available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

The methionine salvage pathway compound 4-methylthio-2-oxobutanate causes apoptosis independent of down-regulation of ornithine decarboxylase

Baiqing Tang [a], *Yuwaraj Kadariya* [a], *Maureen E. Murphy* [b], *Warren D. Kruger* [a,*]

[a] *Division of Population Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, United States*
[b] *Division of Medical Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, United States*

ARTICLE INFO

Article history:
Received 24 April 2006
Accepted 14 June 2006

Keywords:
Methionine
Polyamines
Apoptosis
Methylthioadenosine phosphorylase
Keto-acid

Abbreviations:
MTOB, 4-methylthio-2-oxobutanoic acid
ODC, ornithine decarboxylase
DFMO, difluoromethylornithine
MTAP, methylthioadenosine phosphorylase
7-AAD, 7-amino-actinomycin D
FBS, fetal bovine serum

ABSTRACT

4-Methylthio-2-oxobutanoic acid (MTOB) is the final compound of the methionine salvage pathway that converts the polyamine byproduct methylthioadenosine to adenine and methionine. Here we find that MTOB inhibits growth of several human cell lines in a dose-dependent manner. Growth inhibition was specific for MTOB as we did not observe any inhibition with other chemically related compounds. MTOB treatment causes apoptosis and reduction of ornithine decarboxylase (ODC) activity but not ODC mRNA. To determine if MTOB exerts its effects primarily via ODC inhibition, we compared the effects of MTOB with the ODC-specific inhibitor difluoromethylornithine (DFMO). We found that MTOB was a more potent inducer of apoptosis than DFMO, lacked activation of caspase 3/7, and was able to induce apoptosis in cells lacking p53. Our results show that MTOB-induced growth inhibition and apoptosis is not simply secondary due to ODC inhibition and implies that MTOB activates apoptosis via other mechanisms.

1. Introduction

4-Methylthio-2-oxobutanoic acid (MTOB) is the penultimate compound in the methionine salvage pathway which is used to recycle methylthioadenosine, a by-product of polyamine metabolism, into adenine and methionine (Fig. 1). The initial enzyme in the pathway is methylthioadenosine phosphorylase (MTAP). Loss of MTAP activity is frequently observed in a variety of human tumors, including lung cancer, leukemia, glioblastoma, pancreatic cancer, and melanomas [1–5]. This activity loss is generally due to homozygous deletion of the MTAP gene [6].

* *Corresponding author*. Tel.: +1 215 728 3030; fax: +1 215 214 1623.
E-mail address: warren.kruger@fccc.edu (W.D. Kruger).
0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.06.018

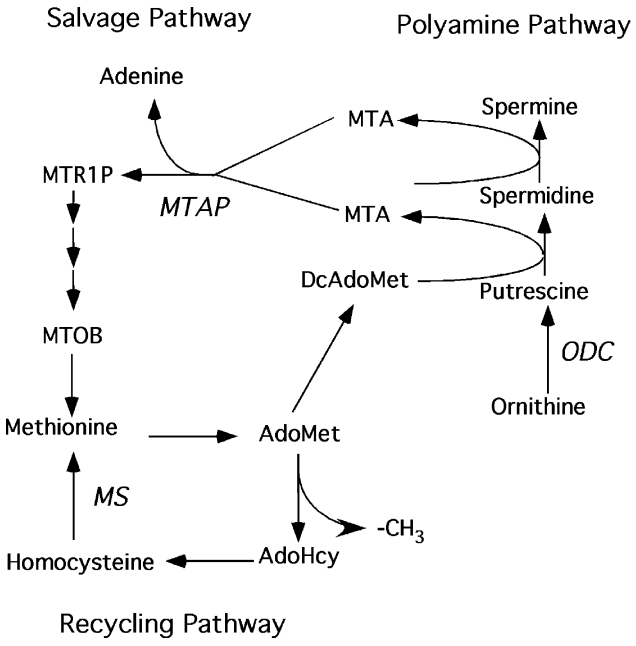

Fig. 1 – Methionine metabolic pathways. Metabolites are interconverted as shown by arrows. Key enzymes are in italics next to arrows. Abbreviations—AdoMet: S-adenosylmethionine; AdoHcy: S-adenosylhomocysteine; DcAdoMet: decarboxylated S-adenosylmethionine; MTA: methylthioadenosine; MTR1P: methylthio-ribose 1-phosphate; MTOB: methylthio-oxo-butanoic acid; MTAP: methylthioadenosine phosphorylase; ODC: ornithine decarboxylase; MS: methionine synthase.

Evidence suggests that loss of MTAP may be important in tumorigenesis. Expression of ectopic MTAP in MTAP-deleted MCF-7 cells inhibits the ability of the cells to grow in soft agar and form tumors in SCID mice [7]. Re-expression of MTAP in Mel Im melanoma cells inhibits migration in collagen [5]. Loss of MTAP is also associated with elevation in polyamines due to increased levels of ornithine decarboxylase (ODC), the initial enzyme in the polyamine biosynthetic pathway [4,7,8]. Elevated ODC and polyamines are observed in many different tumors, and studies have shown that over-expression of ODC is able to transform fibroblasts *in vitro* [9,10]. In addition, transgenic mice with ODC expressed under control of the keratinocyte promoter are more susceptible to skin tumors [10].

Previous work from our lab has suggested that the accumulation of the salvage pathway compound MTOB is responsible for the decrease in ODC activity observed in MTAP expressing cells. Substitution of methionine with MTOB in media causes a significant decrease in ODC levels and activity in both *S. cerevisiae* and human tumor derived cells lines [8]. At concentrations of 100 μM MTOB can substitute for 100 μM methionine as a source of fixed-sulfur for mammalian cell growth [11]. However, at high concentrations (>1 mM) MTOB has been reported to induce apoptosis in at least one cell line [12]. In this paper we explore the effect of MTOB on cell growth and test the hypothesis that MTOB induces apoptosis via its inhibition of ODC.

