PERIODICUM BIOLOGORUM
VOL. 116, No 1, 37–43, 2014

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Metabolism and differentiation

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Key words: Metabolism; Differentiation; Leukemia

Abstract

Textbook biochemical pathways do not usually apply to intermediary metabolism of highly proliferating, differentiating, or tumor cells. Over 80 years ago, Otto Warburg observed that cancer cells, unlike normal cells, favor glycolysis for energy production, even under aerobic conditions, and proposed that this shift in cancer cell metabolism (termed „aerobic glycolysis") was due to mitochondrial dysfunction. Recent studies by several groups suggest that aerobic glycolysis in tumor cells is actually caused by oncogene-directed changes in metabolism that are necessary for both continuous proliferation and a block in cellular differentiation.

Phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) is one of the principal proliferative and anti-apoptotic signaling pathways, which is known to support glycolysis and anabolism. Our previous studies demonstrated the activation of PI3K and Akt in nuclei of leukemia cells during differentiation, and confirmed that the inhibition of proximal components of the pathway inhibits proliferation, but negatively affects differentiative capacity of the cells. In contrast, use of rapamycin, which inhibits mTOR, a more distal component of the pathway, potentiates differentiation along granulocytic pathway. To further investigate the role of upstream regulators of mTOR in leukemia differentiation, we tested the effects of modulators of AMP-activated protein kinase (AMPK). Our results suggest a strong differentiative property of an AMPK activator, AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) in monocytic U937 cells. The mechanism of AICAR-mediated effects will be presented and a possible role of AMPK-modulators in differentiation therapy will be discussed.

Received February 14, 2014.

HEMATOPOIESIS, LEUKEMIA CELL LINES, DIFFERENTIATION AND SIGNALING PATHWAYS

Hematopoiesis is one of the most fascinating physiological processes in a healthy adult that is responsible for the production of almost 10^{12} mature blood cells per day, including thrombocytes *(1).* The whole system is based on the existence of a small number of pluripotent hemopoietic stem cells (HSC) that are quiescent or dormant most of the time, but sometimes divide and become progressively differentiated into more and more committed progenitors of different blood lineages. Some of these progenitors are named after the *in vitro* system that was initially used for their identification. For example, CFU-GM stands for „colony forming unit-granulocyte, monocyte", as the assay for their detection is based on counting of clusters or colonies (units) after 7-14 days of growth of mononuclear cells in a semisolid media containing appropriate cocktails of growth factors. These ready-to-use semisolid media for detection

of CFU-GMs of various species have been commercially available for decades, and 20 years ago, we used them for the detection of murine CFU-GMs as a readout assay to study the mechanism of phorbol myristate acetate (PMA) signaling in murine bone marrow cells *(2).*

The suspension of bone marrow cells that are flushed out from the cavities of murine long bones is actually a very heterogeneous mixture, and purification of specific progenitors using sorting or centrifugal elutriation was too expensive for us at that time. Therefore, we decided to obtain an acute myeloid leukemia cell line and to continue studying the mechanisms on the homogenous population of human leukemia HL-60 cells.

HL-60 cells were initially isolated from peripheral blood of a patient suffering from acute myeloid leukemia or AML-M2 subtype, according to French American British (FAB) classification *(3).* These cells grow indefinitely in RPMI media supplemented with 10% fetal bovine serum with no need for any of the expensive cytokines. When incubated with PMA, cells express monocytic markers and start to attach to the surface of a Petri dish within 24 hours, thus resembling mature macrophages. HL-60 cells are actually bipotential progenitors capable of partial differentiation into cells having some phenotypic characteristics of mature granulocytes or monocytes; *all-trans*-retinoic acid (ATRA) and dimethyl sulfoxide (DMSO) induce maturation along neutrophilic pathways, while interferon-γ (IFN-γ) and vitamin D_3 increase the expression of monocytic markers. For the past 20 years, these cells have provided a valuable model system for our studies aimed to dissect biochemical mechanisms specific to each differentiation pathway. Studies performed in IFN-γ-treated cells revealed a role of the phospholipase A_2/arachidonic acid pathway in mediating IFN-γ-induced sphingomyelin hydrolysis and phenotypic changes associated with differentiation of HL-60 cells along monocytic lineage *(4).* Vitamin D_3-mediated rapid, non-genomic effects on the activity of neutral sphingomyelinase was found to be completely abolished by prior depletion of PKC-α from the cytosol indicating that the effect was not only independent of genomic actions of the steroid hormone but also independent of any putative cell-membrane bound receptors for 1,25(OH)D_3 *(5).* In ATRA-treated cells, an increase in the level of nuclear PtdIns(3,4,5)P_3 and PtdIns(3)P was detected, which was due to the activity of phosphoinositide 3-kinase (PI3K), but no increase in the activity of PI3K-C2β was detected in cells treated with agonists of monocytic differentiation or other inducers of granulocytic phenotype like DMSO or dbcAMP *(6).* Therefore, the aim of the next grant proposal was to dissect the role of PI3K/protein kinase B (Akt) pathway in ATRA-mediated differentiation of leukemia cells.

