Suppressive effects of 3-bromopyruvate on the proliferation and the motility of hepatocellular carcinoma cells

MINORU TOMIZAWA[1], FUMINOBU SHINOZAKI[2], YASUFUMI MOTOYOSHI[3], TAKAO SUGIYAMA[4], SHIGENORI YAMAMOTO[5] and NAOKI ISHIGE[6]

Departments of [1]Gastroenterology, [2]Radiology, [3]Neurology, [4]Rheumatology, [5]Pediatrics and [6]Neurosurgery, National Hospital Organization, Shimoshizu Hospital, Yotsukaido, Chiba 284-0003, Japan

Received August 28, 2015; Accepted October 13, 2015

DOI: 10.3892/or.2015.4370

Abstract. The compound 3-bromopyruvate (3BP) is an analogue of pyruvate, which is the final product of glycolysis that enters the citric acid cycle. The present study aimed to investigate the suppressive effects of 3BP on the proliferation and motility of hepatocellular carcinoma (HCC) cells. HLF and PLC/PRF/5 cells were cultured with 3BP and subjected to an MTS assay. Apoptosis was analyzed by hematoxylin and eosin staining. Cell motility was analyzed using a scratch assay. Real-time quantitative polymerase chain reaction (PCR) was performed to determine the expression levels of cyclin D1 and matrix metalloproteinase (MMP)9. Proliferation of both cell lines was significantly suppressed by 3BP at 100 μM ($P<0.05$). The expression level of cyclin D1 was decreased after 3BP treatment at 100 μM in both cell lines ($P<0.05$). Pyknotic nuclei were observed in the cells cultured with 3BP at 100 μM. These results revealed that 3BP suppressed cell proliferation, decreased the expression of cyclin D1, and induced apoptosis in HCC cells. 3BP significantly suppressed motility in both cell lines ($P<0.05$). The expression level of MMP9 was significantly decreased ($P<0.05$). 3BP suppressed the proliferation and motility of HCC cells by decreasing the expression of cyclin D1 and MMP9.

Introduction

Hepatocellular carcinoma (HCC) is a common liver malignancy and its prognosis is poor despite advancements in treatment (1). The treatment options for HCC include local ablation, surgery, transcatheter arterial chemoembolization, and systemic chemotherapy (2,3). Molecular therapy has been under investigation for the development of a novel treatment for HCC (4).

Glycolysis is more pronounced in cancer cells than in normal cells. Cancer cells require more glucose under conditions of a sufficient oxygen supply (Warburg effect) (5). The Warburg effect is strong in cancer cells, but weak in normal cells. If glycolysis is inhibited, it is expected that cancer cells die while normal cells survive (6). Thus, inhibition of glycolysis is a new treatment strategy for HCC (7).

Pyruvate is the final product of glycolysis and enters the citric acid cycle (8). 3-Bromopyruvate (3BP) is an analogue of pyruvate (9) and is used as an inhibitor of glycolysis. 3BP inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase (10), and unlike other chemotherapeutic agents, it is less toxic to cells as it mimics pyruvate (9). Therefore, 3BP can be used for the treatment of cancer (11).

In the present study, we investigated the suppressive effects of 3BP on the proliferation and motility of HCC cells.

Materials and methods

Cell culture. HCC cell lines, HLF and PLC/PRF/5, were purchased from the Riken Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA). The cells were cultured in 10-cm dishes (Asahi Techno Glass, Funabashi, Japan) with 5% carbon dioxide at 37˚C in a humidified chamber.

Cell proliferation assay. The cells were trypsinized, harvested, and spread on 96-well plates (Asahi Techno Glass) at a density of 1,000 cells/well. The cells were cultured in DMEM supplemented with 10% FBS. 3BP was added at 0, 1, 3, 10, 30, or 100 μM to the culture medium. The cells were cultured for 72 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carb oxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). MTS is reduced by cells to a colored formazan product with an absorbance maximum at 490 nm. The absorbance was measured using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA).

Correspondence to: Dr Minoru Tomizawa, Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido, Chiba 284-0003, Japan
E-mail: nihminor-cib@umin.ac.jp

Key words: matrix metalloproteinase, MTS assay, scratch assay, cyclin D1, apoptosis, 3-bromopyruvate

Table I. Primer sequences used for real-time quantitative PCR.