2. Materials and methods

2.1. Chemicals and reagents

4-Methylthio-2-hydroxyl-butanoic acid (MTHB), MTOB, 2-oxo-glutaric acid (OG), methionine, DFMO, putrescine, spermidine, spermine, mouse monoclonal anti-β-actin were purchased from Sigma Chemical Corp. (St. Louis, MO). Rabbit polyclonal anti-PARP (p85 fragment) antibody was purchased from Upstate (Lake Placid, NY). Mouse monoclonal anti-p53 antibody (Ab-6) was purchased from Calbiochem (San Diego, CA).

2.2. Cell lines and media

MiaPaca-2, MCF-7, and HepG2 cells were purchased from ATCC. All cells except PA-1 were routinely grown in DMEM supplemented with 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml of streptomycin, and 10% dialyzed fetal bovine serum (FBS). This media contains 200 μM methionine and will be referred to as standard media. In the experiment in which ODC activity and mRNA levels were examined (Fig. 4A), the media was identical to that described above except that the DMEM lacked methionine (methionine-free media). PA-1 and PA-1/E6 cells were obtained from Dr. Wafik El-Deiry (University of Pennsylvania). PA-1 cells were cultured in BME media supplemented with 10% dialyzed FBS, 2 mM glutamine, 100 μg/ml of penicillin and 100 μg/ml of streptomycin. PA-1/E6 cells were cultured with same media as used for PA-1 cells with addition of 400 μg/ml G418. F5889 cells were a generous gift of Dr. Gottfried Boers (University Medical Center, Nijmegen, The Netherlands). All media and serum were obtained from the tissue culture facility at Fox Chase Cancer Center.

2.3. Stable transformation of HT1080 cells with hMTAP

The pTRE2hyg:MTAP plasmid was created by inserting the hMTAP containing BamHI/EcoRV fragment from pCR:sMTAP [7] into pTRE2hyg (Clontech, Palo Alto, CA). HT1080 Tet-Off cells (Clontech) were cultured in standard media supplemented with 250 μg/ml G418. Transformations were performed using Fugene6 reagent (Roche, Indianapolis, IN), a nonliposomal transformation agent according to the manufacturer's instructions. Four micrograms of pTRE2hyg:MTAP DNA were used for each transformation. Clones were selected using 250 μg/ml (Sigma). The stable expression of human MTAP gene in the transformed cell line was confirmed by MTAP activity assay and Western blot.

2.4. Cell growth assays

Cell growth was determined with the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (MTS assay, Promega, Madison, WI) following manufacturer's instructions. Cells were plated in a 96 well-plate and cultured in standard media with various indicated supplements. Each treatment was carried out in triplicate. At the designated time, media was replaced by 100 μl standard media containing 15% cell titer 96 Aqueous one solution and plate was incubated at 37 °C for 3 h. The absorbance of the MTS metabolite formazin at 492 nm

was recorded immediately by using a 96-well plate reader, Multiskan Ascent (Thermo Bioanalysis, Santa Fe, NM).

2.5. Measurement of ornithine decarboxylase

Ornithine decarboxylase activity was assayed by measuring the $^{14}CO_2$ formed by decarboxylation of 1-^{14}C-labeled ornithine as previously described [8].

2.6. RNA preparation and real-time PCR

MiaPaca-2 cells were initially seeded in 100 mm Petri-dishes. After 24 h, standard media was replaced with methionine-free media supplemented with the concentration of methionine or MTOB indicated in Fig. 4A. At the end of the treatment period, cells were harvested and total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction.

Human ODC gene-specific probe and primer sets for quantitative assay were obtained from Applied Biosystems (Foster, CA). Quantitative real time PCR was carried out according to the TaqMan Assay-on-Demand one-step protocol of Applied Biosystems with universal thermal condition. Human β-actin primer and probe sets from the same supplier were used as endogenous normalization standard. Each assay was performed in triplicate.

2.7. Measurement of caspase 3/7

Caspase 3/7 activity was measured with Caspase-Glo 3/7 Assay kit from Promega (Madison, WI) following manufacture's instruction. In brief, MiaPaca-2 cells were plated in 96 well-plate and cultured in standard media with specified supplements. Each treatment was carried out in triplicate. At assay time, media was replaced by 200 μl of freshly made reaction solution (one part standard media and one part Caspase-Glo 3/7 reagent), mixed gently, and then incubated at 25 °C for 60 min. The luminescence of each sample was measured by using a plate reader, Fluoroskan Ascent FL (Thermo Bioanalysis, Santa Fe, NM), according instruction of the manufacturer.

2.8. Apoptosis assay and cell-cycle analysis

Annexin V and 7-AAD levels were determined with Guava Nexin kit using a Guava Personal Cell Analysis System (PCAS, Guava Technologies, Hayward, CA) according to the manufacturer's instruction. Cells were cultured in standard media supplemented with various concentrations of MTOB or DFMO for 72 h. Cells were trypsinized and collected by centrifuging at 1000 rpm for 5 min at 4 °C. After washing with ice-cold 1× Nexin buffer, cells were resuspended in the same buffer, labeled with Annexin V-PE and 7-aminoactinomycin D in the dark on ice for 20 min, and then analyzed with the PCAS.

For cell cycle analysis, 1×10^6 cells were collected as described above. After washing with ice-cold PBS, cells were fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed twice with PBS, resuspended in 500 μl staining solution (PBS containing 200 μg/ml of DNase-free RNase, 25 μg/ml of propidium iodide and 0.1% of Triton X-100) and then incubated at 25 °C for 30 min in the dark. The stained cells were sorted and analyzed on the PCAS.

2.9. Western blotting

Western blotting was performed as previously described [7]. Membranes were probed with primary antibodies (1:2000 for rabbit anti-PARP antibody, 1:1000 for mouse anti-p53 antibody, and 1:20,000 for mouse anti-β-actin antibody) with 1% non-fat milk in TTBS. HRP-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) were used at 1:30,000. The SuperSignal West Dura ECL Western Blotting kit (Pierce, Rockford, IL) was used to detect the antigen–antibody complex.

