Synergisitic and Antagonistic AML Cell Type-specific Responses to 5-Aza-2-deoxycitidine and 1-h-D-Arabinofuranoside

ABEER ABD ELMONEIM[1,2,3*], ELISABETH HEUSTON[2*], DANIEL H. WAI[4], TIMOTHY TRICHE[5] and ROBERT J. ARCECI[2]

[1]*Pediatric Department, Sohag Faculty of Medicine, Sohag University, Sohag, Egypt;*
[2]*Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, U.S.A.;*
[3]*Pediatric Department, Taibah University, Madina, Kingdom of Saudi Arabia;*
[4]*Phoenix Children's Research Institute, Phoenix Children's Hospital, Phoenix, AZ, U.S.A.;*
[5]*Department of Pathology, Children Hospital, Los Angeles, CA, U.S.A.*

Abstract. *Background: The search for synergistic drug combinations is critical to the treatment of drug-resistant cancer, such as acute myeloid leukemia (AML). Characterizing RNA expression associated with 5-aza-2´-deoxycytidine (DAC) and 1-h-D-arabinofuranosylcytosine (Ara-C) is a critical step to increase the efficacy of their combinatorial therapies. Materials and Methods: After 72 h of single-dose treatments of AML cells with DAC or Ara-C, the half-maximal effective concentration of DAC and Ara-C and the drug combination index were assessed. Results: Pre-treatment with DAC restores cellular sensitivity in Ara-C-resistant AML cells. In contrast, DAC/Ara-C combinations are antagonistic in other Ara-C-sensitive AML cells. Conclusion: Our results provide an alternative approach for predicting what combinations, dosing and scheduling of drug delivery should be used to better individualize therapy of AML.*

Drug interactions are synergistic, antagonistic or additive. Pharmacologically, synergism and antagonism are defined respectively, as greater or lesser pharmacological effect for a two-drug combination than what would be predicted from the effects of each drug individually (1, 2). In the Chou and Talalay model, a generalized method for analyzing dose-effect relationships was described (3). In acute lymphoblastic leukemia (ALL) of childhood, the percentage of remissions with single agents is 40-50% as compared with 94-95% with combinations of three drugs (4). The need for synergy is important in drug-resistant pediatric acute myeloid leukemia (AML), which has an associated 50-60% overall survival rate. Conventional treatment for resistant AML in children includes polymerase II inhibitor 1-β-D-arabinofuranoside (Ara-C), a deoxycytidine analog that has been used either alone or in combination with other chemotherapeutic agents for the treatment of AML (5), relapsed and refractory ALL (6), and other malignancies (7-9).

Nucleoside analog drugs, such as Ara-C, have low rates of passive diffusion across membranes and enter cells primarily as substrates for specialized nucleoside transporters. Two human nucleoside transporter proteins have been identified: the human equilibrative nucleoside transporters (hENT1 to hENT4; also known as SLC29A1-SLC29A4) and the human concentrative nucleoside transporters (hCNT1 to hCNT3; also known as SLC28A1-SLC28A3). hENT1 is the primary transporter responsible for cellular uptake of Ara-C (10, 11). Ara-C is activated to form Ara-CTP through sequential phosphorylation by deoxycytidine kinase (DCK), dCMP kinase, and nucleoside diphosphate kinase. The cytotoxicity of Ara-C is accomplished by non-productive incorporation of Ara-CTP into nascent DNA or RNA. Decreased expression of *hENT1* mRNA and *DCK* mRNA is associated with reduced DCK activity; both alterations are associated with increased cellular resistance to Ara-C (10). In addition, activation of 5'-nucleotidase and ribonucleotide reductase may affect conversion of Ara-C to the active inhibitor, possibly reducing its therapeutic efficacy (12, 13). The prototypical DNA methyltransferase inhibitor 5-aza-2'-deoxcytidine (DAC), a nucleoside analog, enters cells using hENT1 and hENT2. DAC is then phosphorylated by DCK into the monophosphorylated derivative 5-aza-dCMP then to its active form, 5-aza-dCTP that is incorporated into DNA, inducing demethylation (14). Ara-C also relies on DCK as the initial rate-limiting step for incorporation (10). Ara-C and

*These Authors contributed equally to this work.