In clinical practice, ATRA-based treatment of AML provides the most successful example of differentiation therapy used for the target treatment of cancer. The effects of ATRA are generally attributed to the effects on the fusion protein promyelocytic leukemia (PML)/retinoic acid receptor α (RARα), which is encoded by t(15;17); pharmacological doses of ATRA bind to protein and relieve the differentiation block. Based on the proposed mechanism of action, ATRA-therapy is currently being restricted only to a particular subtype of AML carrying a typical t(15;17) - AML-M3 or acute promyelocytic leukemia (APL) *(7).* However, it should be noted that differentiative properties of ATRA were first discovered in HL-60 cell line *(8),* a line that actually carries no t(15;17) and therefore, paradoxically, would not fulfill the current clinical criteria for ATRA-based treatment. Obviously, ATRA-mediated effects on differentiation could not be solely ascribed to the effects on the fusion protein.

PI3K/Akt pathway is principally considered to transmit proliferative and anti-apoptotic signals *(9).* Our study revealed that ATRA-mediated differentiation was associated with an increase in Akt activity in both HL-60 and NB4 cell lines, the latter containing the typical t(15;17). The increase in the level of nuclear Akt activity did not reflect cellular changes during differentiation that were common to all granulocytic inducers and/or inducers with a strong antiproliferative effect since no changes were observed in cells treated with DMSO or PMA. While the presence of commercially available Akt inhibitors had various effects on ATRA-mediated differentiation, a down-modulation of the expression of Akt protein in HL-60 cells using siRNA reduced the expression of differentiation markers in ATRA-treated cells. These results suggested that PI3K/Akt pathway had some role in differentiative responses of leukemia cells *(10).*

PI3K/AKT PATHWAY AND MAMMALIAN TARGET OF RAPAMYCIN (MTOR); METABOLISM AND DIFFERENTIATION

In physiology textbooks, PI3K/Akt pathway is usually described as a principal signaling pathway initiated downstream of insulin receptors. Guyton's description of insulin signaling is brief; insulin binds to the α-subunit of its receptor, which causes autophosphorylation of the β-subunit and induces tyrosine kinase activity; the activated receptor tyrosine kinase begins a cascade of target phosphorylation, including insulin receptor substrates (IRS) and several enzymes that mediate the effects on glucose, fat and protein metabolism (11). Boron's textbook provides more details; phosphorylated IRS activates PI3K/Akt pathway, Akt induces translocation of glucose transporters GLUT4 into the cell membrane and increases the activity of glycogen synthase (GS) by removing the inhibitory effects of glycogen synthase kinase (GSK). Protein synthesis is stimulated by the activation of mTOR, an evolutionary conserved kinase that, among many substrates, phosphorylates S6K, which then increases mRNA translation and cell growth *(12).* In addition, an activated

S6K phosphorylates and inhibits IRS, and that provides a typical example of a negative feedback loop. Although difficult to memorize, an extensive description of signaling pathways initiated downstream of insulin receptor is necessary for the understanding of the mechanism of peripheral resistance to insulin action that occurs in diabetes mellitus (DM) type II. As shown in Figure 1a, whenever the activity of mTOR is chronically increased, either because of a constant stimulation of insulin-IRS-PI3K-Akt pathway, or because of any other cause for the increase in the level of ATP, the activated S6K would not only increase the protein synthesis but would also phosphorylate and inhibit IRS and other proximal components of insulin signaling. Therefore, the sensitivity of insulin receptors decreases, and the level of insulin outside increases together with the level of plasma glucose *(13)*.

