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The Effects of Ara-C, Simvastatin and combo therapy on energy metabolism of HL-60 promyolocytic leukemia cell lines

[HL-60 lösemi hücrelerinde Ara-C ve Simvastatinin tek tek ve kombine kullanımlarının enerji metabolizması üzerindeki etkileri]

Hakan Boyunağa[1],
Z.Günnur Dikmen[2],
Levent Kenar[3],
Oral Saygun[4],
Hatice Keleş[5],
Ali Ugur Ural[6],
Mustafa Lale[7],
Mehmet Koşargelir[8]

Kirikkale University, Faculty of Medicine, Departments of [1]Biochemistry, [4]Surgery, [5]Internal Medicine, Kirikkale

[2]Hacettepe University, Faculty of Medicine, Department of Biochemistry, Ankara

Gulhane Military Medical School, Departments of [3]Biochemistry, [6] Hematology, Ankara

[7]Advisor to the Secretary General TOBB The Union of Chambers and Commodity Exchanges of Turkey

[8]Haydarpaşa Numune Training and Research Hospital, İstanbul

Yazışma Adresi
[Correspondence Address]

Z.Günnur Dikmen, MD, PhD

Hacettepe University, Faculty of Medicine, Department of Biochemistry
06100 Sıhhiye, Ankara
Phone. +90 312 305 16 52
E-mail. zgunnur@gmail.com

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ABSTRACT

Objective: Cancer cells choose their metabolic pathway depending on the oxygen content and substrate concentration of the medium. A wide spectrum of therapeutic agents regulating the energy metabolism of cancer cells are in still in use. Cytosine arabinoside (Ara-C) is a pyrimidine analogue used in the treatment of acute myeloid leukemia (AML) and simvastatin is an inhibitor of HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, which regulates cholesterol biosynthesis. Thus, this study aimed to assess the energy metabolism of HL-60 promyelocytic leukemia cells and healthy white blood cells, additionally to determine the effects of simvastatin and Ara-C, alone or in combination on the energy metabolism of these cells.

Materials and Methods: Healthy white blood cells, untreated and treated HL-60 promyolocytic leukemia cell lines were incubated for 4 hours with radiolabelled glucose. Following incubation, lactate, which is one of the end products of the carbohydrate catabolism, and radiolabelled CO_2 produced by cells were collected and measured by the liquid scintillation device. In addition, glycogen consumption per hour was determined in each group.

Results and Conclusion: We found that untreated HL-60 promyolocytic cells use anaerobic glycolytic pathway whereas healthy white blood cells use aerobic glycolysis for energy gain. It was concluded that combined use of Ara-C and Simvastatin might lead to significant increase in the rate of aerobic glycolysis of HL-60 promyelocytic cells and the metabolism of these leukemia cells become more similar to the metabolism of healthy white blood cells which they originate from.

Key Words: Warburg effect, HL-60 cell lines, energy metabolism, simvastatin, Ara-C.

Conflict of Interest: Authors declare no conflict of interest.

ÖZET

Amaç: Kanser hücrelerinde metabolik yönelim, ortamın oksijen içeriği ve substrat konsantrasyonuna göre düzenlenmektedir. Kanser hücresinin enerji metabolizmasının düzenlenmesine yönelik çeşitli terapotik ajanlar halen kullanılmaktadır. Sitozin arabinosid (Ara-C) akut myeloid lösemi (AML) tedavisinde kullanılan bir pirimidin analogudur, simvastatin ise HMG-Co A (3-hidroksi-3-metil-glutaril-KoA) redüktaz inhibitörü olup kolesterol biyosentezini regüle etmektedir. Bu çalışmada, HL-60 promyelositik lösemi hücreleri ile sağlıklı beyaz kan hücrelerinin enerji metabolizmalarını belirlemek, ayrıca simvastatin ile Ara-C'nin tek tek ve kombine kullanımlarının hücrelerin enerji metabolizması üzerindeki etkilerini incelemek amaçlandı.

