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Original Article

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Changes in *IDH2*, *TET2* and *KDM2B* Gene Expression After Treatment With Classic Chemotherapeutic Agents and Decitabine in Myelogenous Leukemia Cell Lines

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Abstract

Background: Hematological malignancies are a heterogeneous group of tumors with increased proliferative and auto-replicative capacity. Despite treatment advances, post-treatment quality of life remains highly affected. Studies addressing the molecular mechanisms of these diseases are critical for the development of effective, rapid and selective therapies, since few therapeutic strategies succeed in being effective without triggering high-grade toxicities or debilitating late effects. Our aim of this study was to verify changes in the expression of genes involved in the malignant phenotype of hematological malignancies, by treating human cell lines *in vitro* with classic chemotherapeutic agents and the demethylating agent, decitabine.

Methods: KASUMI-1 and K-562 human myeloid leukemia cell lines were plated at a density of 3×10^4 cells/well and treated with increasing concentrations of different chemotherapeutic agents commonly used in the clinical setting. After 24 and 48 h of treatment, cell viability was tested, and RNA was extracted. Complementary DNA (cDNA) was synthesized and quantitative real-time polymerase chain reaction (qPCR) was performed to evaluate the gene expression of *IDH2*, *TET2* and *KDM2B*.

Results: A modulation in gene expression was observed before and after treatment with classic chemotherapeutic agents. It was possible to demonstrate a difference in gene expression when cells were treated with chemotherapeutic agents or decitabine alone when compared to chemotherapeutic agents in association with decitabine.

Conclusions: The genes tested, and the modulation of their expression during *in vitro* treatments suggest that *IDH2*, *TET2*, and *KDM2B* should be further investigated as potential biomarkers for ongoing treatment response and follow-up for patients diagnosed with hematological malignancies of the myeloid lineage.

Keywords: Myeloid leukemia; Gene expression biomarkers; Chemotherapy; Decitabine

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Introduction

Acute myeloid leukemia (AML) is a hematological malignancy characterized by uncontrolled proliferation of myeloblasts that accumulate in the bone marrow and peripheral blood. This proliferation leads to high levels of malignant and immature leukocytes, and to an abnormal production of platelets and erythrocytes [1], culminating in anemia, thrombocytopenia, and neutropenia [2].

Typically, treatment is divided into three phases: induction to remission, consolidation, and maintenance. In induction to remission, cytotoxic chemotherapy is employed to eliminate circulating and bone marrow leukemic cells until complete remission of the disease is achieved. During consolidation therapy, if a bone marrow transplant is deemed unnecessary, treatment is similar or less intense than the induction therapy, aiming at eliminating any residual malignant cells. Maintenance therapy varies according to the patients' overall well-being and age [1].

The standard therapy includes 3 days of combined anthracycline treatment with 7 days of cytarabine treatment (Ara-C) [2, 3], aiming to achieve complete remission and prolongation of patient survival. About 70% of patients under 60 years of age achieve complete remission when using this combination [4]; however, older patients and those with comorbidities are often considered ineligible for standard induction therapy, and their treatment options are guided by disease biology, performance status, and clinical limitations, which hinder the efficacy of therapy, or the patient's ability to tolerate the toxic side effects of induction therapy [2, 3].

Another hematological malignancy with predominance in patients over 60 years of age is chronic myeloid leukemia (CML), responsible for up to 20% of all cases of newly diagnosed leukemia in adults [5]. It is characterized by the excessive proliferation of cells from the myeloid lineage, especially

granulocytes in the bone marrow [6] and by the accumulation of these cells in peripheral blood [7].

The main treatment is based on the use of tyrosine kinase inhibitors (TKIs), such as imatinib, for patients holding the reciprocal translocation t(9;22)(q34; q11) [8, 9]. TKIs can achieve durable cytogenetic and molecular remissions, improve survival of most patients, and demonstrate a reasonable disease remission rate, since a high percentage of patients achieve a good molecular response [10]. Although the side effects with TKIs are less frequent and milder than other chemotherapeutic agents, they still require frequent patient follow-ups. Moreover, resistance to TKIs and important treatment side-effects have already been reported in the literature [11-13].