Correspondence to: Abeer Abd Elmoneim, MD, Associate Professor of Pediatrics, Pediatric Department, Sohag University, Sohag, Egypt. Tel: +96 6535351061, e-mail: abeeraaa6@gmx.de

Key Words: Drug interaction, myeloid leukemia, pediatric cancer.

DAC utilize the same molecular pathways to enter the cell and activate the pro-drug, and both are incorporated specifically into DNA during S-phase of the cell cycle.

Our current study aimed to detect the effect of pre-treatment of Ara-C-resistant AML cells with DAC on the response of these cells to Ara-C therapy and to predict what combinations, dosing and scheduling of drug delivery should be used to better individualize therapy.

Materials and Methods

Cells and drugs. Molm13 cells are M5a leukemia, NB4 cells are M3 leukemia cells (15, 16), both were cultured in RPMI-1640 media (Mediatech, Herndon,VA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). UT7-epo cells are M7 leukemia cells (17) and cultured in Isocove's modified Dulbecco's medium (IMDM; Sigma-Aldrich) supplemented by 10% FBS, L-glutamine and 1 μl of erythropoietin (Epogen; Amagen, Thousand Oaks, CA, USA) per 1 ml IMDM. DAC (Sigma-Aldrich) was freshly dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all experiments did not exceed 0.08%. Ara-C (Sigma-Aldrich) was freshly dissolved in distillated water.

Cytotoxic assays and graphical analysis. Cell suspensions were aliquoted (1×10^6/ml for NB4 and Molm13 cells and 0.5×10^6/ml for UT7epo cells) into 6-well plates in a volume of 2 ml/well. The following drug concentrations were used for both DAC and Ara-C: 0, 0.02, 0.2, 2, 20 and 50 μM. Cells were incubated for 72 h at 37˚C in a CO_2 incubator and analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) (18). Graphical analysis using Prism 5 (GraphPad Software, San Diego, CA, USA) to calculate the concentration of drug causing death of 50% of leukemia cells or 20% of leukemia cells (EC_{50} and EC_{20}) values were carried out.

Fractional analysis. The combination effect of DAC and Ara-C on Molm13, NB4 and UT7-epo myeloid leukemia cells were estimated based on the combination index (CI) values (3). The drug combination experiment was designed and analyzed as previously described (19).

Figures were drawn using Prism 5 (GraphPad Software) where f values were represented on X axis and CI values on Y axis, based on the median-effect method (3), to calculate the CI for each f. A CI<1 indicates the combination is synergy, CI=1 the combination is additive, and CI>1 the combination is antagonism.

Results

In this study, we investigated the effect of different drug combinations on three different AML cell lines that have different responses to Ara-C and DAC. Molm13 and NB4 cells both are resistant to DAC and Ara-C. UT7-epo cells are resistant to Ara-C while maintaining sensitivity to DAC. Table I shows the EC_{50} for DAC and Ara-C in these three leukemia cell lines, as measured using the MTS assay.

UT7-epo cells exhibited resistance to DAC or Ara-C after 72 h of treatment. Pretreatment of UT7-epo cells with DAC for 72 h sensitized the cells to the secondary treatment with either DAC (DAC/DAC) or Ara-C (DAC/Ara-C). UT7-epo cells were resistant to simultaneous treatment with both DAC and Ara-C (DAC+Ara-C) (Figure 1a).

Table I. *The concentration of drug causing death of 50% of leukemia cells or 20% of leukemia cells ($EC_{50/20}$) of 5-aza-2'-deoxcytidine (DAC) and1-β-D-arabinofuranoside (Ara-C) in selected human acute myeloid leukemia (AML) cell lines. Values are presented as the mean value of two independent experiments each in duplicate.*

	Molm13		NB4		UT7-epo	
	DAC	Ara-C	DAC	Ara-C	DAC	Ara-C
EC_{50} (μM)	0.158	0.014	0.074	0.8	0.378	9.21
EC_{20} (μM)	0.038	0.0054	0.009	0.33	0.143	2.446

Molm13 cells were resistant to 72 h treatment with either DAC or Ara-C. Pre-treatment with DAC did not sensitize the cells to the effect of secondary treatment. Molm13 cells were sensitive to simultaneous treatment with both DAC and Ara-C only after 144 h (Figure 1b). NB4 cells exhibited sensitivity to simultaneous treatment with DAC and Ara-C. Pre-treatment of NB4 cells with DAC for 72 h increased cells sensitivity to secondary treatment with Ara-C (DAC/Ara-C) (Figure 1c).