According to the scheme depicting the role of mTOR in Figure 1a, one can also presume that the opposite will happen in a case of mTOR inhibition. Namely, if an increase in mTOR activity decreases the activity of Akt, then a decrease in mTOR activity would probably increase the activity of Akt, as shown in Figure 1b. As we had not tested the effects of hyperactivation of Akt on ATRA-mediated differentiation (which requires use of methods like transfection of constitutively activated myristoylated Akt), we decided to test the effects of pharmacological inhibition of mTOR as an affordable way to increase the level of Akt. Rapamycin, a commercially available mTOR inhibitor, has been used for decades as an immunosupressive drug.

Experiments aimed to test the effects of mTOR inhibition soon revealed that rapamycin enhanced ATRA-mediated differentiation. At that time, Nishioka *et al*. reported similar findings *(14)*, which forced us to test further for the effects of combined inhibition of mTOR and proximal components of the pathway. That simple study finally led to the conclusion that combined inhibition of PI3K and mTOR exerted synergistic antiproliferative effect, but diminished differentiative properties of rapamycin in acute myeloid leukemia cells *(15)*. Then, the effects of rapamycin on leukemia cell differentiation in response to agents that differ from ligands of the nuclear receptor family were tested. As mentioned earlier, our previous study *(10)* had demonstrated that ATRA and DMSO, although being potent inducers of granulocytic differentiation, had not had the same effects on Akt; ATRA had increased Akt activity in nuclei and DMSO had had no effects. In accordance with these results, rapamycin enhanced CD11b expression in ATRA-treated cells but had no effects on DMSO-mediated increase. However, rapamycin had some beneficial effects since it enhanced DMSO-mediated growth arrest and apoptosis *(16)*.

AMP-ACTIVATED KINASE (AMPK) AND DIFFERENTIATION

As shown in Figure 1, the level of cellular ATP is an important regulator of mTOR activity. The link between the level of ATP and mTOR is provided by AMPK, an evolutionary conserved nutrient-sensitive protein kinase.