The carcinogenic process initiates at the molecular level, with gene mutations and epigenetic alterations in key genes which participate in cell cycle regulation and proliferation. These changes are used as targets for therapies and markers for diagnosis, prognosis, and treatment response evaluation [14, 15]. Genes such as *IDH2* (isocitrate dehydrogenase 2), *TET2* (Ten Eleven Translocation 2), and *KDM2B* (lysine demethylase 2B) are considered epigenetic regulators [16]. Changes in expression of *IDH2* and *TET2* have already been related to decreased myeloid differentiation patterns [17]. The *KDM2B* gene is a histone demethylase that favors oncogenesis in the lymphoid line and suppresses oncogenesis the myeloid lineage [18].

Decitabine is a potent demethylating agent capable of inducing hypomethylation, demonstrating the ability of reactivating several genes. It is used for the treatment of myelodysplastic syndrome (MDS) and AML, with promising, but not fully curative, results. Presently, decitabine shows limitations in about half of the patients treated, being that less than half of the patients treated with this drug achieve full remission [19, 20]. Clinical studies have shown that the combination of classic therapeutic regimens and decitabine may yield satisfactory results [21, 22]. Moreover, at the present date, there are 58 ongoing clinical trials for different types of leukemia using decitabine combined with other drugs [23].

AML and CML are dynamic diseases with established treatments according to the characteristics found during the diagnostic process. However, the disease may present changes over time and adaptations to treatment are necessary for treatment success. In this way, studies that can identify tumor adaptations and/or modifications are important for disease control [24]. The aim of this study was to verify whether treatment with classic chemotherapeutic agents, decitabine, or a combination of both was able to modify the expression of *IDH2*, *TET2* and *KDM2B* in two commercially available human myelogenous leukemia cell lines.

Materials and Methods

Cell lines and reagents

American Type Culture Collection (ATCC) AML (KASUMI-1) and CML (K-562) human cell lines were obtained via a donation from the Laboratory of Bone Marrow and Stem Cells of the Federal University of Sso Paulo, UNIFESP. Cells were grown in 25 cm^2 flasks containing 7 mL of RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS, Nutricell) and 1% antifungal/antibiotic solution (Sigma-Aldrich; St. Louis, USA) and maintained at 37 °C in a humidified incubator with 5% atmospheric CO_2. Cytarabine (Ara-C) and methotrexate (MTX) (Libbs; Sao Paulo, Brazil); etoposide (VP-16) (Blau Farmaceutica; Cotia, Brazil) and vincristine (VCR) (Zodiac; Pindamonhangaba, Brazil) were donated by Oncosinos, located in Sao Leopoldo, Brazil. Doxorubicin (Doxo) was obtained from Sigma-Aldrich (St. Louis, USA) and decitabine (Deci) from Caymann (Ann Arbor, USA).

Evaluation of cellular proliferation

Cells were seeded in 4- and 24-well plates suitable for growth of cells in suspension at a density of 3×10^4 cells/well. Cells were then treated with high (IC_{70}) or half maximal inhibitory concentration (IC_{50}) doses of Ara-C (25 µM or 10 µM), MTX (100 µM or 50 µM), VP-16 (25 µM or 1 µM), VCR (2.5 µM or 1 µM), Doxo (25 µM or 1 µM) or Deci 100 nM. Cells were also treated with a combination of each chemotherapeutic agent in its IC_{50} dose with Deci 100 nM. Twenty-four and 48 h later, 10 µL of cellular suspension was homogenized 1:1 with 0.4% trypan blue solution (Sigma-Aldrich). Cells were counted in a hemocytometer and viability was determined by the trypan blue exclusion test. Results are expressed as absolute cell counts.

Total RNA extraction

Forty-eight hours post-treatment, after cells were counted, the culture medium containing cells in suspension was removed from each well and centrifuged for 10 min at 1,000 g. The supernatant was discarded and RNA from each cell pellet was extracted using the Trizol™ reagent (Invitrogen), according to the manufacturer's instructions. The final RNA pellet was resuspended in water previously treated with diethylpyrocarbonate (DEPC).