3. Results

3.1. MTOB is a specific growth inhibitor in a variety of cell lines

We examined the effect of MTOB on cell growth in five different human cell lines, three tumor derived cell lines and two primary non-transformed cell lines (Fig. 2A). The tumor

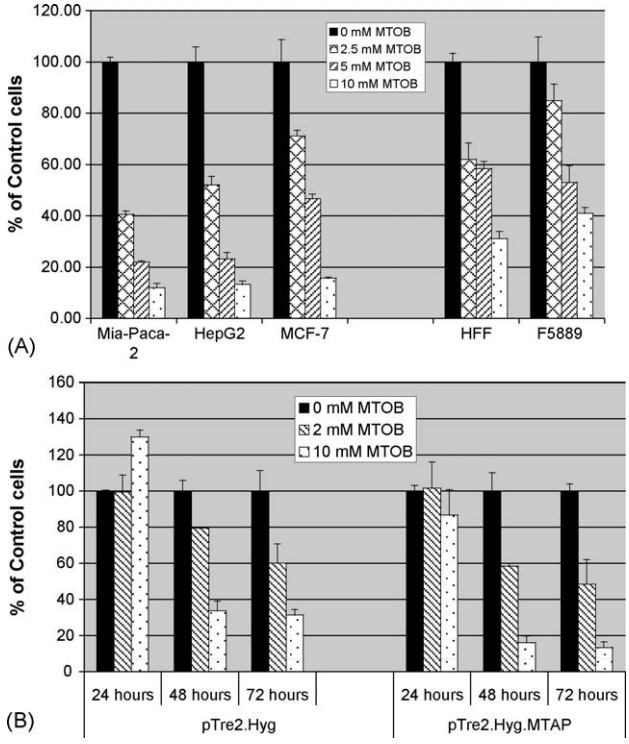

Fig. 2 – Growth inhibition by MTOB. (A) 5×10^3 of the indicated cell types were seeded in triplicate in 96-well plates in standard media supplemented with the indicated amount of MTOB. After 72-h growth was assessed by MTS assay. Error bars show standard deviation. (B) Growth inhibition in isogenic MTAP$^+$ and MTAP$^-$ HT1080 cells. Cells stably transformed with either the empty vector (pTre2.Hyg) or an MTAP expressing plasmid (pTre2.Hyg.MTAP) were assessed for growth in standard media supplemented with the indicated amount of MTOB. Growth was assessed by MTS assay.

derived cell lines included a human pancreatic adenocarcinoma derived cell line (MiaPaca-2), a human hepatocellular carcinoma line (HepG2) and a breast adenocarcinoma derived line (MCF-7). The two primary lines included early-passage human foreskin fibroblasts (HFF) and a skin-derived fibroblast line derived from a patient with cystathionine beta-synthase deficiency (F5889). Using normal methionine containing media, we found that supplementation with high concentrations of MTOB caused growth inhibition in all the lines tested. The IC_{50} for the tumor derived Mia-Paca-2, HepG2, and MCF-7 cells was 2.1, 2.7 and 4.6 mM, respectively. The IC_{50} for the non-transformed HFF and F5889 cells was 6.2 and 6.8 mM. The mean IC_{50} value after 72 h of growth for the three tumor derived cell lines was 3.1 mM compared to a mean of 6.4 mM for the non-tumor derived cell lines ($p < 0.05$). These results suggest that transformed cells are somewhat more sensitive to MTOB-induced growth inhibition than non-transformed cells.

Since all of the tumor cell lines we tested were MTAP-deficient, while the primary cell lines were not, we examined directly if MTAP status affects MTOB sensitivity in isogenic $MTAP^+$ and $MTAP^-$ cells. HT1080 is a MTAP-deleted human osteosarcoma derived cell line. We stably transfected this line with either an empty vector plasmid (pTRE2hyg) or an MTAP expression plasmid (pTRE2hygMTAP). Expression of MTAP in these cells was confirmed by Western blot analysis (data not shown). As shown in Fig. 2B, at all three time points examined the $MTAP^-$ cells exhibited greater growth than $MTAP^+$ cells. These results show that MTAP-expressing cells are more sensitive to MTOB.

To determine if growth inhibition of MTOB was specific, we examined the growth inhibitory effects of other structurally related compounds (Fig. 3A). MTHB is identical to MTOB with the exception that there is a hydroxyl, as opposed to a keto-group, present on the alpha carbon. OG, like MTOB, is an oxoacid, but substitutes a second carboxylic acid group for the methylthio group in MTOB. Unlike MTOB, we found that neither MTHB nor OG exhibited significant growth inhibition when added to standard media at concentrations up to 20 mM on HepG2 cells (Fig. 3B). We also failed to observe any growth inhibition by methionine itself both in HepG2 cells as well as MiaPaca-2 cells and MCF-7 cells (data not shown). From these studies we conclude that growth inhibition by MTOB is specific and requires both a methylthio-group and a oxoacid moiety.

3.2. *Post-transcriptional inhibition of ODC by MTOB*

Previously we had shown that the addition of MTOB to methionine-free media resulted in down-regulation of ODC activity in *S. cerevisiae* and two mammalian cell lines [8]. To reconfirm this observation, we placed MiaPaca-2 cells in media containing either 100 μM methionine, no methionine, 800 μM MTOB or 8 mM MTOB for 48 h and then isolated protein and RNA. We first examined ODC activity using a radiometric assay (Fig. 4A, white bars). We found that cells exposed to methionine-free media had about half the ODC activity (normalized to total protein) of cells exposed to standard media. Surprisingly, cells exposed to 800 μM MTOB actually had significantly increased ODC activity compared to cells

