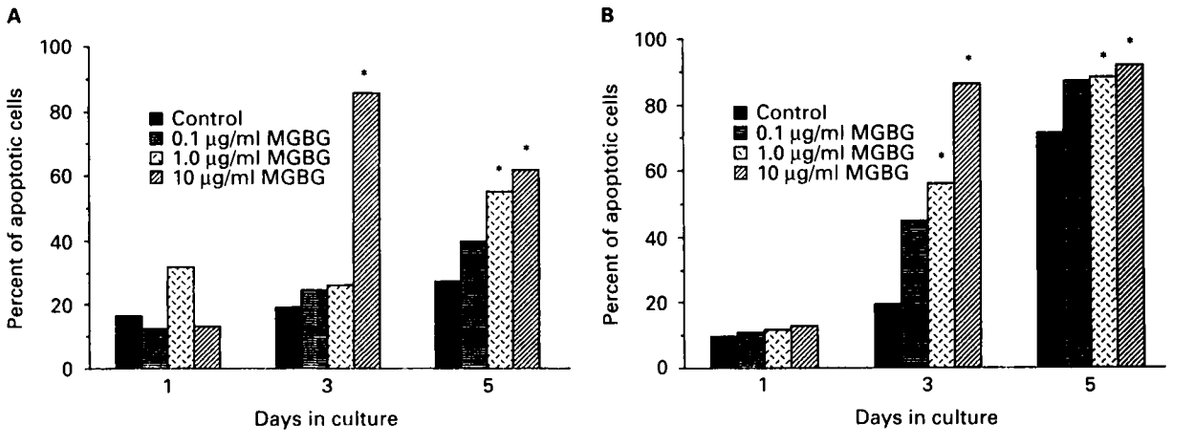

Figure 2. Quantitation of apoptosis in Ramos cells by (A) TdT fluorescence and (B) flow cytometry. *$p < 0.5$.

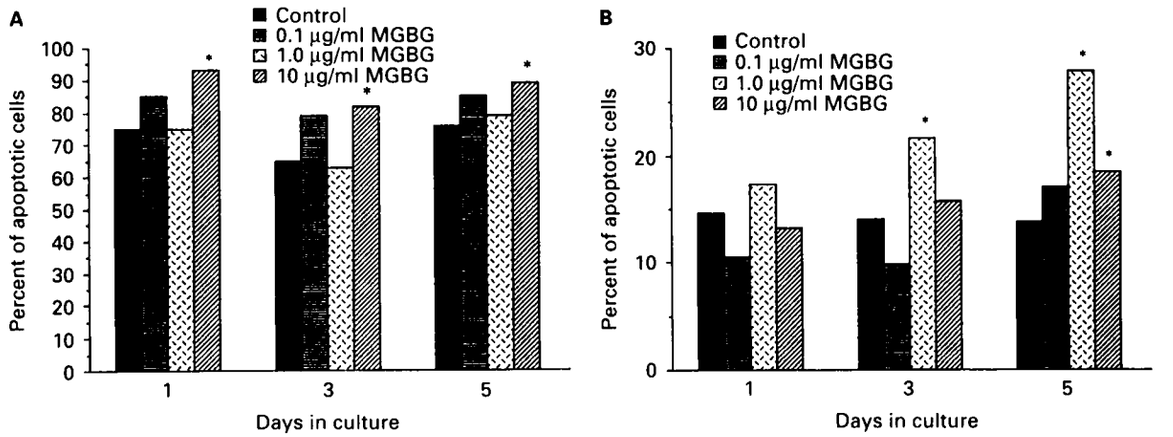

Figure 3. Quantitation of apoptosis in Daudi cells by (A) TdT fluorescence and (B) flow cytometry. *$p < 0.5$.