Primer name	Sequence	Description	Product size (bp)	Annealing temperature (°C)	Cycle	GenBank
OMC355	5'-AGAGGCGGAGGAGAACAAACAG-3'	Cyclin D1, forward	180	60	40	NM_053056
OMC356	5'-AGGCGGTAGTAGGACAGGAAGTTG-3'	Cyclin D1, reverse				
OMC749	5'-CCTGGGCAGATTCCAAACCT-3'	MMP9, forward	89	60	40	NM_004994
OMC750	5'-GCAAGTCTTCCGAGTAGTTTTGGAT-3'	MMP9, reverse				
OMC321	5'-CGAATGCCAGAGAAGGTCAC-3'	RPL19, forward	157	60	40	BC095445
OMC322	5'-CCATGAGAATCCGCTTGTTT-3'	RPL19, reverse				

MMP9, matrix metalloproteinase 9; RPL19, ribosomal protein L (*RPL*) 19.

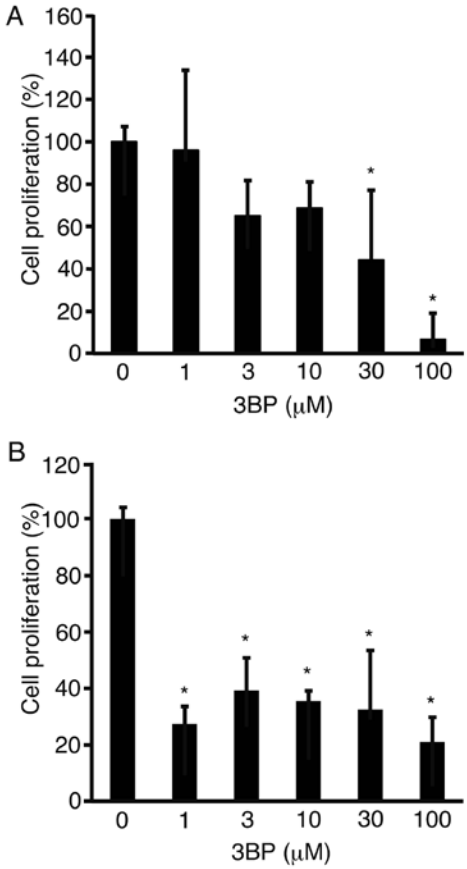

Figure 1. MTS assay. HLF (A) and PLC/PRF/5 cells (B) were subjected to an MTS assay (see Materials and methods) 72 h after incubation with 3-bromopyruvate (3BP) at 0, 1, 3, 10, 30, or 100 μM. Error bar, standard deviation, *P<0.05 compared with 0 μM, n=3.

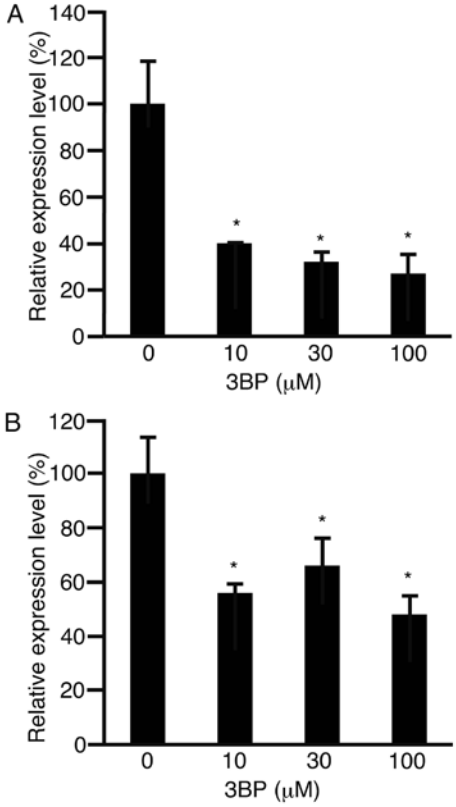

Figure 2. Real-time quantitative PCR of cyclin D1. HLF (A) and PLC/PRF/5 cells (B) were incubated with 3-bromopyruvate (3BP) at 0, 10, 30, or 100 μM. Error bar, standard deviation, *P<0.05 as compared with 0 μM, n=3.