Fractional analysis of UT7-epo response to DAC and Ara-C combination, (Figure 2a) showed that simultaneous treatment with both drugs resulted in antagonistic interaction, while sequential treatment of UT7epo cells with both drugs, (Figure 2b) resulted in synergistic interaction at any ratio of drug combinations.

For Molm13 cells (Figure 2c and d), fractional analysis showed that DAC and Ara-C combination at any drug ratio had antagonistic interaction whether simultaneously or sequentially. Fractional analysis of DAC and Ara-C combination effect on NB4 cells showed that sequential DAC and Ara-C combination treatment at any ratio had antagonistic interaction (Figure 2e) but in simultaneous treatment (Figure 2f), DAC and Ara-C combination had synergistic interaction when the Ara-C ratio was between 90-100%, while at an Ara-C ratio of 80%, the drug combination showed additive interaction; from an Ara-C ratio of 75% or lower DAC and Ara-C combination treatment the interaction was antagonistic.

Discussion

In the prsent study, we hypothesized that drug interaction between DAC and Ara-C may change the way that AML cells respond to each of these drugs. We also aimed to provide an approach for predicting what combinations,

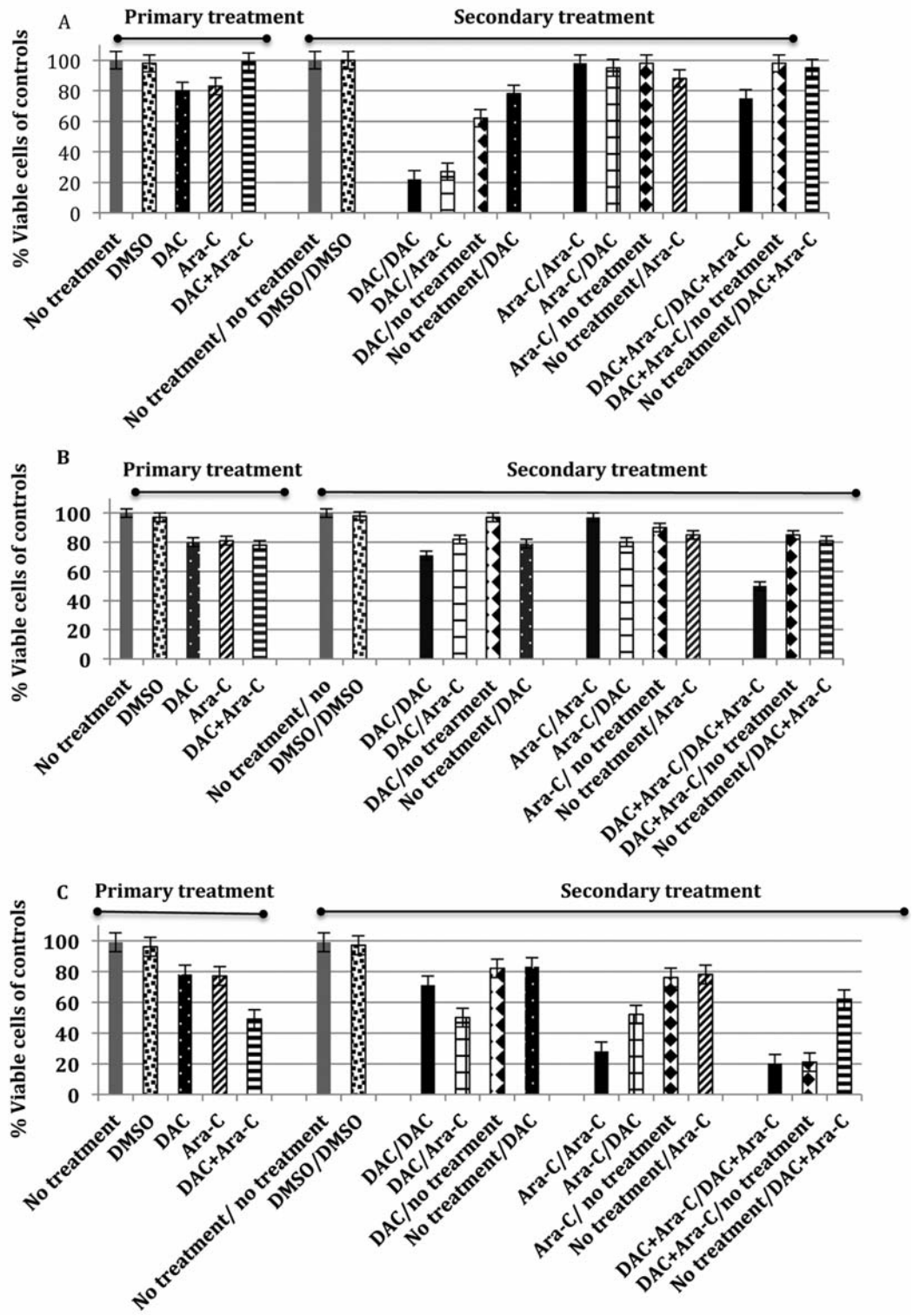

Figure 1. *3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay of UT7-epo (a) , Molm13 (b) and NB4 (c) cells after primary and secondary treatment with 5-aza-2'-deoxcytidine (DAC) and 1-β-D-arabinofuranoside (Ara-C) either sequentially or simultaneously. Cells were treated with either individual or combination doses of DAC and ARA-C at the concentration of drug causing death of 20% of leukemia cells (EC_{20}) for 72 h (primary treatment). Viability was subsequently determined and cells were replated in fresh media and again with either individual or combination doses of DAC and Ara-C at EC_{20}, and allowed to grow for an additional 72 h (secondary treatment). The percentage of viable cells was subsequently measured. Results are the mean of two independent experiments.*