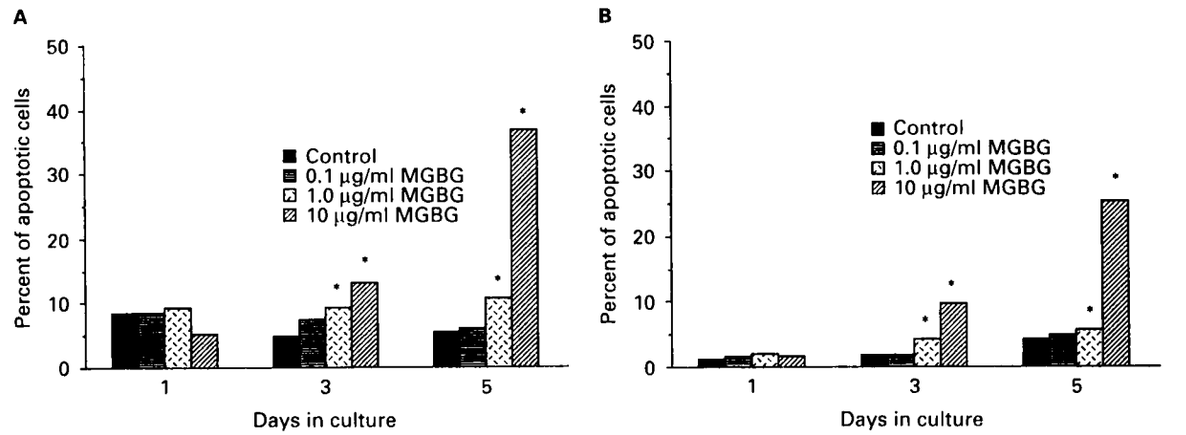

Figure 4. Quantitation of apoptosis in MPC3 cells by (A) TdT fluorescence and (B) flow cytometry. *$p < 0.5$.

significant apoptosis from day 3 ($p < 0.01$ and $p < 0.01$, respectively, on day 3 with apoptosis quantitated by flow cytometry) (Figures 5 and 6). The mutation of the p53 suppressor oncogene in the VM4K cell lines did not prevent apoptosis induction by mitoguazone.

Discussion

The present study has demonstrated that mitoguazone induces apoptosis in three different human lymphoma cell lines (Raji, Ramos and Daudi), a human prostate carcinoma cell line (MPC 3) and two human breast cancer cell lines (MCF7 and VM4K). The induction of apoptosis by mitoguazone was both concentration and time dependent. Mitoguazone at the concentration of 0.1 μg/ml was not effective in inducing apoptosis; however, mitoguazone at the concentration of 1 μg/ml induced a significant degree of apoptosis from day 3. At the highest concentration of 10 μg/ml, the degree of apoptosis was more extensive than at the concentration of 1 μg/ml. Interestingly, p53 suppressor oncogene status did not influence the induction of apoptosis by mitoguazone in the human breast cancer cell lines. Apoptosis was evident in MCF7 cell line with p53 wild-type following treatment with mitoguazone. Apoptosis was also observed in VM4K breast cancer cells line with mutated p53.

It has been demonstrated that polyamines interact and stabilize DNA, and that polyamine-depleted cells