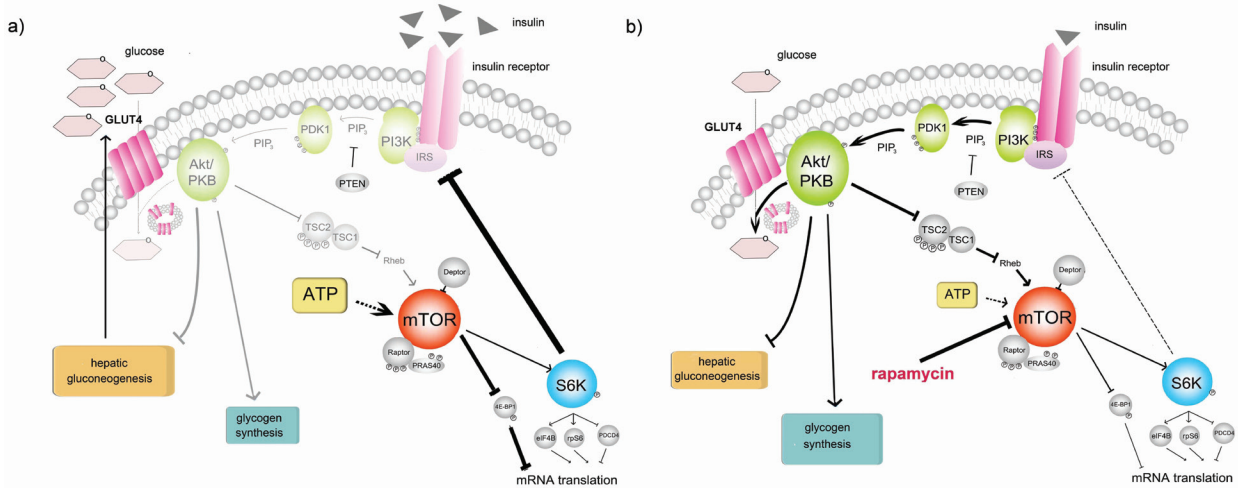

Figure 1. *The role of mTOR in a negative feedback mechanism that represses insulin/PI3K/Akt signaling. a) Chronic activation of mTOR down-regulates insulin receptor/PI3K/Akt signaling via a negative feedback loop b) The effect of rapamycin-mediated inhibition of mTOR on the activity of PI3K/Akt signaling pathway. Abbreviations used (alphabetically): 4E-BP1 – eukaryotic translation initiation factor 4E-binding protein 1; Akt/PKB – Akt/protein kinase B; eIF4B - eukaryotic translation initiation factor 4B; GLUT4 – glucose transporter type 4; IRS – insulin receptor substrate; mTOR – mammalian target of rapamycin; PDCD4 – programmed cell death protein 4; PDK1 – phosphoinositide-dependent kinase-1; PI3K – phosphoinositide 3-kinase; PIP_3 – phosphatidylinositol (3,4,5)-triphosphate; PRAS40 – proline-rich AKT substrate 40; PTEN – phosphatase and tensin homolog; Rheb – Ras homolog enriched in brain; rpS6 – ribosomal protein S6; S6K – S6 kinase; TSC1/2 – tuberous sclerosis complex 1/2.*

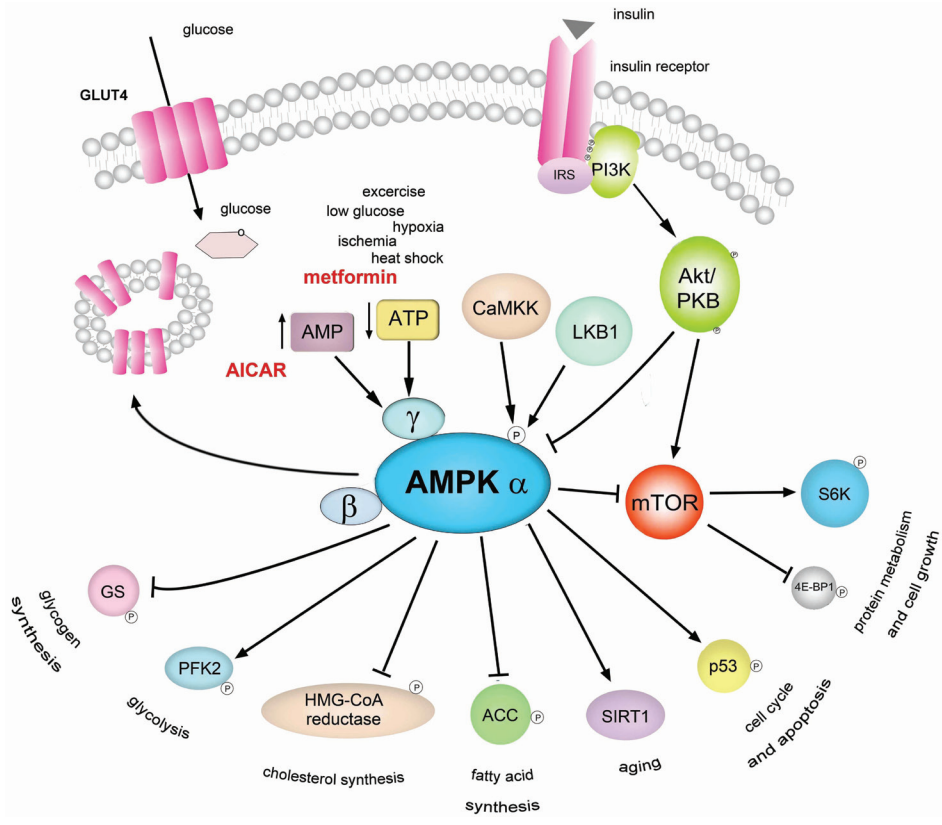

Figure 2. *Changes in cellular energy state regulate multiple cell functions through AMPK-activation. An increase in AMP/ATP ratio activates AMPK, which then preserves cellular energy levels by inhibiting anabolic and activating catabolic processes. In addition, AMPK has a role in processes like aging, cell growth and apoptosis. Abbreviations used (alphabetically): 4E-BP1 – eukaryotic translation initiation factor 4E-binding protein 1; ACC – acetyl-coenzyme A carboxylase; Akt/PKB – Akt/protein kinase B; AICAR (AICA-riboside) – 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK – AMP-activated protein kinase; CaMKK – calmodulin-dependent protein kinase kinase; GLUT4 – glucose transporter type 4; GS – glycogen synthase; HMG-CoA reductase – 3-hydroxy-3-methylglutaryl-CoA reductase; IRS – insulin receptor substrate; LKB1 – liver kinase B1; mTOR - mammalian target of rapamycin; p53 – protein 53; PFK2 – phosphofructokinase 2; PI3K – phosphoinositide 3-kinase; SIRT1 – sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae); S6K – S6 kinase.*