Real-time quantitative polymerase chain reaction. Total RNA (5 μg), which was isolated using Isogen (Nippon Gene, Tokyo, Japan), was used for the first-strand cDNA synthesis with SuperScript III and oligo(dT) according to the manufacturer's instructions (Life Technologies). Real-time quantitative PCR was performed using Fast SYBR Green Master Mix (Life Technologies) with Mini Opticon (Bio-Rad). The results were analyzed using the Mini Opticon system (Bio-Rad). Real-time quantitative PCR was performed for 40 cycles, with 5 sec of denaturation and 5 sec of annealing-extension. Table I shows primer sequences used. RPL19 was used as an internal control since it is a housekeeping gene that is constitutively expressed (12).

Scratch assay and hematoxylin and eosin staining. The cells were plated on 4-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA). When the cells reached confluency, they were scratched with a sterile razor. The cells were incubated with 3BP (0 or 100 μM) for 48 h and stained with hematoxylin and eosin. The cells were plated in 4-well chamber slides (Becton Dickinson) for the analysis of apoptosis. The

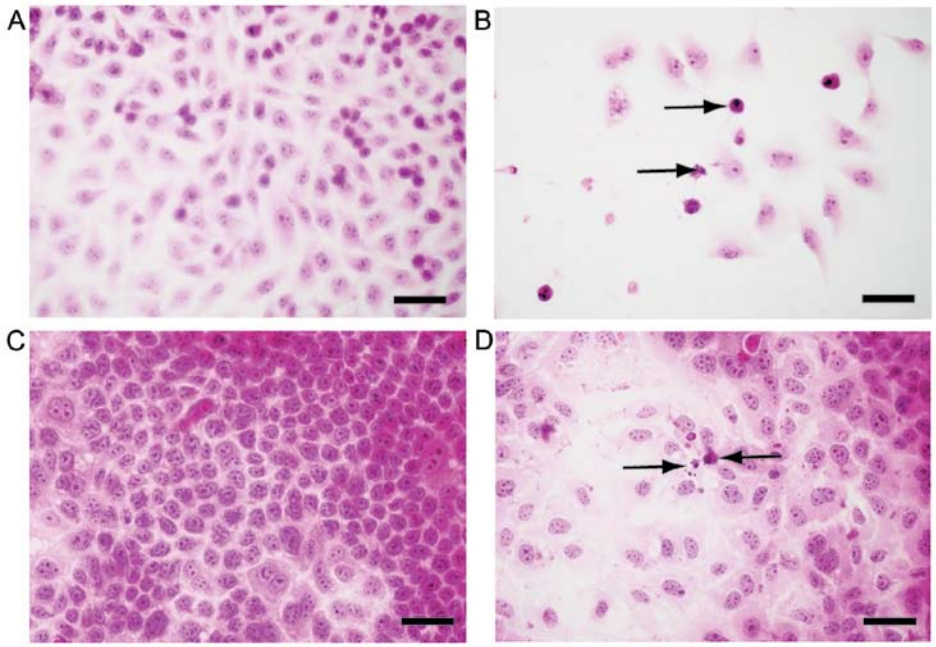

Figure 3. Hematoxylin and eosin staining. HLF (A and B) and PLC/PRF/5 cells (C and D) were subjected to hematoxylin and eosin staining after 48 h of incubation with 3-bromopyruvate at 0 μM (A and C) or 100 μM (B and D). Arrows, pyknotic nuclei; original magnification, x400; scale bar, 50 μM.

cells were incubated with 3BP (0 or 100 μM) for 48 h and stained with hematoxylin and eosin. The stained slides were observed under an AX80 microscope (Olympus, Tokyo, Japan) for the analysis of apoptosis and scratch assay. For the scratch assay, the distance between the scratched line and the growing edges of the cells was measured at five different points.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) and statistical analysis was carried out with JMP 10.0.2 software (SAS Institute, Cary, NC, USA). P-values <0.05 were determined to be statistically significant.