Complementary DNA (cDNA) synthesis

cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System for qPCR kit (Thermo Fisher) utilizing oligodT primers, according to the manufacturer's instructions. Briefly, 500 ng of RNA was utilized for a reaction containing 1 µL 10 mM deoxyribonucleoside triphosphates (dNTPs), 50 µM oligo (dT)20, and DEPC-treatd water in a final reaction volume of 10 µL. After a 5-min incubation period at 65 °C, 10 µL buffer containing 25 mM $MgCl_2$, 0.1 M DTT, 1 µL RNAseOUT™ and 1 µL SuperScript III RT™ was added. Samples were then incubated at 50 °C for 50 min for synthesis, which was terminated at 85 °C for 5 min. A sample included with the kit was used as a positive control, and a sample without RNA was included as a negative control.

Table 1. Forward and Reverse Primers Used for Gene Amplification

Gene	Primers 5' - 3'	Temperature (°C)	Size (bp)
IDH2	Forward: ATGCCATCCAGAAGAAATGG	59.89	175
	Reverse: TGAGCCACCATGTCATCAAT	59.93	
TET2	Forward: TTGCAATGAGATACCCCACA	59.92	200
	Reverse: TGCAAACCAACAAAGATGGA	60.09	
KDM2B	Forward: ACAACAAGGAAGGGCAGGAA	59.44	196
	Reverse: CCAGGTTTGAGCCGCTTG	59.04	
ACTB	Forward: AAACTGGAACGGTGAAGGTG	60.01	171
	Reverse: AGAGAAGTGGGGTGGCTTTT	60.11	
GAPDH	Forward: CTTTGTCAAGCTCATTTCCTGG	54.20	133
	Reverse: TCTTCCTCTTGTGCTCTTGC	54.90	

Real-time PCR

Quantitative real-time PCR reactions were performed in a StepOne Real Time PCR System (AppliedBiosystems), with the primers described in Table 1 and utilizing the conditions described in Tables 2 and 3, for K562 and KASUMI-1 cell lines, respectively. Data were analyzed using the comparative Ct method. PCR efficiency from the exponential phase was calculated for each individual amplification plot with the LinRegPCR software version 11.0. In each plate, the average of PCR efficiency (Eff) for each amplicon was determined and used in future calculations. Ct values of *β-actin* and *GAPDH* were used to normalize Ct values for each gene tested. Gene expression was calculated based on the ratio to *β-actin* and *GAPDH*. Each data point corresponds to three true biological replicate samples.

Statistical analysis

Data were analyzed with descriptive statistics using SPSS software (Statistical Package of Social Sciences, IBM Inc.) version 19.0. Normality tests were applied to verify whether data distribution was parametric or non-parametric. If parametric, the results were expressed as mean ± standard deviation (SD) of the mean. If non-parametric, results were expressed as median and their interquartile ranges (25% and 75%). For parametric results, further analysis was then carried out via one-way ANOVA, followed by the Tukey *post-hoc* test to verify if there were differences between groups. For non-parametric results, a Kruskal-Wallis test was employed, followed by the Dunn's multiple correlation test. The significance level adopted was 5%, with values of $P < 0.05$ being considered significant.

The work described here does not contain any studies with human participants or animals.

Results

After establishing cell growth curves and optimizing cellular density for each plate, dose curves were performed for each chemotherapeutic agent and for decitabine in the KASUMI-1

Table 2. Standardization of qPCR Reaction for *IDH2*, *TET2* and *KDM2B* Genes in the K-562 Cell Line