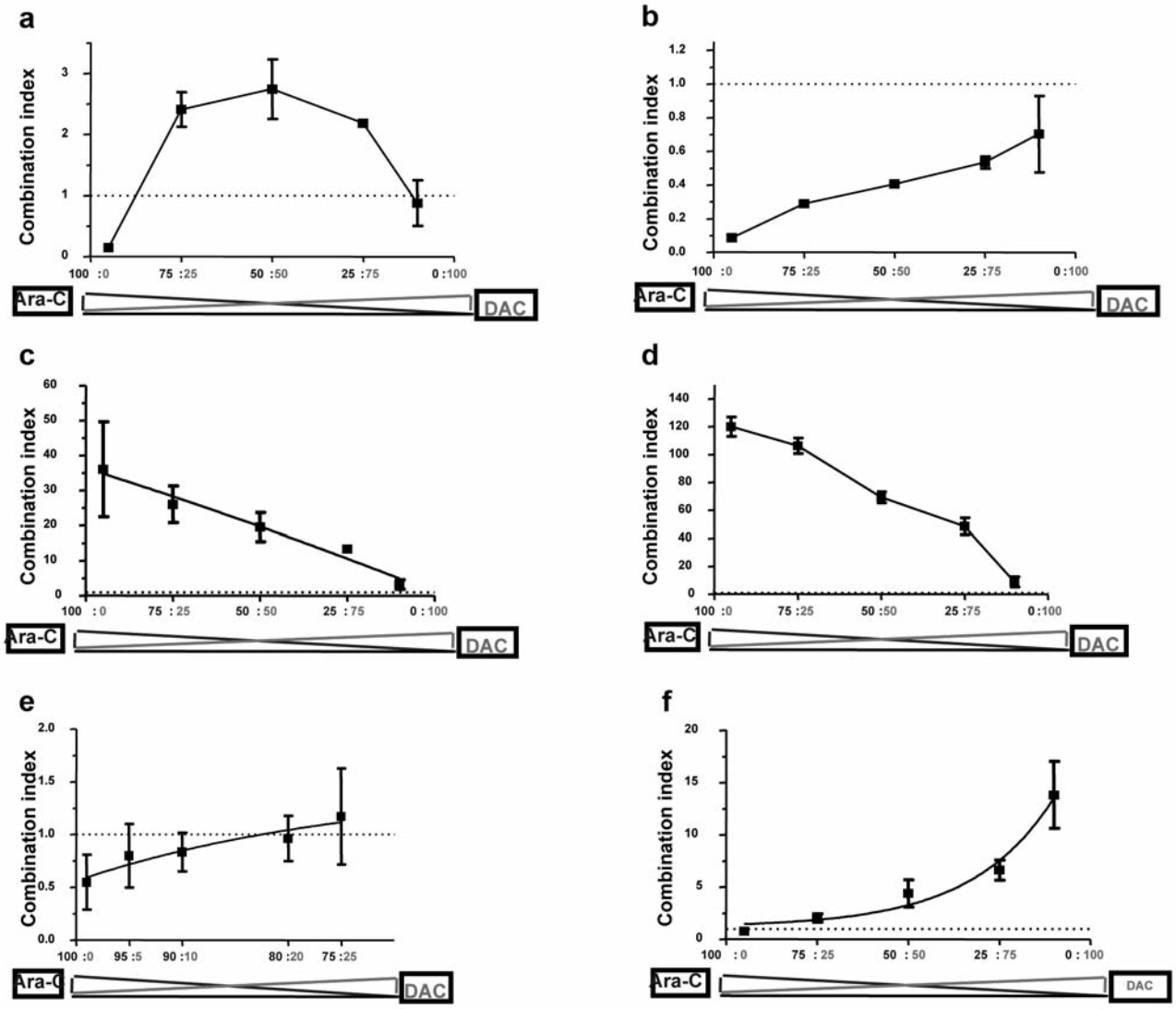

Figure 2. *Fractional analysis after simultaneous (a, c and e) and sequential (b, d and f) treatment with 5-aza-2'-deoxcytidine (DAC) and 1-β-D-arabinofuranoside (Ara-C) of UT7epo (a, b), Molm13 (c, d) and NB4 (e, f) cells. X-axis represents treatment ratio of Ara-C to DAC, Y-axis represents the combination index (CI). Following 72 h of the indicated treatment, viable cells were counted and the Chou–Talalay equation (3) was applied to determine the CI of each drug mixture. Drug interactions are as described: CI >1 is synergistic, CI <1 is antagonistic, CI=1 is additive. Results are the mean of two independent experiments.*

dosing and scheduling of drug delivery should be used to better individualize therapy. We used the UT7-epo cell line, which is an erythropoietin-dependent megakaryoblastic leukemia (M7) cell line that is resistant to Ara-C while maintaining sensitivity to DAC. We also used other AML cell lines with differing sensitivity to DAC and Ara-C.

Our results showed that pre-treatment of UT7-epo cells with DAC for 72 h can pre-sensitize these Ara-C-resistant cells to the effect of secondary treatment with Ara-C, while simultaneous treatment of UT7-epo cells with DAC and Ara-C had no effect on their sensitivity to both drugs. Fractional analysis supports our MTS findings where sequential treatment of UT7-epo cells with DAC followed by Ara-C resulted in a synergistic drug effect at all drug ratios, while simultaneous treatment of UT7-epo cells with DAC and Ara-C results in antagonistic drug effect. hENT1 has been shown to be the main transporter responsible for cellular uptake of Ara-C. Once inside the cells, Ara-C relies on DCK as the initial activation step (10). Decreased expression of *hENT1* mRNA and *DCK* mRNA is associated with a decrease in intracellular Ara-C uptake and reduced DCK activity, respectively. Consequently, both alterations are associated

with increased cellular resistance to Ara-C in cancer cells (20). *DCK* mutations have been associated with high levels of Ara-C resistance (21, 22). Conversely, introduction of *DCK* cDNA into $DCK^{-/-}$ cells increased cellular sensitivity to Ara-C (23). In addition, alternatively spliced DCK mRNAs have been detected in leukemia blasts from patients with resistant AML but not in those with sensitive AML, suggesting that these inactive *DCK* mRNAs might play a role in Ara-C resistance *in vivo* (24).