Results

To address the possibility that 3BP suppresses cell proliferation, HLF (Fig. 1A) and PLC/PRF/5 cells (Fig. 1B) were incubated with 3BP. After 72 h of incubation, the cells were subjected to MTS assay. Proliferation of both cell lines was significantly suppressed (P<0.05).

The expression levels of cyclin D1 were analyzed with real-time quantitative PCR, as this gene plays a role in cell proliferation (Fig. 2) (13). The expression levels of cyclin D1 were decreased in both cell lines (P<0.05).

We hypothesized that apoptosis may be involved in the suppression of cell proliferation. To clarify the role of apoptosis, hematoxylin and eosin staining was performed in the HLF (Fig. 3A and B) and PLC/PRF/5 cells (Fig. 3C and D) after a 48-h incubation with 3BP at 0 μM (Fig. 3A and C) or 100 μM (Fig. 3B and D). Pyknotic nuclei were observed in the cells cultured with 3BP at 100 μM (arrows).

In order to investigate cell motility, a scratch assay was performed in the HLF (Fig. 4A and B) and PLC/PRF/5 cells (Fig. 4C and D) after a 48-h incubation with 3BP at 0 μM (Fig. 4A and C) or 100 μM (Fig. 4B and D). The distance between the growing edges of the cells and the scratched line was measured (Fig. 4E). Cell motility was significantly suppressed in both cell lines (P<0.05).

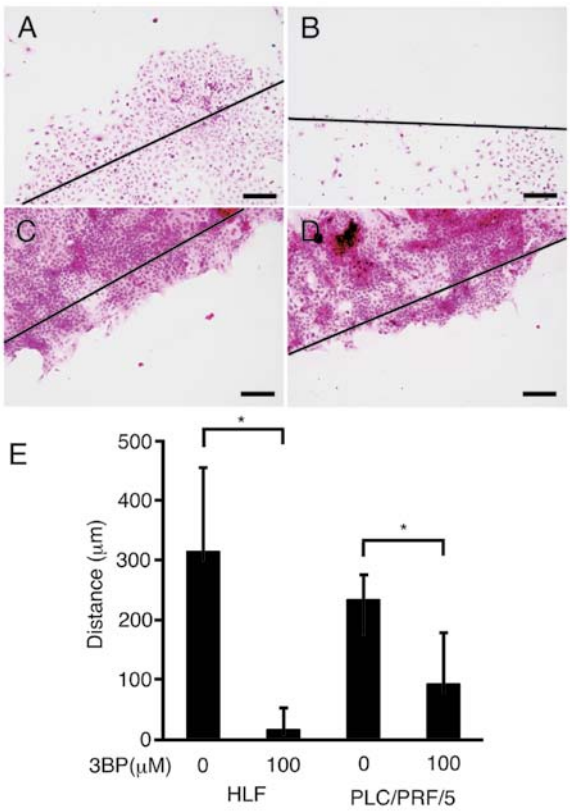

Figure 4. Scratch assay. HLF (A and B) and PLC/PRF/5 cells (C and D) were subjected to scratch assay to investigate the suppression of motility with 3-bromopyruvate (3BP) at 0 μM (A and C) or 100 μM (B and D). The distance was measured between the growing edges of the cells and the scratched line (solid line) (E). Original magnification, x100; scale bar, 200 μm; error bar, standard deviation, *P<0.05 compared with 0 μM, n=3.

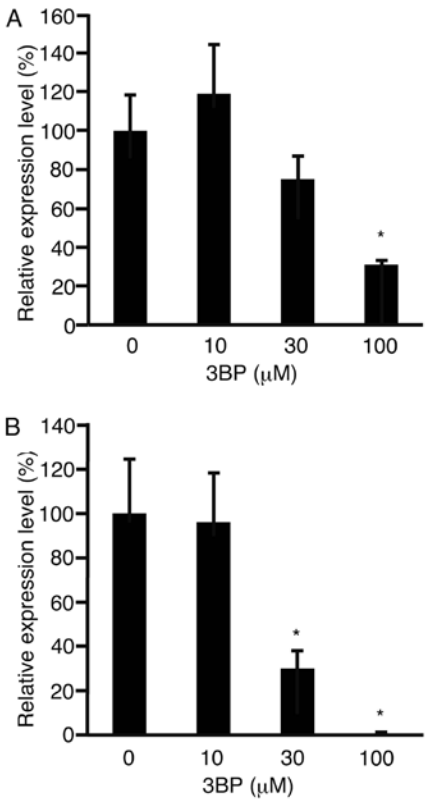

Figure 5. Real-time quantitative PCR of matrix metalloproteinase 9. HLF (A) and PLC/PRF/5 cells (B) were cultured with 3-bromopyruvate (3BP) at 0, 10, 30, or 100 μM. Error bar, standard deviation, *P<0.05 as compared with 0 μM, n=3.