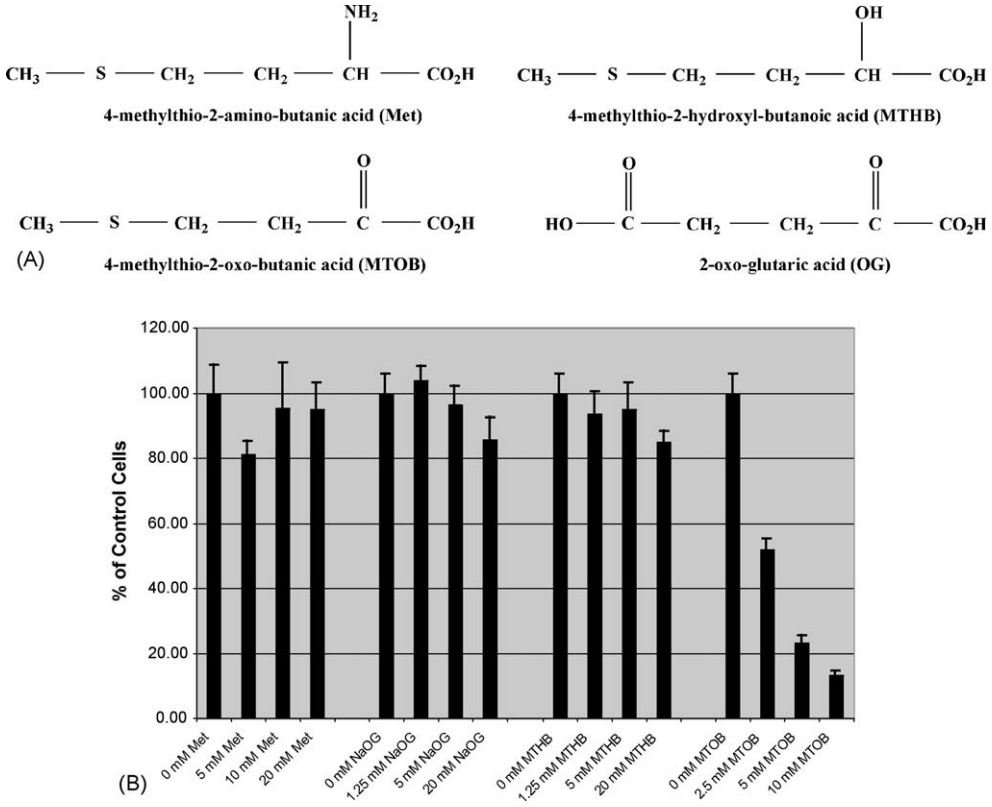

Fig. 3 – Growth effects of MTOB related compounds. (A) Chemical structures of compounds used in this study. (B) HepG2 cells were seeded as described above in standard media supplemented with the indicated compounds. Growth was assessed by MTS assay.

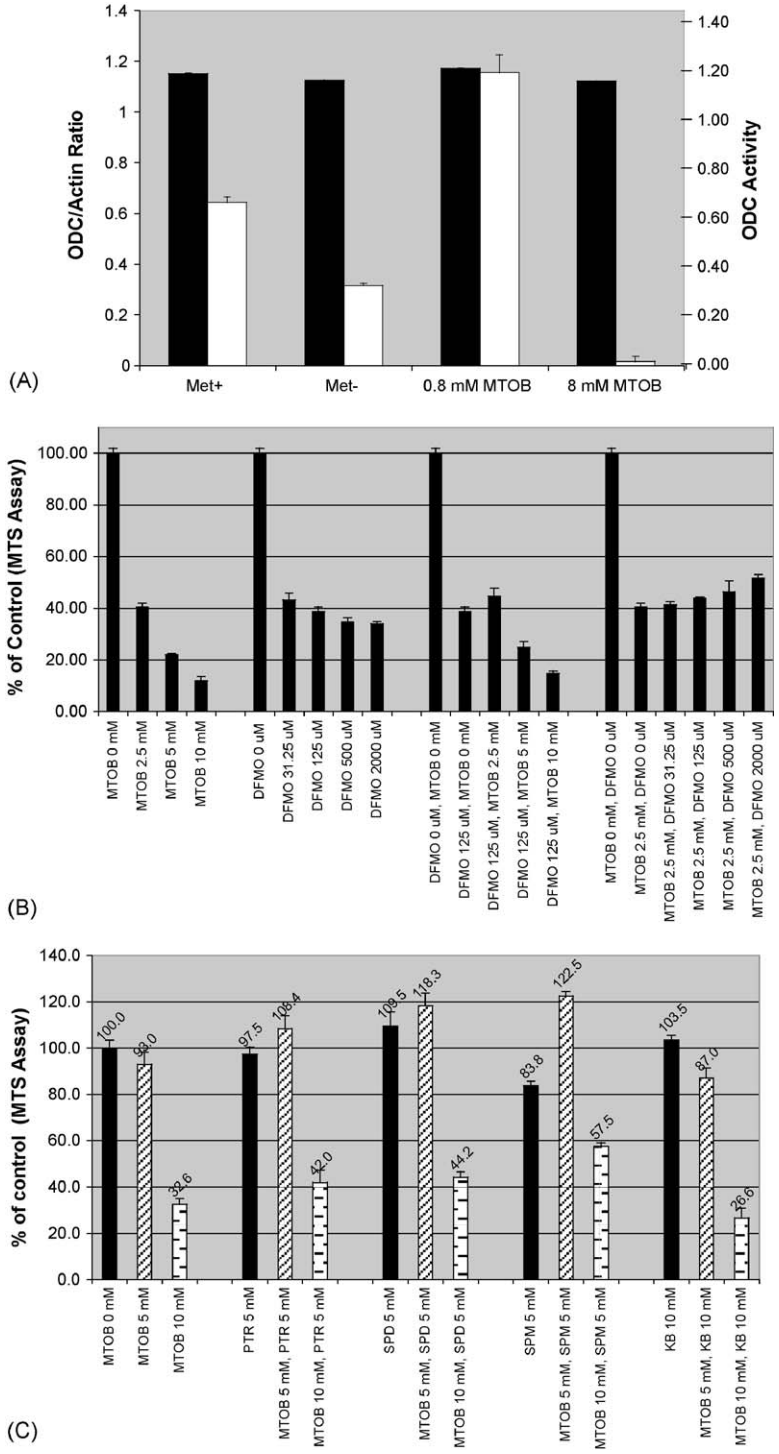

Fig. 4 – MTOB effects on polyamine pathway. (A) Effect of MTOB on ODC mRNA and enzyme activity. MiaPaca-2 cells were grown in methionine-free media supplemented with either methionine or MTOB at the indicated concentration. After 48 h cells were harvested and ODC mRNA and ODC activity were determined as described in Section 2. Black bars show relative level of ODC mRNA as compared to actin mRNA using quantitative real time PCR. White bars show ODC enzyme activity in the same cells. Measurements were performed in triplicate for qRT-PCR and quadruplicate for the enzyme assay. Standard error is shown as indicated by the bars. (B) Comparison of growth inhibition of MTOB and DFMO. MiaPaca-2 cells were plated at a density of 3000 cells per well in 96-well plates in triplicate in standard media supplemented with the indicated levels of MTOB and DFMO and allowed to grow for 72 h. Growth was measured by MTS assay. Bars indicate the standard error. (C) Effect of exogenous polyamines. MiaPaca-2 cells were plated in standard media containing the indicated supplements and allowed to grow for 48 h. Growth was assessed using the MTS assay. Experiment was done in triplicate and standard error is shown.

grown in 100 μM methionine. However, when a higher concentration of MTOB was used, we observed a 30-fold decrease in ODC activity. These findings confirm that high levels of MTOB can inhibit ODC activity.