Reagent	*IDH2* (60 °C)	*TET2* (59 °C)	*KDM2B* (60 °C)
Ultrapure water	3.85 µL	3.85 µL	3.5 µL
Buffer (10 ×)	2 µL	2 µL	2 µL
$MgCl_2$ (50 nM)	1.2 µL (3 nM)	1.2 µL (3 nM)	1.5 µL (3.75 nM)
dNTPs (10 mM)	-	-	0.1 µL (0.05 mM)
dNTPs (5 mM)	0.1 µL (0.025 mM)	0.1 µL (0.025 mM)	-
Primer foward (10 mM)	0.4 µL (0.2 mM)	0.4 µL (0.2 mM)	0.4 µL (0.2 mM)
Primer reverse (10 mM)	0.4 µL (0.2 mM)	0.4 µL (0.2 mM)	0.4 µL (0.2 mM)
SYBR Green	2 µL	2 µL	2 µL
Taq Platinum (5 U/µL)	0.05 µL	0.05 µL	0.1 µL
cDNA (1:15)	10 µL	10 µL	10 µL
Total	20 µL	20 µL	20 µL

qPCR: quantitative real-time polymerase chain reaction; dNTPs: deoxyribonucleoside triphosphates; cDNA: complementary DNA.

Table 3. Standardization of qPCR Reaction for *IDH2*, *TET2* and *KDM2B* Genes in the KASUMI-1 Cell Line

Reagent	*IDH2* (60 °C)	*TET2* (59 °C)	*KDM2B* (60 °C)
Ultrapure water	3.6 µL	3.6 µL	3.5 µL
Buffer (10 ×)	2 µL	2 µL	2 µL
$MgCl_2$ (50 mM)	1.2 µL (3 mM)	1.2 µL (3 mM)	1.5 µL (3.75 mM)
dNTPs (10 mM)	0.1 µL (0.05 mM)	0.1 µL (0.05 mM)	0.1 µL (0.05 mM)
Primer forward (10 mM)	0.5 µL (0.25 mM)	0.5 µL (0.25 mM)	0.4 µL (0.2 mM)
Primer reverse (10 mM)	0.5 µL (0.25 mM)	0.5 µL (0.25 mM)	0.4 µL (0.2 mM)
SYBR Green	2 µL	2 µL	2 µL
Taq Platinum (5 U/µL)	0.1 µL	0.1 µL	0.1 µL
cDNA (1:15)	-	-	10 µL
cDNA (1:45)	10 µL	10 µL	-
Total	20 µL	20 µL	20 µL

qPCR: quantitative real-time polymerase chain reaction; dNTPs: deoxyribonucleoside triphosphates; cDNA: complementary DNA.

and K-562 cell lines to verify each reagent's cytotoxic activity. The chemotherapeutic doses were selected according to the IC_{50} and IC_{70} for each agent. IC_{50} concentrations for each chemotherapeutic agent were: Doxo 1 µM, VP-16 1 µM, VCR 1 µM, Ara-C 10 µM, and MTX 50 µM. Decitabine is considered a demethylating agent and showed a cytotoxic effect on both cell lines tested, reducing cellular proliferation in a dose-dependent manner (Fig. 1). For the combination treatments, Deci was employed at 100 nM, which is a concentration that does not affect cellular proliferation significantly (data not shown).

With regards to the KASUMI-1 cell line, all chemotherapeutic agents tested at the IC_{70} and IC_{50} concentrations yielded a significant decrease in cellular proliferation when compared to decitabine alone after 48 h post-treatment. No differences were found in cellular proliferation when comparing chemotherapeutic agents alone and associated with Deci 100 nM (Fig. 2). In the K-562 cell line, all chemotherapeutic agents tested at the IC_{70} and IC_{50} concentrations yielded a significant decrease in cellular proliferation when compared to decitabine alone after 24- and 48-h post-treatment (Fig. 3). When comparing each chemotherapeutic agent alone as well as associated with decitabine, there were significant differences only in the treatments with etoposide ($P < 0.05$) (Fig. 3b) and vincristine ($P < 0.0001$) (Fig. 3c).

Gene expression levels of *TET2*, *IDH2* and *KDM2B* in both untreated cell lines were compared to expression levels of *TET2*, *IDH2* and *KDM2B* after treatment with each chemotherapeutic agent alone, decitabine alone, or an association of both in order to verify whether any of these treatments could modulate gene expression. *IDH2* gene expression in the KASUMI-1 cell line was significantly elevated ($P < 0.0001$ for all combinations) when treated with a combination of chemotherapeutic agents in association with decitabine, when compared to chemotherapy alone (Fig. 4), except in the case of vincristine (Fig. 4d). In the K-562 cell line, *IDH2* gene expression increased after treatment with an association of decitabine and etoposide ($P < 0.001$) (Fig. 4g), vincristine ($P < 0.0001$) (Fig. 4h), and methotrexate ($P < 0.01$) (Fig. 4j) when compared to each chemotherapeutic agent alone.