MMP9 is involved in cell motility (14). RNA was isolated from the HLF (Fig. 5A) and PLC/PRF/5 cells (Fig. 5B) and subjected to real-time quantitative PCR. The expression levels of MMP9 were significantly decreased in both cell lines (P<0.05).

Discussion

3BP reduces HCC cell activity and induces apoptosis (15). Ganapathy-Kanniappan *et al* found that 3BP at 150 μM suppressed the proliferation of Hep3B cells (16). In our study, 3BP at 100 μM induced the apoptosis of HLF and PLC/PRF/5 cells. Although the cells were different, the concentration of 3BP was almost the same between the previous report and our study. These reports and our data suggest that 3BP induces apoptosis in HCC cells at 100-150 μM.

Our results clearly showed that proliferation of HCC cells was suppressed by 3BP, and 3BP also inhibited the cell cycle (17). No reports exist on the effects of 3BP on cyclin D1 expression. In the present study, the expression of cyclin D1 decreased after 3BP treatment. This result indicated that cyclin D1 was involved in the suppression of cell proliferation by 3BP. However, the detailed mechanism underlying the decrease in the expression of cyclin D1 is yet to be elucidated.

3BP in combination with 2-deoxyglucose, which is another inhibitor of glycolysis, was found to suppress the motility of breast cancer cells (18). In the present study, 3BP alone successfully suppressed the motility of HLF and PLC/PRF/5 cells. Moreover, the expression levels of MMP9 decreased. The previous report and our results clearly showed that 3BP suppressed the motility of cancer cells by decreasing the expression of matrix metalloproteinase with or without the combination of other agents.

One major limitation of 3BP is that it exerts limited antitumor effects in *in vivo* animal models (19). To overcome this limitation, a combination of 3BP and other agents could be employed (20). Furthermore, it is proposed that 3BP should be administered to patients in combination with other chemotherapeutic agents during transcatheter arterial chemoembolization (21). No harmful effects were reported in an animal model (22).

In conclusion, 3BP suppressed the proliferation and motility of HCC cells by decreasing the expression of cyclin D1 and matrix metalloproteinase.