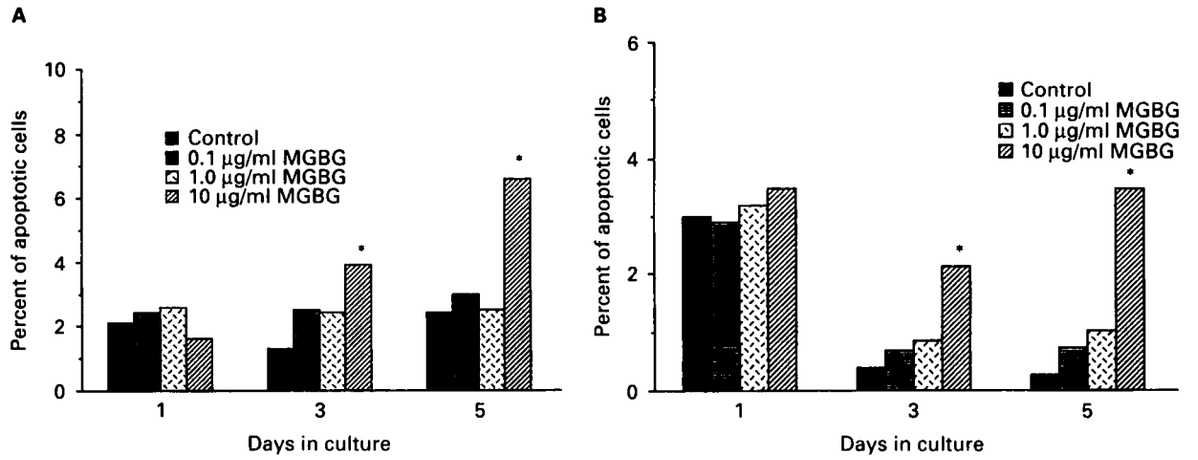

Figure 5. Quantitation of apoptosis in MCF7 cells by (A) TdT fluorescence and (B) flow cytometry. *p < 0.5.

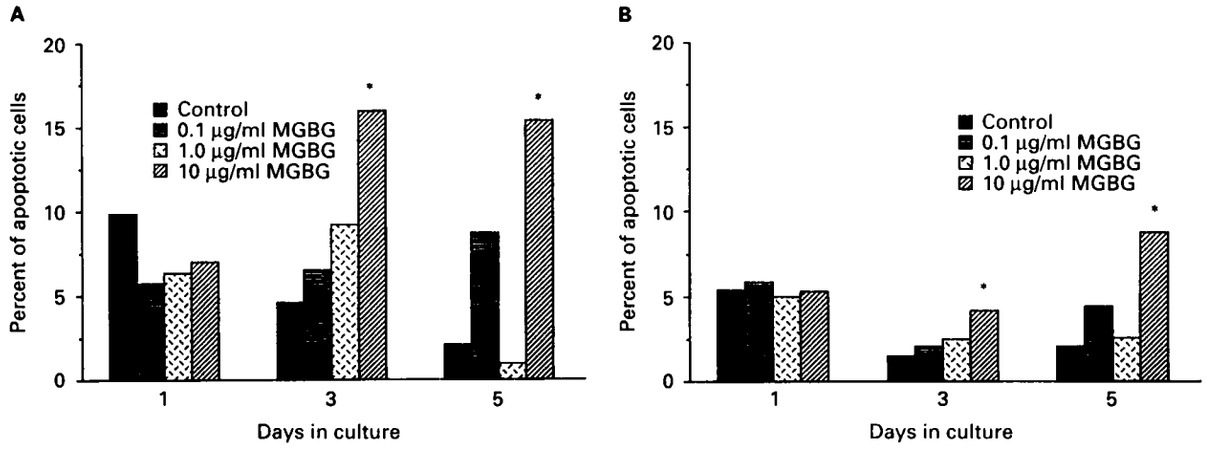

Figure 6. Quantitation of apoptosis in VM4K cells by (A) TdT fluorescence and (B) flow cytometry. *p < 0.5.

undergo changes in chromatin structure.[6] Previous reports have shown that a decline in polyamine levels induced apoptosis in thymocytes, and addition of exogenous spermine and spermidine prevented endonucleases activation and apoptosis in these cells.[19] The presence of spermine, spermidine and putrescine prevented apoptosis in a concentration-dependent manner in neurons.[20] Mitoguazone has previously been shown to induce apoptosis in human lung carcinoma cell lines and human colon carcinoma cell lines.[21] It was also observed that exogenous spermine protected these human cancer cell lines against mitoguazone-induced apoptosis.[21] These results provide evidence that the loss of the interactions between polyamines and DNA may trigger programmed cell death. We have shown that mitoguazone-induced apoptosis does not require an intact p53 function. It has already been suggested that p53 status did not affect cell lines from undergoing apoptosis with polyamine depletion. Indeed, various selenium derivatives were able to induce apoptosis by disruption of the polyamines pathway via a p53-independent mechanism.[21]

Conclusion

Our data demonstrated that mitoguazone induces apoptosis in various cancer cell lines. Mitoguazone produces apoptosis in human breast cancer cell lines by disruption of the polyamines pathway via a p53-independent mechanism. Depletion of polyamine pools seems to trigger p53-independent programmed cell death and inhibitors of polyamine synthesis such as mitoguazone provide a unique class of agents with potential antitumor properties on p53 mutant tumors.