Two distinct mechanisms in Ara-C-resistant leukemia cell lines have been shown (25). Firstly, Ara-C-resistant leukemia cells show complete loss of function of hENT, and silencing of a single gene copy of *hENT1* gene in leukemia cells may provide significant cellular resistance and concomitant growth advantage during treatment with Ara-C. Secondly, Ara-C-resistant cells exhibit obvious loss of function mutations in the *DCK* gene, which is absolutely required for phosphorylation of Ara-C, a process necessary for Ara-C-mediated inhibition of RNA and DNA synthesis. Moreover, partial (50%) loss of DCK function may be sufficient to cause clinically relevant Ara-C *in vitro* resistance in NALM-6 human B leukemia cell line (12). Depending on the second observation, our results can be explained by pretreatment of UT7epo, an AML cell line that resistant to Ara-C, with DAC. A hypomethylation agent, DAC, can reactivate silenced (hypermethylated) *DCK* gene, subsequently restoring cellular sensitivity to Ara-C through increase of intracellular activation of Ara-C by re-activated DCK. This may explain the synergistic effect observed when we applied DAC and Ara-C sequentially for the treatment of Ara-C-resistant leukemia cells. Similarly, the antagonistic effect of simultaneous treatment of UT7epo, Ara-C-resistant, AML cells results from competition of both DAC and Ara-C for DCK in order to be activated (phosphorylated) inside the cells.

Molm13 cells are AML cells resistant to both DAC and Ara-C treatment, whether sequential or simultaneous. Fractional analysis of the effect of DAC and Ara-C combinations on these cells showed that the combination had an antagonistic effect at all possible drug ratios. This indicates the need for further molecular studies that distinguish the underlying molecular mechanisms resulting in this pattern of cell resistance.

NB4 cells are AML cells resistant to DAC and Ara-C when each drug is given separately, while being sensitive to both drugs given simultaneously. Analysis of the effect of DAC and Ara-C combinations on NB4 cells showed that the sequential drug combination had an antagonistic effect, while simultaneous drug combinations at certain concentrations had a synergistic effect and at others an additive effect, which may indicate that different underlying molecular mechanisms may explain this different cellular response to different ratios of drugs in combination.

Overall, our results indicate that drug combinations can be of benefit in treating resistant leukemia cells but an understanding of the underlying molecular mechanisms in each type of leukemia cell is important to select the best drug combination and the correct ratio of each drug in order to achieve the maximum required synergistic effect and avoid unwanted antagonistic effects.

Future studies. A detailed study of the distinct molecular patterns of the AML cell lines used here will be carried on in order to better understand the antagonistic *versus* synergistic effects of drug combinations in AML-resistant cell lines.

Conflicts of Interest

The Authors declare they have no conflicts of interest in regard to this work.

Acknowledgements

The Authors dedicate this work to the late Professor Robert Arceci.

References

1 Greco W, Bravo G and Parson J: The search for synergy: A critical review from a response surface perspective. Pharmacol Rev *47*: 331-385, 1995.

2 Berenbaum M: What is Synergy? Pharmacol Rev *41*: 93-141, 1989.

3 Chou T and Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*: 27-55, 1984.

4 Pui C, Relling M, Pharm D and Downing J: Acute Lymphoblastic Leukemia. N Engl J Med 350: 1535-1548, 2004.

5 Kern W and Estey E: High-dose cytosine arabinoside in the treatment of acute myeloid leukemia: review of three randomized trials. Cancer *107*: 116-24, 2006.

6 Tedeschi A, Montillo M, Strocchi E, Cafro A, Tresoldi E, Intropido L, Nichelatti M, Marbello L, Baratè C, Camaggi C and Morra E: High-dose idarubicin in combination with Ara-C in patients with relapsed or refractory acute lymphoblastic leukemia: a pharmacokinetic and clinical study. Cancer Chemother Pharmacol *59*: 771-9, 2007.

7 Oki Y, Ogura M, Kato H, Kikuchi A, Taji H, Kagami Y, Oshiro A, Tsujimura A, Yamamoto K and Morishima Y: Phase II study of a salvage regimen using cyclophosphamide, high-dose cytarabine, dexamethasone, etoposide, and rituximab in patients with relapsed or refractory B-cell non-Hodgkin's lymphoma. Cancer Sci *99(1)*: 179-84, 2008.

8 Mey U, Orlopp K, Flieger D, Strehl J, Ho A, Hensel M, Bopp C, Gorschlüter M, Wilhelm M, Birkmann J, Kaiser U, Neubauer A, Florschütz A, Rabe C, Hahn C, Glasmacher A and Schmidt-Wolf I: Dexamethazone, high-dose cytarabine and cisplatin in combination with rituximab as salvage treatment for patients with relapsed or refractory aggressive non-Hodgkin's lymphoma. Cancer Invest *24*: 593-600, 2006.