When the energy level in the cell is low and the ratio of AMP to ATP is increased, AMP activates AMPK and thus contributes to the shift of intermediate metabolism to energy conservation; AMPK stimulates ATP-generating pathways (glycolysis and fatty acid oxydation), and inhibits ATP-consuming pathways (gluconeogenesis and synthesis of fatty acids and cholesterol) through both direct phosphorylation of enzymes and alteration in gene expression (Figure 2). Any kind of metabolic stress leading to a decrease in cellular ATP, such as hypoxia, ischemia or starvation, will increase the AMP/ATP ratio and activate AMPK. For example, exercise decreases ATP and activates AMPK, which induces translocation of GLUT4 irrespective of insulin, and that helps to explain a well-known phenomenon that diabetics need less insulin for plasma glucose control during physical activity. Among many other substrates, activated AMPK inhibits the activity of mTOR, which provides a link between a decrease in intracellular energy levels and the regulation of protein synthesis *(17).*

If mTOR inhibition enhances differentiation, and activated AMPK inhibits mTOR, than one would expect that AMPK activators stimulate leukemia cell differentiation. Various agents have been found to activate AMPK, including commonly used drugs like barbiturates or peroral antidiabetics like biguanides (metformin, phenformin) and thiazolidinediones (troglitazone). Most of these agents cause indirect activation of AMPK by decreasing cellular ATP due to inhibition of the respiratory chain. AICA-riboside (AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside, acadesine) is a cell-permeable nucleoside that increases the activity of AMPK by intracellular conversion into an AMP mimetic. As shown in Figure 3, AICAR shares some structural similarities with adenosine. Within the cell, AICA-riboside is phosphorylated by adenosine kinase into AICA-ribotide or ZMP, and ZMP then binds to γ-subunit and allosterically activates AMPK *(17, 18).*

AICAR was first developed to block adenosine reuptake in the ischemic heart *(19),* but became popular since

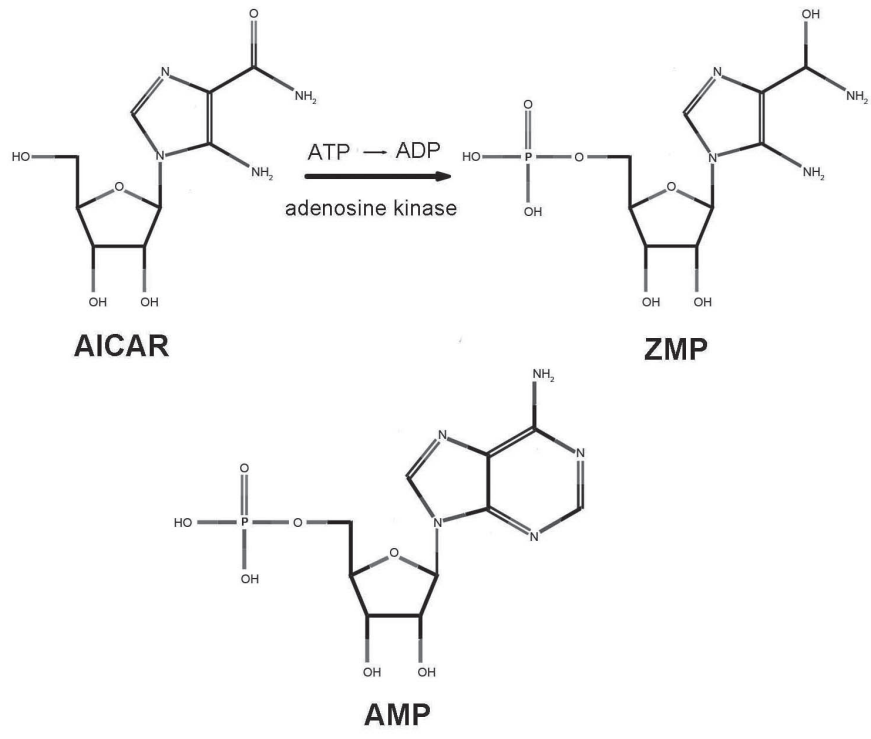

Figure 3. *AICAR is a pharmacological agent that is intracellularly transformed into an AMP analog. AICAR or AICA-riboside is intracellularly phosphorylated by adenosine kinase into ZMP or AICA-ribotide. Abbreviations used (alphabetically): AICAR (AICA-riboside) – 5-aminoimidazole-4-carboxamide ribonucleoside; ZMP (AICA-ribotide) – 5-aminoimidazole-4-carboxamide ribonucleotide.*