References

1. Warrel RP, Burchenal JH. Methylglyoxal-bis(guanylhydrazone)(methyl GAG): current studies and future prospects. *J Clin Oncol* 1983; **1**: 52-65.
2. Cabanillas F, Hagemeister FB, McLaughlin P, *et al.* Results of MIME salvage regimen for recurrent or refractory lymphoma. *J Clin Oncol* 1987; **5**: 407-12.
3. Levine AM, Tulpule A, Tessman D, *et al.* Mitoguazone therapy in patients with refractory AIDS-related lymphoma: results from a multicenter phase II trial. *J Clin Oncol* 1997; **15**: 1094-103.
4. Von Hoff DD. MGBG: teaching an old drug new tricks. *Ann Oncol* 1994; **5**: 487-93.
5. Pine MJ, DiPaolo J. The antimitochondrial actions of 2-chloro-4',4''-bis(2-imidazolin 2-yl)-terephtalanilide and methylglyoxal-bis(guanylhydrazone). *Cancer Res* 1966; **26**: 18-25.
6. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* 1988; **48**: 759-74.
7. Corti A, Dave C, Williams-Ashman HG, Mihich E, Schenone A. Specific inhibition of the enzymatic decarboxylation of 5-adenosylmethionine by methylglyoxal-bis(guanylhydrazone) and related substances. *Biochem J* 1974; **139**: 351-7.
8. Hyvonen T, Seiler N, Persson L. Characterization of a COS cell line deficient in polyamine transport. *Biochim Biophys Acta* 1994; **1221**: 279-85.
9. Heby O, Persson L. Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends Biol Sci* 1990; **15**: 153-8.
10. Pegg AE, Poulin R, Coward JK. Use of aminopropyltransferase inhibitors and of non-metabolizable analogs to study polyamine regulation and function. *Int J Biochem Cell Biol* 1995; **27**: 425-42.
11. Marton LJ, Pegg AE, Polyamines as targets for therapeutic intervention. *Annu Rev Pharmacol Toxicol* 1995; **35**: 55-91.
12. Lowe SW, Ruley HE, Jacks T, Housman DE. P53-dependent apoptosis modulates the cytotoxicity of anticancer drugs. *Cell* 1993; **74**: 957-67.
13. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell* 1994; **78**: 539-42.
14. Ohnuki Y, Marnell MM, Babcock MS, Lechner JF, Kaighn ME. Chromosomal analysis of human prostatic adenocarcinoma cell lines. *Cancer Res* 1980; **40**: 524-34.
15. Moore MR. An insulin effect on cytoplasmic estrogen receptor in the human breast cancer cell line MCF-7. *J Biol Chem* 1981; **256**: 3637-40.
16. Morris D, Jorstad C, Seyfried CE. Inhibition of the synthesis of polyamines and DNA in activated lymphocytes by a combination of methylornithine and methylglyoxal-bis(guanylhydrazone). *Cancer Res* 1977; **37**: 3169-72.
17. Burk D, Evans W, Hunter J, Woods M. Primary inhibition of respiration by methylglyoxal-bis(guanylhydrazone) in L1210 leukemia and other cells, with implications for multiple chemotherapy. *Proc Am Ass Cancer Res* 1962; **3**: 308.
18. Rizzo J, Levine AM, Weiss GR, *et al.* Pharmacokinetic profile of mitoguazone (MGBG) in patients with AIDS related non-Hodgkin's lymphoma. *Invest New Drug* 1996; **14**: 227-34.
19. Desiderio MA, Grassilli E, Bellesia E, Salomoni P, Franceschi C. Involvement of ornithine decarboxylase and polyamines in glucocorticoid-induced apoptosis in rat thymocytes. *Cell Growth Different* 1995; **6**: 505-13.
20. Harada J, Sugimoto M. Polyamines prevent apoptotic cell death in cultured cerebellar granule neurons. *Brain Res* 1997; **753**: 251-9.
21. Redman C, Xu MJ, Peng YM, *et al.* Involvement of polyamines in selenomethionine induced apoptosis and mitotic alterations in human tumor cells. *Carcinogenesis* 1997; **18**: 1195-202.

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