We then used quantitative RT-PCR to determine whether the drop in ODC activity was due to decreased ODC mRNA levels. We found that the number of cycles required to amplify the ODC mRNA did not vary significantly between the samples, and that the ratio of ODC mRNA to actin mRNA was essentially identical (Fig. 4A, black bars). These findings indicate that the reduction in ODC activity by MTOB must be occurring at the post-transcriptional level.

3.3. Growth inhibition by MTOB and DFMO

Since inhibition of ODC is known to inhibit cell growth, we compared the effect of MTOB and the specific ODC inhibitor DFMO on the growth of MiaPaca-2 cells [13]. As shown in Fig. 4B, both MTOB and DFMO inhibited MiaPaca-2 cell growth after 72 h of exposure. However, DFMO appears to be less potent than MTOB. Growth inhibition with DFMO exhibited at plateau at 35–40% of wild-type, while MTOB inhibition exhibited a strong linear response over the entire dose range. Interestingly, when DFMO and MTOB were combined we did not see any indication of additivity, but rather the addition of DFMO actually blunted some of the inhibitory effect of MTOB. These findings suggest that although DFMO and MTOB can inhibit ODC, their effects on cell growth are quite different.

3.4. MTOB induces morphological changes and apoptosis

To determine exactly why MTOB resulted in growth inhibition of cells, we examined the effect of MTOB on cell morphology of MiaPaca-2 cells. We found that cells exposed to high concentrations of MTOB seemed to contract in surface area and leave behind long thin pseudopodia jutting out from the cell's body. We also observed many dead cells with the characteristic blebbing observed in cells undergoing apoptosis (Fig. 5A). These observations suggested that growth inhibition observed in MTOB exposed cells was caused by increased apoptosis.

To confirm that MTOB was inhibiting growth via apoptosis, we examined molecular markers of apoptosis in MTOB treated cells as well as cells treated with DFMO. For these experiments, MiaPaca-2 cells were plated in media containing either nothing, 5 mM MTOB, 10 mM MTOB, 1 mM DFMO, or 5 mM DFMO for 72 h and apoptosis was assessed and examined in two different ways. First, cells were stained for two markers of apoptosis, annexin V and 7-AAD, and assessed by flow cytometry (Fig. 5B). Annexin V positive 7-AAD negative cells are in the early stages of apoptosis, while Annexin V positive 7-AAD positive cells are in the late stage of apoptosis. Treatment of cells with MTOB resulted in a four-fold increase in the number of early stage apoptotic cells, and a two-fold increase in the number of late stage apoptotic cells. Treatment of cells with DFMO resulted in a weaker response with a 1.5-fold increase in the number of early stage cells and a 1.6-fold increase in the number of late stage cells. In the same cells we also examined DNA content by flow cytometry to determine the percentage of cells with less than G1 DNA content, which is indicative of apoptosis (Fig. 5C). We found that treatment with MTOB caused a 2.6-fold increase in the number of cells in the pre-G1 (apoptotic) fraction, while treatment with DFMO caused only a 1.6-fold increase in the pre-G1 fraction. We did not notice any significant differences in the ratio of cells in G1 and G2, suggesting that neither MTOB or DFMO resulted in a specific cell cycle arrest point. From these experiments we conclude that MTOB induces apoptosis and is more potent than DFMO.

3.5. Activation of caspase 3/7 by DFMO but not MTOB

Cells treated with MTOB and DFMO were also assessed for activation of caspases 3 and 7, the so-called "executioner" caspases, by incubating cells with a luminogenic substrate that is activated by caspases 3 or 7 dependent cleavage. As shown in Fig. 6A, when normalized for cell number we observed a significant increase in caspase 3/7 activity in cells treated with DFMO, but failed to see any increase in activity in MTOB treated cells. Our results show that the apoptotic program initiated by MTOB does not involve caspase 3/7, while DFMO induced apoptosis does.

In a separate experiment, we looked for the presence of cleaved poly (ADP-ribose) polymerase-1 (p85 PARP), a known target of caspase 3/7. As shown in Fig. 6B, we observed a significant increase in p85 PARP in cells that were exposed to high doses of either MTOB or DFMO.

3.6. MTOB growth inhibition is partially rescued by addition of polyamines

The data shown above clearly indicated that growth inhibition and apoptosis by MTOB was not due simply to inhibition of ODC, but did not exclude the possibility that ODC inhibition was contributory. To examine the importance of ODC inhibition in the MTOB response, we asked whether polyamines could rescue the growth inhibition and apoptotic phenotype. We exposed MiaPaca-2 cells to 5 and 10 mM MTOB in the presence of the three main polyamines, putrescine, spermidine, and spermine (Fig. 4C). Addition of 5 mM of either putrescine, spermidine, or spermine resulted in a modest increase in growth, with spermine working the best. However, in no case did addition of polyamines fully alleviate MTOB induced growth inhibition. These findings suggest that ODC inhibition does not play a major role in MTOB-induced growth suppression and apoptosis.

3.7. MTOB growth inhibition is p53 independent

The p53 protein plays an important role in controlling cell growth and apoptosis. We compared the p53 dependence of growth inhibition and apoptosis by MTOB and DFMO using PA-1 ovarian teratocarcinoma cells either lacking or expressing the HPV16 E6 protein, which targets p53 for degradation [14]. PA-1 control cells or E6 expressing cells were exposed either to 2 and 4 mM MTOB or 0.2 and 1 mM DFMO for 48-h. As shown in Fig. 7A, wild-type PA-1 cells show similar growth inhibition with both DFMO and MTOB. In contrast, PA-1/E6 cells show no growth inhibition with either concentration of DFMO, but substantial growth inhibition occurred with 2 or 4 mM MTOB.