the discovery that it acts as „exercise in a pill" and increases endurance in sedentary mice by genetically reprogramming muscle metabolism *(20).* The compound is widely used as an AMPK-agonist in numerous studies related to metabolism and insulin signaling pathways, and now is becoming a putative anticancer agent. Namely, recent findings that diabetics treated with metformin, another AMPK agonist, are less prone to cancer *(21)* has lead to numerous studies showing anti-tumor activity of AMPK agonists in solid tumors, particularly breast and prostate cancer *(22).* In hematological malignancies, antiproliferative and proapoptotic effects of both metformin and AICAR have been reported *(23, 24),* but possible beneficial effects of AICAR on AML differentiation have not been investigated.

The results of our current study confirm that AICAR induces apoptosis and reduces the number of viable AML cells. In addition, AICAR enhances ATRA-mediated differentiation of HL-60 and NB4 cells. In monocytic U937 cells, AICAR alone induces the expression of cell surface markers associated with mature monocytes and macrophages, and these effects seem to be independent of the level of AMPK and associated with an increase in the activity of mitogen-activated protein kinase (MAPK). This is, to our knowledge, the first report showing that AICAR has some differentiating properties in leukemia cells *(25).*

What is the possible mechanism of AICAR-induced changes in U937 cells? As shown in Figure 2, activated AMPK has profound effects on many biochemical pathways, and AICAR is known to affect multiple AMPK-dependent and independent targets. Textbook biochemical pathways do not usually apply to highly proliferating, differentiating or tumor cells. Over 80 years ago, Otto Warburg observed that cancer cells, unlike normal cells, „ferment" glucose into lactate even in the presence of sufficient oxygen, and proposed that this shift in cancer cell metabolism (termed „aerobic glycolysis") was due to mitochondrial dysfunction *(26).* The role of glycolysis and mitochondrial metabolism in leukemia cells proliferation and differentiation remains to be determined, but several studies pointed to the role of mitochondrial translation and oxidative phosphorylation *(27)* or fatty acid oxydation *(28)* in AML cells. We have not yet measured the level of glucose uptake, lactate production, ATP or mitochondrial potential, but what we have observed in AICAR-treated U937 cells was a consistent shift in autofluorescence, and that may be due to a change of cellular NAD/NADH levels. NAD-dependent histone deacetylase SIRT1, which is regulated by AMPK (Figure 2), was reported to have some role in neutrophil differentiation of APL cells *(29),* and our preliminary data using Sirt-inhibitors suggest a possible role in AICAR-mediated effects. In addition, AICAR exerts some effects in cells with

siRNA-mediated down-regulation of AMPK, suggesting possible AMPK-independent effects *(25)*. Autophagy was recently proposed to be important for AMPK-independent effects in acadesine-treated B-CLL *(30),* and induction of autophagy was found to be important during monocyte-macrophage differentiation induced by GM-CSF *(31)*. Metabolic changes that are necessary for lineage-specific differentiation seem to represent an interesting research topic, and we are currently trying to define the full mechanism responsible for AICAR-mediated effects in leukemia cells.

Concluding remarks

Signaling pathways that control proliferation, growth and metabolism are involved in differentiation of leukemia cells. The most successful recent proof-of-concept that inhibitors targeting an „oncometabolite" could have potential applications as a differentiation therapy for AML was reported in cells carrying mutation of isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2). Somatic mutations in IDH1 and IDH2 genes occur frequently in AML and confer a gain-of-function that facilitates the reduction of α-ketoglutarate to D-2-hydroxyglutarate (2HG), an „oncometabolite" that leads to epigenetic reprogramming and promote leukemogenesis. Treatment with pharmacological inhibitors of the tumor-associated mutant IDH2/R140Q induces differentiation of primary human acute myelogenous leukemia cells *in vitro (32).*

Acknowledgements: *This work was supported by the Ministry of Science, Education and Sport of the Republic of Croatia, grants No. 108-1081347-1448 (to D. V.) and 108-1081347-0173 (to H. B.)*

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