Gereç ve Yöntemler: Bu amaçla, ilaç kullanılan ve kullanılmayan tüm HL-60 akut promyelositik lösemi hücre hatları, radyoaktif glukoz ile 4 saat inkübasyona bırakıldı. İnkübasyon sonrası, karbonhidrat katabolizmasının son ürünlerinden laktat ve hücreler tarafından üretilen radyoaktif işaretli CO_2 toplanarak likit sintilasyon cihazında ölçüldü. Ayrıca her bir grupta saatteki glikojen tüketimi hesaplandı.

Bulgular ve Tartışma: Çalışmanın sonucunda enerji eldesinde HL-60 promyelositik hücrelerinin anaerobik glikolizi, sağlıklı lökositlerin ise aerobik glikolizi kullandıkları saptandı. Ara-C ile Simvastatinin kombine kullanımı sonucu, HL-60 promyelositik hücrelerinde aerobik glikoliz oranlarınının belirgin şekilde arttığı ve bu hücrelerin metabolizmalarını köken aldıkları beyaz kan hücrelerininkine benzer hale geldiği sonucuna varıldı.

Key words: Warburg etkisi, HL-60, enerji metabolizması, simvastatin, Ara-C.

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Many structural and metabolic changes take place during carcinogenic transformation of a normal cell under the influence of chemical agents, radiation and/or microbial carcinogens [1]. Metabolic changes are principally seen at the pathways cells use for energy gain. It was first reported by Warburg in 1930, at a publication named "Tumor Metabolism", that the rate of anaerobic glycolysis is high in cancer cells [2]. Other studies performed in the following years also reported varying degrees of anaerobic glycolysis in cells undergoing cancer transformation, thus this condition was named as "the Warburg Effect" [3-5]. The choice of metabolic pathway depends mainly on the oxygen and nutrient concentration of the medium. When the oxygen concentration is insufficient, anaerobic glycolytic pathway, which has less energy yield than aerobic glycolysis, is activated so that cells can continue to survive under low oxygen concentrations. Depending on the low oxygen concentration, the choice of anaerobic pathway for ATP production is called the "Pasteur Effect" [6-8]. Apart from the "Pasteur Effect", the nutrient concentration of the medium also affects the choice of the metabolic pathway even though the oxygen concentration is sufficient [9-12]. This characteristic is named as the "Crabtree Effect" leading these cells choose the anaerobic pathway under high nutrient concentrations and the aerobic glycolytic pathway under low nutrient concentrations [13-14]. Tumor cells usually choose anaerobic glycolytic pathway for energy gain as it is shorter, with the aim yielding their high metabolism while rapidly consuming the sources of the organism. However, when the nutrient concentration of the medium decreases the tumor cells choose the more energy yielding aerobic glycolytic way in order to survive [15-17]. Thus, knowing whether the tumor cell displays the Pasteur or Crabtree Effects is important in terms of the success of anti-cancer therapy.

Acute myeloid leukemia (AML) is one of the most common types of leukemia in adults. In younger patients, the prognosis is better than for their older counterparts. Cytosine arabinoside (Ara-C) is a deoxycytidine (dCyd) analog that remains one of the most effective drugs used in the treatment of acute leukemia as well as other hematopoietic malignancies. More recent work has attempted to improve the patient outcomes by adding targeted therapies to the current induction chemotherapy [18]. Simvastatin (Sim) is one of the statins, which are commonly used for treating hypercholesterolemia but also have some unexpected benefits such as reducing the overall cancer incidence and inducing apoptosis [19].

In this study, the effects of simvastatin, Ara-C, and combination of Simvastatin + Ara-C therapy over the energy metabolism of HL-60 acute promyelocytic leukemia cells and untreated control white blood cells were investigated.

Materials and Methods

Chemicals and Biomaterials:

D-(6-C^{14}) Glucose (Amersham) was used for radiolabeling, hexokinase and glucose-6-phosphate dehydrogenase enzymes were obtained from Boehringer. All other chemicals used were analytically purified. HL-60 acute promyelocytic leukemia cells were from the Karolinska Institute, Hematology Department, Huddinge, Sweden and they were maintained at 37°C, at the RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM L-Glutamine, 100 μg/mL streptomycin and 100 u/mL penicillin, in a medium containing 5 % CO_2.