Gene expression of *KDM2B* in the KASUMI-1 cell line was significantly increased ($P < 0.0001$) after combined treatment of decitabine and doxorubicin (Fig. 5a), etoposide (Fig. 5b) and cytarabine (Fig. 5d) when compared to each chemotherapeutic agent alone. Treatment with vincristine (Fig. 5c) and methotrexate (Fig. 5e) significantly decreased the expression of *KDM2B* ($P < 0.0001$) when in combination with decitabine, as compared to chemotherapy alone. In the K-562 cell line, *KDM2B* gene expression was significantly decreased after treatment with all chemotherapeutic agents in association with decitabine when compared to chemotherapy alone (Fig. 5), except for etoposide (Fig. 5g), which presented a significant increase ($P < 0.0001$) in gene expression.

With regards to *TET2* gene expression, when comparing each chemotherapeutic agent associated with decitabine versus treatment with chemotherapy alone, a decrease in gene expression ($P < 0.0001$) was observed for doxorubicin (Fig. 6a), vincristine (Fig. 6c), and methotrexate (Fig. 6e) in the KASUMI-1 cell line. Etoposide (Fig. 6b) and cytarabine (Fig. 6d) treatments increased *TET2* gene expression when associated with decitabine as compared to treatment with chemotherapy alone ($P < 0.0001$). In the K-562 cell line, treatment with doxorubicine (Fig. 6f), etoposide (Fig. 6g), and methotrexate (Fig. 6j) in association with decitabine increased *TET2* gene expression when compared to each chemotherapeutic agent alone ($P < 0.0001$). *TET2* gene expression was significantly decreased after treatment with decitabine and vincristine (Fig. 6h) or cytarabine (Fig. 6i) when compared to treatment with vincristine or cytarabine alone ($P < 0.0001$).

Discussion

The present study evaluated the effects of combining decitabine with classical chemotherapeutic agents used in the treatment of different onco-hematological diseases in the expression of epigenetic regulatory genes which play a part in leukemogenesis: *IDH2, TET2*, and *KDM2B*. As expected, the