References

1. Tejeda-Maldonado J, García-Juárez I, Aguirre-Valadez J, González-Aguirre A, Vilatobá-Chapa M, Armengol-Alonso A, Escobar-Penagos F, Torre A, Sánchez-Ávila JF and Carrillo-Pérez DL: Diagnosis and treatment of hepatocellular carcinoma: An update. World J Hepatol 7: 362-376, 2015.
2. Lencioni R, Petruzzi P and Crocetti L: Chemoembolization of hepatocellular carcinoma. Semin Intervent Radiol 30: 3-11, 2013.
3. Kim HY and Park JW: Clinical trials of combined molecular targeted therapy and locoregional therapy in hepatocellular carcinoma: Past, present, and future. Liver Cancer 3: 9-17, 2014.
4. Chen C and Wang G: Mechanisms of hepatocellular carcinoma and challenges and opportunities for molecular targeted therapy. World J Hepatol 7: 1964-1970, 2015.
5. Vaitheesvaran B, Xu J, Yee J, Q-Y L, Go VL, Xiao GG and Lee WN: The Warburg effect: A balance of flux analysis. Metabolomics 11: 787-796, 2015.
6. Jang M, Kim SS and Lee J: Cancer cell metabolism: Implications for therapeutic targets. Exp Mol Med 45: e45, 2013.
7. Granja S, Pinheiro C, Reis RM, Martinho O and Baltazar F: Glucose addiction in cancer therapy: Advances and drawbacks. Curr Drug Metab 16: 221-242, 2015.
8. Ngo DC, Ververis K, Tortorella SM and Karagiannis TC: Introduction to the molecular basis of cancer metabolism and the Warburg effect. Mol Biol Rep 42: 819-823, 2015.
9. Shoshan MC: 3-Bromopyruvate: Targets and outcomes. J Bioenerg Biomembr 44: 7-15, 2012.
10. Ganapathy-Kanniappan S, Geschwind JF, Kunjithapatham R, Buijs M, Vossen JA, Tchernyshyov I, Cole RN, Syed LH, Rao PP, Ota S, *et al*: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is pyruvylated during 3-bromopyruvate mediated cancer cell death. Anticancer Res 29: 4909-4918, 2009.
11. Cardaci S, Desideri E and Ciriolo MR: Targeting aerobic glycolysis: 3-bromopyruvate as a promising anticancer drug. J Bioenerg Biomembr 44: 17-29, 2012.
12. Davies B and Fried M: The L19 ribosomal protein gene (RPL19): Gene organization, chromosomal mapping, and novel promoter region. Genomics 25: 372-380, 1995.
13. Casimiro MC, Velasco-Velázquez M, Aguirre-Alvarado C and Pestell RG: Overview of cyclins D1 function in cancer and the CDK inhibitor landscape: Past and present. Expert Opin Investig Drugs 23: 295-304, 2014.
14. Vandooren J, Van den Steen PE and Opdenakker G: Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): The next decade. Crit Rev Biochem Mol Biol 48: 222-272, 2013.
15. Ganapathy-Kanniappan S, Geschwind JF, Kunjithapatham R, Buijs M, Syed LH, Rao PP, Ota S, Kwak BK, Loffroy R and Vali M: 3-Bromopyruvate induces endoplasmic reticulum stress, overcomes autophagy and causes apoptosis in human HCC cell lines. Anticancer Res 30: 923-935, 2010.
16. Ganapathy-Kanniappan S, Kunjithapatham R, Torbenson MS, Rao PP, Carson KA, Buijs M, Vali M and Geschwind JF: Human hepatocellular carcinoma in a mouse model: Assessment of tumor response to percutaneous ablation by using glyceraldehyde-3-phosphate dehydrogenase antagonists. Radiology 262: 834-845, 2012.

17. Liu XH, Zheng XF and Wang YL: Inhibitive effect of 3-bromopyruvic acid on human breast cancer MCF-7 cells involves cell cycle arrest and apoptotic induction. Chin Med J (Engl) 122: 1681-1685, 2009.
18. Feng X, Wang P, Liu Q, Zhang T, Mai B and Wang X: Glycolytic inhibitors 2-deoxyglucose and 3-bromopyruvate synergize with photodynamic therapy respectively to inhibit cell migration. J Bioenerg Biomembr 47: 189-197, 2015.
19. Jae HJ, Chung JW, Park HS, Lee MJ, Lee KC, Kim HC, Yoon JH, Chung H and Park JH: The antitumor effect and hepatotoxicity of a hexokinase II inhibitor 3-bromopyruvate: In vivo investigation of intraarterial administration in a rabbit VX2 hepatoma model. Korean J Radiol 10: 596-603, 2009.
20. Yu SJ, Yoon JH, Yang JI, Cho EJ, Kwak MS, Jang ES, Lee JH, Kim YJ, Lee HS and Kim CY: Enhancement of hexokinase II inhibitor-induced apoptosis in hepatocellular carcinoma cells via augmenting ER stress and anti-angiogenesis by protein disulfide isomerase inhibition. J Bioenerg Biomembr 44: 101-115, 2012.
21. Liapi E and Geschwind JF: Interventional oncology: new options for interstitial treatments and intravascular approaches: targeting tumor metabolism via a loco-regional approach: a new therapy against liver cancer. J Hepatobiliary Pancreat Sci 17: 405-406, 2010.
22. Geschwind JF, Ko YH, Torbenson MS, Magee C and Pedersen PL: Novel therapy for liver cancer: Direct intraarterial injection of a potent inhibitor of ATP production. Cancer Res 62: 3909-3913, 2002.