MTT assay

The potential growth-inhibitory and cytotoxic effects of Ara-C (0.001-10 μM) and Simvastatin (0.0001-100 μM) in HL-60 cell line were evaluated using the the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide) assay according to the study of Mosmann et al [20]. Cells, at a concentration of 3×10^5 cells/ml, were exposed to a concentration range of drugs and incubated at 37°C for 72 hr. Cell viability was expressed as IC_{50}, which was defined as the concentration of drug causing 50% growth inhibition.

Radioactive incubation and analysis of end products:

HL-60 cells were seeded at $1'10^5$ cells/mL in growth medium in 96-well microtiter plates (Costar Co., Cambridge, MA) and were divided into 4 groups. Control cells remained untreated, whereas Simvastatin (0.0001-100 μM) and Ara-C (0.001-10 μM) were added to treatment groups. The combo treatment regimen including both Simvastatin (0.1μM) and Ara-C (0.01μM), the doses equivalent to IC_{50} values, was also tested on the cells. All the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 5 days which was the commonly used incubation time reported in similar studies.

White blood cell isolation was performed on the blood samples of healthy donors and was used for the aerobic control group. Then, all cells were incubated with radioactive glucose [D-(6-C^{14}) Glucose], at 37°C for 4 hours. The radiolabeled glucose was taken by the cells during the incubation and catabolized by the glycolytic pathway, finally converted to end products like lactate, acetate, and propionate. Following the incubation, the radioactive CO_2 left in the equipment was hold in the scintillation vials via the nitrogen. Then, the rest of the content was centrifuged at 3000g for 10 minutes. The supernatant and the cell pellet were collected into different tubes. Lactate, as the end product of anaerobic glycolysis was measured in the supernatants collected in the scintillation vials by anion exchange chromatography. The data was calculated with Microsoft excel hardware with standard graphics. The protein content of the cell pellets was measured by the modified Lowry

method [21]. The glycogen amount in the cell pellet was measured with the enzymatic method of Hassid and Abraham [22].

Statistical Analysis

Mann-Whitney U-test was used to compare groups. P values less than 0.05 were considered significant.

Results

The effects of Ara-C and combination therapy on cell proliferation

The effect of different concentrations of Sim, Ara-C and combination therapy was determined in HL-60 cell line by MTT assay. Ara-C (0.001-10 μM) and Sim (0.0001 to 100 μM) alone inhibited cell proliferation in a concentration- dependent fashion in the range of concentrations used. IC_{50} value for Simvastatin was found as 0.1μM and 0.01μM for Ara-C. Co-incubation of Sim (0.1 μM) with Ara-C (0.01 μM) resulted in a marked and significant inhibition of proliferation of HL-60 cells compared with Sim and Ara-C alone ($p<0.001$ for each) as determined by MTT assay and trypan blue dye exclusion assay run based on previous related studies [23, 24]**.**

The effects on energy metabolism

We first compared the CO_2 and lactate production and glycogen consumption of healthy white blood cells (WBC) and the untreated HL-60 cells, and we observed statistically significant difference between their energy metabolisms. The results showed that the healthy white blood cells use the aerobic glycolytic pathway whereas the untreated HL-60 promyelocytic cell cultures principally use the anaerobic glycolytic pathway for energy gain. When HL-60 leukemia cells treated with Simvastatin or Ara-C or Sim + Ara-C were compared with the untreated HL-60 leukemia cells or healthy white blood cell cultures, the difference in CO_2 and lactate production, in addition to glycogen consumption was also statistically significant (Table 1).