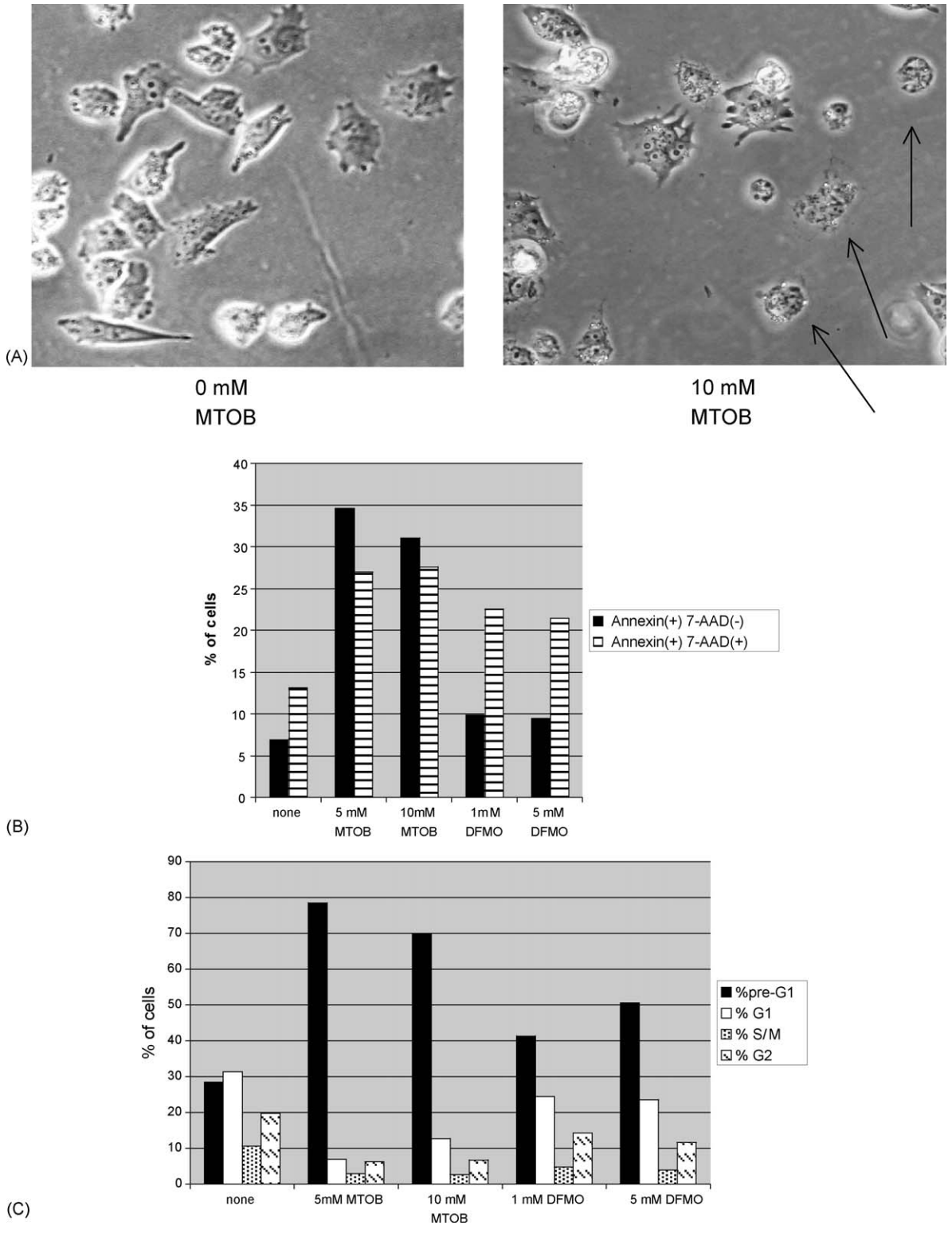

Fig. 5 – Induction of apoptosis by MTOB. (A) Morphology of MiaPaca-2 cells in standard media supplemented with either 0 or 10 mM MTOB for 48 h. Magnification is 200×. (B) MiaPaca-2 cells grown in standard media exposed to either DFMO or MTOB for 72 h were assessed for apoptosis by staining with annexin V and 7-AAD as described in Section 2. (C) Cell cycle analysis of cells exposed to DFMO or MTOB for 72 h.

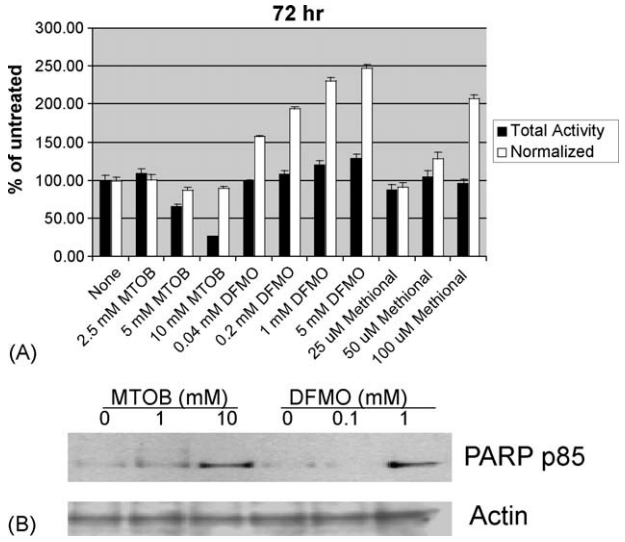

Fig. 6 – Caspase activation. (A) MiaPaca-2 cells grown in standard media supplemented with either MTOB, DFMO, or methional were assessed for caspase 3/7 activity using a luminogenic substrate as described in Section 2. Black bars show total caspase 3/7 activity. White bars show activity when normalized for cell number. Experiments were done in triplicate and standard error is shown by bars. (B) MiaPaca-2 cells grown in standard media supplemented with the indicated amount of either MTOB or DFMO for 72 h were harvested and assessed by immunoblot for levels of p85 PARP and actin.