9 Lassaletta A, Perez-Olleros P, Scaglione C, Sirvent S, De Prada I, Perez-Martinez A, Ruiz-Hernandez A and Madero L: Successful treatment of intracranial ependymoma with leptomeningeal spread with systemic chemotherapy and intrathecal liposomal cytarabine in a two-year-old child. J Neurooncol *83*: 303-306, 2007.

10 Hubeek I, Stam R, Peters G, Broekhuizen R, Meijerink J, van Wering E, Gibson B, Creutzig U, Zwaan C, Cloos J, Kuik D, Pieters R and Kaspers G: The human equilibrative nucleoside transporter 1 mediated *in vitro* cytarabine sensitivity in childhood acute myeloid leukemia. Br J Cancer *93*: 1388-1394, 2005.

11 Zhang J, King K, Baldwin S, Young J and Case C: The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs. Cancer Metastasis Rev *26*: 85-110, 2007.

12 Kanno S, Hiura T, Ohtake T, Koiwai K, Suzuki H, Ujibe M and Ishikawa M: Characterization of resistance to cytosine arabinoside (Ara-C) in NALM-6 human B leukemia cells. Clin Chim Acta *377*: 144-149, 2007.

13 Galmarini C, Thomas X, Calvo F, Rousselot P, El Jafaari A, Cros E and Dumontet C: Potential mechanisms of resistance to cytarabine in AML patients. Leuk Res *26*: 621-629, 2002.

14 Qin T, Jelinek J, Si J, Shu J and Issa J: Mechanisms of resistance to 5-aza-2-deoxycytidine in human cancer cell lines. Blood *113(3)*: 659-666, 2009.

15 Matsuo Y, Macleod R, Uphoff C, Drexler H, Nishizaki C, Katayama Y, Kimura G, Fujii N, Omoto E, Harada M and Orita K: Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL–AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). Leukemia *11*: 1469-1477, 1997.

16 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R: NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood *77(5)*: 1080-1086, 1991.

17 Komatsu N, Yamamoto M, Fujita H, Miwa A, Hatake K, Endo T, Okano H, Katsube T, Fukumaki Y and Sessa S: Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7. Blood *82(2)*: 456-464, 1993.

18 Cory A, Owen T, Barltrop J and Cory J: Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun *3(7)*: 207-212, 1991.

19 Straetemans R, O'Brien T, Wouters L, Van Dun J, Janicot M, Bijnens L, Burzykowski T and Aerts M: Design and analysis of drug combination experiments. Biom J *47(3)*: 299-308, 2005.

20 Stam R, den Boer M, Meijerink J, Ebus M, Peters G, Noordhuis P, Janka-Schaub G, Armstrong S, Korsmeyer S and Pieters R: Differential mRNA expression of ARA-C metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood *101*: 1270-1276, 2003.

21 Stegmann A, Honders M, Hagemeijer A, Hoebee B, Willemze R and Landegent J: *In vitro*-induced resistance to the deoxycytidine analogues cytarabine (Ara-C) and 5-aza-2'-deoxycytidine (DAC) in a rat model for acute myeloid leukemia is mediated by mutations in the deoxycitidine kinase (DCK) gene. Ann Hematol *71*: 41-47, 1995.

22 Stegmann A, Honders M, Willemze R and Landegent J: *De novo* induced mutations in the deoxycytidine kinase (DCK) gene in rat leukemic clonal cell lines confer resistance to cytarabine (Ara-C) and 5-aza-2'-deoxycitidine (DAC). Leukemia *9*: 1032-1038, 1995.

23 Stegmann A, Honders M, Willemze R, Ruiz van Haperen V and Landegent J: Transfection of wild-type deoxycytidine kinase (DCK) cDNA into an Ara-C and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity. Blood *85*: 1188-1194, 1995.

24 Veuger M, Honders M, Landegent J, Willemze R and Barge R: High incidence of alternatively spliced forms of deoxycytidine kinase in patients with resistant acute myeloid leukemia. Blood *96*: 1517-1524, 2000.

25 Cai J, Damaraju V, Groulx N, Mowles D, Peng Y, Robins M, Cass C and Gros P: Two distinct molecular mechanisms underlying cytarabine resistance in human leukemia cells. Cancer Res *68(7)*: 2349-2357, 2008.

Received November 15, 2015
Revised January 11, 2016
Accepted January 12, 2016