Then, HL60 promyelocytic cells were treated with Ara-C, Simvastatin and both Ara-C + Sim for 5 days and the effects of the drugs on energy metabolism were compared. No significant difference was observed with the single use of Simvastatin and Ara-C in terms of lactate production and glycogen consumption ($p>0.05$). Interestingly, following 5 days of treatment, simvastatin caused statistically significant increase in CO_2 production ($p=0.002$) favoring simvastatin use in the treatment of AML as seen in Figure 1. When glycogen consumption and glycolysis end products such as CO_2 and lactate and were compared between 3 treatment groups, a statistically significant difference was observed in groups of Ara-C and Ara-C + Sim groups ($p=0.018$, $p=0.003$, $p=0.002$, respectively). Similarly, the differences between Simvastatin and Ara-C + Sim treatment groups were statistically significant ($p=0.0018$, $p=0.004$, $p=0.007$, respectively).

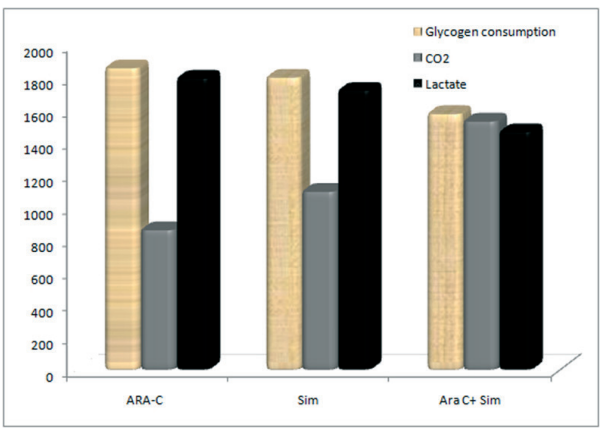

Figure 1: The amounts of CO_2 and lactate production (pmol glucose / h / μg protein) and glycogen consumption (nmol glucose / h / μg protein) of HL-60 leukemia cells after treatments for 5 days.

Table 1. The amounts of glycogen consumptions and end-products of untreated control cells and treatment groups (n=7).

	Glycogen consumption *(nmol glucose / h / μg protein)*	**CO_2 Production** *(pmol glucose /h / μg protein)*	**Lactate production** *(pmol glucose /h / μg protein)*
Leukocyte culture (WBC)	1026 ± 27	3522 ± 276	463 ± 57
HL-60 Control	2870 ± 152	1530 ± 379	43205 ± 6761
Ara-C	1854 ± 154	858 ± 276	1790 ± 112
Simvastatin	1796 ± 168	1094 ± 632	1715 ± 124
Ara-C + Simvastatin	1572 ± 81	1526 ± 268	1461 ± 101

Discussion

Increased anaerobic glycolysis rate of cancer cells was first reported by Warburg in 1930 [2]. Then new studies were planned to investigate the pathways for obtaining ATP during carcinogenesis, with the aim of developing more effective anti-cancer drugs. The results showed that cancer cells use different metabolic pathways for energy gain when compared with the normal cells they are stemmed from. Furthermore, as Warburg suggested, high anaerobic glycolysis rate can not be generalized for all cancer cells, in some cancer types like Morris Hepatoma, the glycolysis rate is normal and the mitotic division rate is low [25,26]. However, most cancer types principally use anaerobic glycolysis with varying degrees and the mitotic division rate is very high. Under normal conditions, the choice of metabolic pathway is related with the oxygen and nutrient concentration of the medium. When the ambient oxygen concentration is decreased and the glycogen and lipid depots of the organism are also rapidly consumed, cells which have undergone cancer transformation chose anaerobic glycolysis instead of aerobic glycolysis, which is known as the Pasteur Effect [27-33].