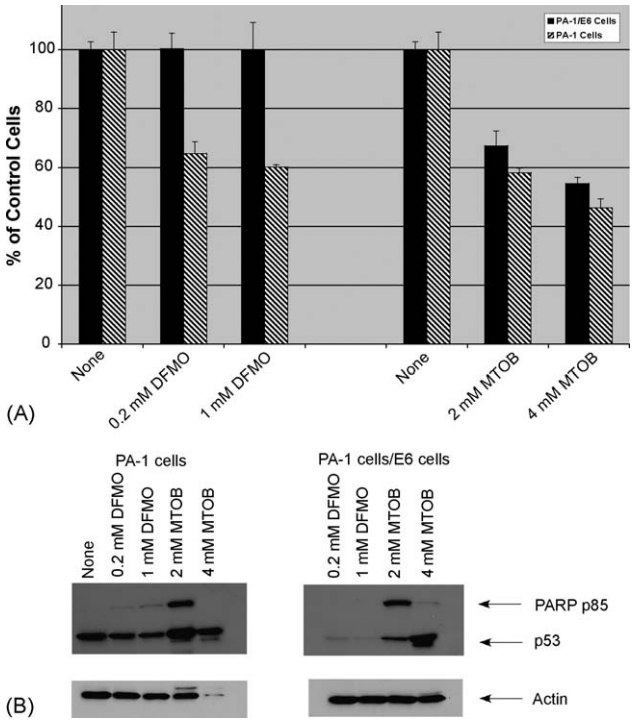

Fig. 7 – Effect of p53 on MTOB and DFMO induced growth inhibition and apoptosis. (A) PA-1 and PA-1/E6 cells were plated and incubated in BME media containing the various supplements for 48 h. Growth was then measured using the MTS assay in triplicate. (B) Cell lysates made from cells grown in parallel with panel A. were immunoblotted and probed for p85 PARP, p53, and actin.

We also examined apoptosis in these cells by examining the appearance of p85 PARP. As shown in Fig. 7B, PA-1 cells show substantially increased levels of p85 PARP when treated with DFMO or 2 mM MTOB. We observed less p85 when cells were exposed to 4 mM MTOB, but actin levels were also reduced suggesting that most of the PARP expressing cells had already completed apoptosis. In sharp contrast, we fail to observe cleaved PARP in PA-1/E6 cells treated with DFMO, but see high levels of cleaved PARP with MTOB treated cells. Interestingly, MTOB also appears to cause elevated p53 levels in PA-1/E6 cells, suggesting that MTOB somehow interferes with E6-dependent degradation of p53. Taken together, these experiments show that growth inhibition and apoptosis caused by MTOB is largely independent of p53 function, in contrast to DFMO-induced apoptosis.

3.8. *Conversion of MTOB to methional is not involved in MTOB induced apoptosis*

Our results from above indicate that most of MTOBs effect on cell growth and apoptosis is not due to its inhibition of ODC. An alternative mechanism that has been proposed involves the conversion of MTOB to methional by the action of branched-chain oxoacid dehydrogenase complex (BCOADC) [12]. If this were the case, one would expect that methional would cause similar effects as MTOB. Although we do observe growth inhibition and apoptosis with methional (data not shown), we see a strong difference in behavior with regard to the activation of caspase 3/7 (see Fig. 6A). Methional, unlike MTOB, results in activation of caspase 3/7, whereas MTOB does not. In addition, if MTOB is being converted to methional via BCOADC we would expect that co-incubation of MTOB with another BCOADC substrate such as α-ketobutyrate would inhibit the formation of methional by competing for BCOADC activity [12]. We found that 10 mM α-ketobutyrate did not rescue the growth inhibition of MTOB in MiaPaca-2 cells (Fig. 4C). In additional studies using HeLa cells we failed to see any protection from MTOB with concentrations of α-ketobutyrate up to 40 mM (data not shown). Our findings do not support the view that MTOB conversion to methional is the mechanism of MTOB-induced growth inhibition and apoptosis.

4. Discussion

In this report we show that MTOB can inhibit growth and induce apoptosis in a variety of different human tumor cell lines. Growth inhibition by MTOB requires relatively high concentrations of compound, with IC_{50} values ranging between 2 and 6 mM. These high external levels of MTOB are probably required in order to raise the internal MTOB concentrations to levels sufficient to observe growth inhibition. This is supported by the fact that cells expressing MTAP are more sensitive to MTOB than MTAP-deleted cells. MTOB

inhibits cell growth of both transformed and non-transformed cells, but transformed cells appear to be more sensitive. The toxic effect of MTOB is quite specific. We failed to observe any significant inhibitory effect with a variety of structurally related compounds at very high concentrations including MTHB, OG, and methionine. The lack of response to these compounds is somewhat surprising given the high degree of structural similarity among these molecules. Methionine and MTHB both share the methylthio group of MTOB, while OG has the oxoacid moiety. Thus, neither of these two functional groups alone is sufficient to explain the pro-apoptotic function of MTOB. Both must be present to induce apoptosis.

In earlier work, our lab has shown that cells lacking MTAP have elevated levels of polyamines due to increased ODC activity [8]. Presumably, these MTAP deleted cells lack MTOB, as MTAP is upstream in the methionine salvage pathway. We had also demonstrated that addition of exogenous MTOB could inhibit ODC activity both in *S. cerevisiae* and in human tumor cell lines. Although we reconfirmed our previous observation that MTOB reduced levels of ODC activity, we did not observe any difference in the level of ODC mRNA. This finding implies that the inhibition of ODC activity by MTOB occurs at the post-transcriptional level. Post-transcriptional regulation of ODC has been well studied with regard to its regulation by the polyamines via the production of the antizyme protein [15]. Antizyme targets ODC to the proteosome for degradation. Whether antizyme is involved in MTOB-induced inhibition of ODC is not yet known and is an area for future study.

Comparisons of the effects of MTOB and the ODC inhibitor DFMO show that MTOBs effects are not primarily due to inhibition of ODC. MTOB was more potent than DFMO in promoting growth inhibition and apoptosis. Furthermore, whereas DFMO strongly stimulated caspase 3/7 activity, we saw no such stimulation with MTOB. Finally, whereas DFMO-induced growth inhibition and apoptosis were largely dependent of p53 function, the effects of MTOB were not dependent on p53. However, we also found that addition of polyamines could lessen some of the growth inhibitory effects of MTOB, suggesting that ODC inhibition may play a small role in the MTOB effect.