The Crabtree Effect which is not seen at normal cells but observed at cancer cells may suggest that the substrate concentration of the environment plays a principal role in the selection of metabolic pathway. As long as the substrate concentration is rich and sufficient, cancer cells use anaerobic glycolysis for obtaining ATP as seen frequently depending on the marked overexpression of type II hexokinase (HKII). In anaerobic glycolysis process, one mole of glucose is degraded into lactate and the net energy gain is 2 moles of ATP. Furthermore, the increase in lactate concentration causes a decrease in pH of the medium. Thus, in aerobic glycolysis, ATP gain is 36-38 moles for every mole of glucose, and the substrate consumption is 19 times higher than seen in physiological conditions. When the nutrient concentration of the medium is extremely decreased, the cell that have undergone tumor transformation chose aerobic glycolysis and start to use the nutrient content of the medium more effectively [34-39]. The change in the aerobic or anaerobic pathway limits the effect of chemotherapy which depends on the energy metabolism of cancer cells.

In recent years, increasing evidence suggests that statins have antiproliferative, antiangiogenic, and antimetastatic properties in addition to their well-described antiatherosclerotic, antithrombotic, and anti-inflammatory properties. Statins have been shown to inhibit the growth of a variety of tumor cell types, including prostate, gastric, and pancreatic carcinoma, as well as colon adenocarcinoma, neuroblastoma, glioblastoma, mesothelioma, melanoma, and acute myeloid leukemia cells. Statins have also been shown to activate caspase proteases involved in programmed cell death, additionaly upregulate proapoptotic protein expression and decrease antiapoptotic protein expression. Statins also impair the metastatic potential of tumor cells by inhibiting cell migration, attachment to the extracellular matrix, and invasion of the basement membrane. Interestingly, normal cells appear to be more resistant to the antiproliferative effects of statins relative to tumor cells [40]. Therefore, statins may thus represent a novel therapeutic approach for cancer prevention and treatment when combined with other cytotoxic agents.

In this study, we aimed to determine how statins effect the energy metabolism of HL60 cells when used single or in combination with Ara-C therapy. We observed that the healthy white blood cells produced CO_2 at a rate of 88.38 % and the rate of lactate production was 11.62 %, showing that they principally use aerobic glycolysis. On the contrary, when the end products of the untreated HL-60 promyelocytic cells was taken into consideration, the rate of lactate production was 96.58 % whereas CO_2 production was 3.42 % showing that they principally use anaerobic glycolysis as shown in Table 1.

When HL-60 cells were incubated with only Ara-C, lactate production was 67.6 % and CO_2 production was 32.4 %; when only Simvastatin was given to the cells these rates were 61.06 % and 38.94 %, respectively. In case of a combination therapy (Ara-C + Sim), lactate production rate decreased to 47 % and CO_2 amount increased to 51 % showing that combo therapy lowered the rate of anaerobic glycolysis. When CO_2 and lactate production in addition to glycogen consumption of treatment groups were compared with healthy white blood cell cultures and untreated HL-60 leukemia cells, the difference was statistically significant in both groups ($p>0.001$), ($p=0.002$). The present data showed that combined drug use increased the rate of aerobic glycolysis, additionally the metabolism of these cells become more similar to the metabolism of healthy white blood cells they originate from.

When CO_2 and lactate production in addition to glycogen consumption of Ara-C and Sim + Ara-C groups were compared, there was a statistically significant difference in between these three indicators ($p=0.018$, $p=0.003$, $p=0.002$, respectively). The difference between Simvastatin group and combo therapy groups also had statistical significances ($p=0.0018$, $p=0.004$, $p=0.007$, respectively).

When the effects of these drugs on energy metabolism was compared, it was observed that single use of Simvastatin and Ara-C did not show any statistically significant difference either in glycogen consumption or lactate production ($p>0.05$), Thus, Simvastatin treatment given to HL-60 promyelocytic cells significantly lead to aerobic glycolysis, especially in terms of CO_2 production. Especially, when compared with Ara-C treatment, a statistically significant difference was observed favoring Simvastatin in terms of CO_2 production ($p=0.002$).

Our results also showed that combination of Sim and

Ara-C produced an antigrowth interaction which was more active than the individual compounds on inhibiting cell growth. According to these results, it is possible to conclude that combined drug use increase the rate of aerobic glycolysis of HL-60 cells, and the metabolism of these cells become more similar to the metabolism of healthy white blood cells they originate from. Based on these observations, we can conclude that Simvastatin enhances the therapeutic efficacy of Ara-C, therefore Simvastatin and Ara-C may be suggested as the promising agents which can be used together in routine chemotherapy protocols for hematological malignancies, however different clinical studies are required to assess the promising results of these agents.

The authors declare no conflict of interest.

References

[1] Fischer CP, Bode BP, Souba WW. Adaptive Alterations in Cellular Metabolism With Malignant Transformation. Ann Surg 1998; 227:627-34.

[2] Warburg O. Metabolism of Tumors. Arnold Constable, 1930; London, UK

[3] Newsholme EA, Board M. Application of metabolic-control logic to fuel utilization and its significance in tumor cells. Adv Enzyme Regul 1991; 31:225-46.

[4] Arora KK, Pedersen PL. Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intra-mitochondrially generated ATP. J Biol Chem 1988; 263:17422-28.

[5] Board M, Humm S, Newsholme EA. Maximum activities of key enzymes of glycolysis, glutaminolysis, pentose phosphate pathway and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. Biochem J 1990; 265:503-9.

[6] Tielens AGM. Energy generation in parasitic helminths. Parasitology Today 1994; l0 (9):346-52.

[7] Tielens AGM, Pas FAM, Heuvel JM, Van den Bergh SG. The aerobic energy metabolism of Schistosoma mansoni miracidia. Mol Biochem Parasitol 1991; 181-84.

[8] Summers JE, Ratcliffe RG, Jackson MB. Anoxia tolerance in the aquatic monocot Potamogeton pectinatus absence of oxygen stimulates elongation in association with an unusually large pasteur effect. J Exp Bot 2000; 51:1413-22.

[9] Boyunaga H, Schmitz MGJ, Brouwers JFHM, Van Hellemond JJ, Tielens AGM. Fasciola hepatica miracidia are dependent on respiration and endogenous glycogen degradation for their energy generation. Parasitology 2001; 122:169-73.

[10] Melo RF, Stevan FR, Campello AP, Carnieri EG, de Oliveira MB, *et al.* Occurrence of the Crabtree effect in HeLa cells. Cell Biochem Funct 1998; 16:99-105.

[11] Golshani-Hebroni SG, Bessman SP. Hexokinase binding to mitochondria: a basis for proliferative energy metabolism. J Bioenerg Biomembr 1997; 29:331-38.

[12] Barron JT, Kopp SJ, Tow J, Parrillo JE. Substrate- dependent alteration in O_2 consumption and energy metabolism in vascular smooth muscle. Am J Physiol 1996; 270:1869-77.

[13] Wojtczak L, Teplova VV, Bogucka K, Aneta Czyż A, Makowska A, *et al.* Effect of glucose and deoxyglucose on the redistribution of calcium in ehrlich ascites tumour and Zajdela hepatoma cells and its conseouences for mitochondrial energetics. Further arguments for the role of Ca^{2+} in the mechanism of the crabtree effect. Eur J Biochem 1999; 263:495-501.

[14] Gibbs J, Morrell S, Valdez A, Setter TL, Greenway H. Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. J Exp Bot 2000; 51:785-96.

[15] Aronen HJ, Pardo FS, Kennedy DN, Belliveau JW, Packard SD *et al.* High microvascular blood volume is associated with high glucose uptake and tumor angiogenesis in human gliomas. Clin Cancer Res 2000; 6:2189-2200.

[16] Robinson SP, Howe FA, Stubbs M, Griffiths JR. Effects of nicotinamide and carbogen on tumour oxygenation, blood flow, energetics and blood glucose levels. Br J Cancer 2000; 82:2007-14.

[17] Minn H, Clavo AC, Fisher SJ, Wahl RL. Effect of nitroimidazole sensitizers on in vitro glycolytic metabolism of hypoxic squamous cell carcinoma. Acta Oncol 2000; 39:199-205.

[18] Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res. 1998; 72:197-233.

[19] Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer 2005; 5:930–42.