What other mechanism might explain the pro-apoptotic properties of MTOB? Quash and colleagues have speculated that the conversion of MTOB to methional by the action of BCOADC might be important [12]. Alpha-ketobutyrate has a three-fold higher affinity for BCOADC compared to MTOB, and therefore would shunt BCOADC away from the metabolism of MTOB. In their study, they showed that addition of α-ketobutyrate to the media could alleviate growth inhibition by 2 mM MTOB in HeLa cells. However, we failed to see any effect of α-ketobutyrate on HeLa cells or the MiaPaca2 cells. Furthermore, whereas methional treatment clearly induced caspase 3/7, we did not observe induction by MTOB. Therefore, our data do not support the idea that conversion of MTOB to methional via BCOADC explains its unique effects, and favor the view that other as yet unknown mechanisms may be involved.

The lack of p53 dependence on growth inhibition and apoptosis is somewhat unusual. The vast majority of apoptosis promoting drugs are far more effective in a p53 null setting [16]. Many compounds that show the least dependence on p53 cause lysosomal membrane permeabilization (LMP) resulting in release of lysosomal proteases into the cytosol. Interestingly, many of these LMP-causing compounds have the unusual property that at high concentration they cause a reduction in caspase 3 dependent cleavage of cytokeritan-18. This observation is consistent with the lack of caspase 3 activation we observed in MTOB treated cells.

The finding that MTOB inhibits ODC and can induce apoptosis in a variety of tumor cell lines suggests the possibility that MTOB might be a useful cancer treatment or prevention. Human clinical trials have shown that low protein diets containing MTOB and other keto-acids are well tolerated and did not increase mortality of patients with chronic renal disease [17]. In rats, MTOB can substitute for methionine, allowing animals to grow and gain weight as efficiently as methionine [18]. Given the results described here, it would be worthwhile to determine if MTOB could retard tumor formation and progression in an appropriate rodent model.

Acknowledgments

This work was supported by American Cancer Society grant RSG0315701, NIH Grants CA100422, CA06927 and by a grant from the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions. We wish to thank the Biosample Repository of Fox Chase Cancer Center for their assistance.

REFERENCES

[1] Schmid M, Malicki D, Nobori T, Rosenbach MD, Campbell K, Carson DA, et al. Homozygous deletions of methylthioadenosine phosphorylase (MTAP) are more frequent than p16INK4A (CDKN2) homozygous deletions in primary non-small cell lung cancers (NSCLC). Oncogene 1998;17:2669–75.

[2] M'Soka TJ, Nishioka J, Taga A, Kato K, Kawasaki H, Yamada Y, et al. Detection of methylthioadenosine phosphorylase (MTAP) and p16 gene deletion in T cell acute lymphoblastic leukemia by real-time quantitative PCR assay. Leukemia 2000;14:935–40.

[3] Brat DJ, James CD, Jedlicka AE, Connolly DC, Chang E, Castellani RJ, et al. Molecular genetic alterations in radiation-induced astrocytomas. Am J Pathol 1999;154:1431–8.

[4] Subhi AL, Tang B, Balsara BR, Altomare DA, Testa JR, Cooper HS, et al. Loss of methylthioadenosine phosphorylase and elevated ornithine decarboxylase is common in pancreatic cancer. Clin Cancer Res 2004;10:7290–6.

[5] Behrmann I, Wallner S, Komyod W, Heinrich PC, Schuierer M, Buettner R, et al. Characterization of methylthioadenosin phosphorylase (MTAP) expression in malignant melanoma. Am J Pathol 2003;163:683–90.

[6] Nobori T, Takabayashi K, Tran P, Orvis L, Batova A, Yu AL, et al. Genomic cloning of methylthioadenosine phosphorylase: a purine metabolic enzyme deficient in multiple different cancers. Proc Natl Acad Sci USA 1996;93:6203–8.

[7] Christopher SA, Diegelman P, Porter CW, Kruger WD. Methylthioadenosine phosphorylase, a gene frequently

codeleted with p16(cdkN2a/ARF), acts as a tumor suppressor in a breast cancer cell line. Cancer Res 2002;62:6639–44.
[8] Subhi AL, Diegelman P, Porter CW, Tang B, Lu ZJ, Markham GD, et al. Methylthioadenosine phosphorylase regulates ornithine decarboxylase by production of downstream metabolites. J Biol Chem 2003;278:49868–73.
[9] Moshier JA, Dosescu J, Skunca M, Luk GD. Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. Cancer Res 1993;53:2618–22.
[10] O'Brien TG, Megosh LC, Gilliard G, Soler AP. Ornithine decarboxylase overexpression is a sufficient condition for tumor promotion in mouse skin. Cancer Res 1997;57: 2630–7.
[11] Tang B, Li YN, Kruger WD. Defects in methylthioadenosine phosphorylase are associated with but not responsible for methionine-dependent tumor cell growth. Cancer Res 2000;60:5543–7.
[12] Quash G, Roch AM, Chantepie J, Michal Y, Fournet G, Dumontet C. Methional derived from 4-methylthio-2-oxobutanoate is a cellular mediator of apoptosis in BAF3 lymphoid cells. Biochem J 1995;305:1017–25.
[13] Meyskens Jr FL, Gerner EW. Development of difluoromethylornithine (DFMO) as a chemoprevention agent. Clin Cancer Res 1999;5:945–51.
[14] Wu GS, El-Diery WS. p53 and chemosensitivity. Nat Med 1996;2:255–6.
[15] Coffino P. Regulation of cellular polyamines by antizyme. Nat Rev Mol Cell Biol 2001;2:188–94.
[16] Erdal H, Berndtsson M, Castro J, Brunk U, Shoshan MC, Linder S. Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis. Proc Natl Acad Sci USA 2005;102:192–7.
[17] Walser M, Hill SB, Ward L, Magder L. A crossover comparison of progression of chronic renal failure: ketoacids versus amino acids. Kidney Int 1993;43:933–9.
[18] Chow KW, Walser M. Substitution of five essential amino acids by their alpha-keto analogues in the diet of rats. J Nutr 1974;104:1208–14.