[20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65 (1-2):55-63.

[21] Bensadoun A, Weinstein D. Assay of proteins in the presence of interfering materials. Anal Biochem 1976; 70:241-50.

[22] Hassid WZ, Abraham S. Chemical procedures for analysis of polysaccharides. In: Methods in Enzymology, Eds: Colowick SP, Kaplan NO, 1957;37-42, Academic Press, New York,

[23] Zhang W, Konopleva M, Shi YX, McQueen T, Harris D, Ling X, Estrov Z, Quintás-Cardama A, Small D, Cortes J, Andreeff M. Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. J Natl Cancer Inst. 2008; 100(3):184-198.

[24] Pratz KW, Cho E, Levis MJ, Levis MJ, Karp JE, *et al.* A pharmacodynamic study of sorafenib in patients with relapsed and refractory acute leukemias. Leukemia 2010; 24(8):1437-44.

[25] Morris HP. Chemically induced hepatoma cell line. Recent Results in Cancer Res 1954; 44:103-14.

[26] Guminska M, Ignacak J. Electrophoretic pattern of cytosolic pyruvate kinase fractions A and B (type L and M2) from normal rat liver and Morris hepatoma. BBA *1996;* 1292:99-105.

[27] Bustamante E, Morris HP, Pedersen PL. Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. J Biol Chem1981; 256:8699-8704.

[28] Chesney J, Mitchell R, Benigni F, Bacher M, Spiegel L. An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect. Proc Natl Acad Sci 1999; 96:3047-52.

[29] Hibi K, Liu Q, Beaudry GA, Madden SL, Westra WH. Serial analysis of gene expression in non-small cell lung cancer. Cancer Res 1998; 58:5690-94.

[30] Hue L, Rousseau GG. Fructose 2,6-bisphosphate and the control of glycolysis by growth factors, tumor promoters and oncogenes. Adv Enzyme Regul 1993; 33: 97-110.

[31] Kress S, Stein A, Maurer P, Weber B, Reichert J, *et al.* Expression of hypoxia-inducible genes in tumor cells. J Cancer Res Clin Oncol 1998; 124:315-20.

[32] Mathupala SP, Rempel A, Pedersen PL. Glucose catabolism in cancer cells. Isolation, sequence, and activity of the promoter for type II hexokinase. J Biol Chem 1995; 270:16918-925.

[33] Mazurek S, Boschek CB, Eigenbrodt E. The role of phosphometabolites in cell proliferation, energy metabolism, and tumor therapy. J Bioenerg Biomembr 1997; 29:315-30.

[34] Wojtczak L. The Crabtree effect: a new look at the old problem. *Acta Biochim Pol 1996;* 43:361-68.

[35] Rempel A, Mathupala SP, Perdersen PL. Glucose catabolism in cancer cells: regulation of the Type II hexokinase promoter by glucose and cyclic AMP. FEBS Lett 1996; 385:233-37.

[36] Rempel A, Mathupala SP, Griffin CA, *et al.* Glucose catabolism in cancer cells: amplification of the gene encoding type II hexokinase. Cancer Res 1996; 56:2468-71.

[37] Sanchez-Martinez C, Aragon JJ. Analysis of phosphofructokinase subunits and isozymes in ascites tumor cells and its original tissue, murine mammary gland. FEBS Lett 1997; 409:86-90.

[38] Lee RB, Urban JP. Evidence for a negative Pasteur effect in articular cartilage. *Biochem J 1997;* 321:95-102.

[39] Ishida Y, Takagi-Ohta K. Lactate production of mammalian intestinal and vascular smooth muscles under aerobic and hypoxic conditions. *J Smooth Muscle Res 1996;* 32:61-7.

[40] Hochachka PW. Mechanism and evolution of hypoxia-tolerance in humans. *Journal of Experimental Biology 1998;* 201:1243-54.

[41] Hindler K, Cleeland CS, Rivera E, Collard CD. The Role of Statins in Cancer Therapy. The Oncologist 2006; 11:306–15.