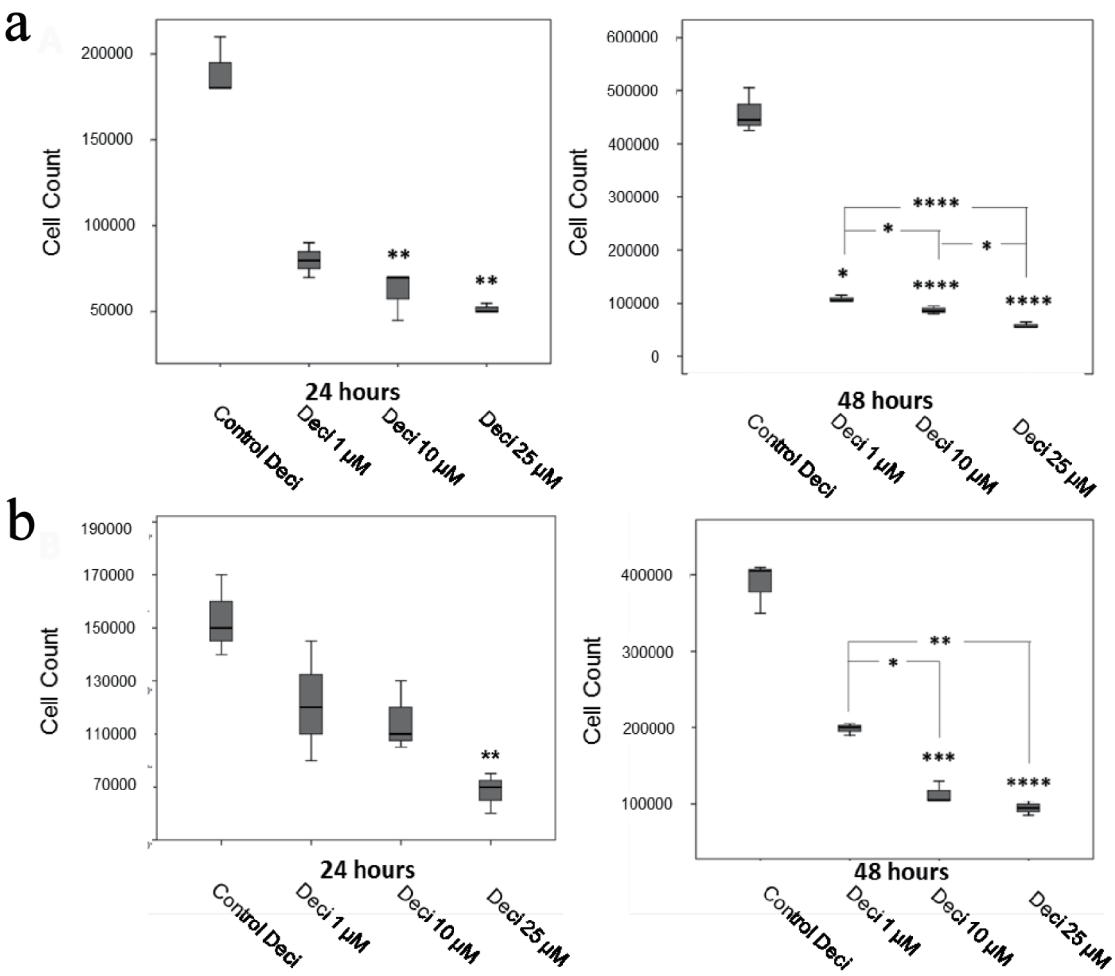

Figure 1. Decitabine dose curves in the KASUMI-1 cell line (a) and in the K-562 cell line (b). P values were determined using one-way ANOVA, followed by the Tukey *post-hoc* test (parametric), or using Kruskal-Wallis test, followed by Dunn's multiple correlations test (non-parametric), using $P < 0.05$ for statistically significant differences. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Results are expressed as absolute cell counts.

chemotherapeutic agents demonstrated cytotoxic effects in both cell lines used (KASUMI-1 and K-562). We aimed at associating low-dose decitabine with intermediate doses of each chemotherapeutic agent in such a way that this association would not alter cellular proliferation. In this way, we would be able to observe whether there were any changes at the molecular level that preceded manifestations at the cellular level. Here we report that the association of decitabine with classical chemotherapeutic agents modulates gene expression of *IDH2, TET2* and *KDM2B* when compared to treatment with each chemotherapeutic agent alone. For the most part, these changes in gene expression occurred all the while not altering cellular proliferation.

Here we also evaluated the expression levels of *IDH2*, *TET2* and *KDM2B* after treatment with decitabine alone, and compared it to untreated controls, to treatment with each chemotherapeutic agent alone, and to combination treatments of chemotherapy plus low-dose decitabine. These comparisons may indicate in which situations the association may modify the expression of each gene tested, more so than with each drug by itself. It is important to emphasize that decitabine is already employed for the treatment of certain hematological malignancies; however its use has not been proven efficient as monotherapy. For this reason, this discussion will focus on the modulation of gene expression as a result of the association of decitabine with other classical chemotherapeutic agents.

In neoplastic cells, decitabine can reactivate silenced genes and inhibit DNA methylation in low doses; cytotoxic effects have been observed in high doses. Patients with MDS/AML, CML and ALL received different doses of decitabine, ranging 5- to 30-fold lower than the maximum tolerated dose. Responses were observed at all doses studied, however low-dose decitabine was as effective, and, at times, more effective than high-dose decitabine [25]. Kopp and collaborators (2013) [26] showed that decitabine treatment presents a biphasic response with regards to hypomethylation and cytotoxicity. Decitabine affects the expression of activating and inhibitory receptors in natural killer cells at low doses during cellular pro-

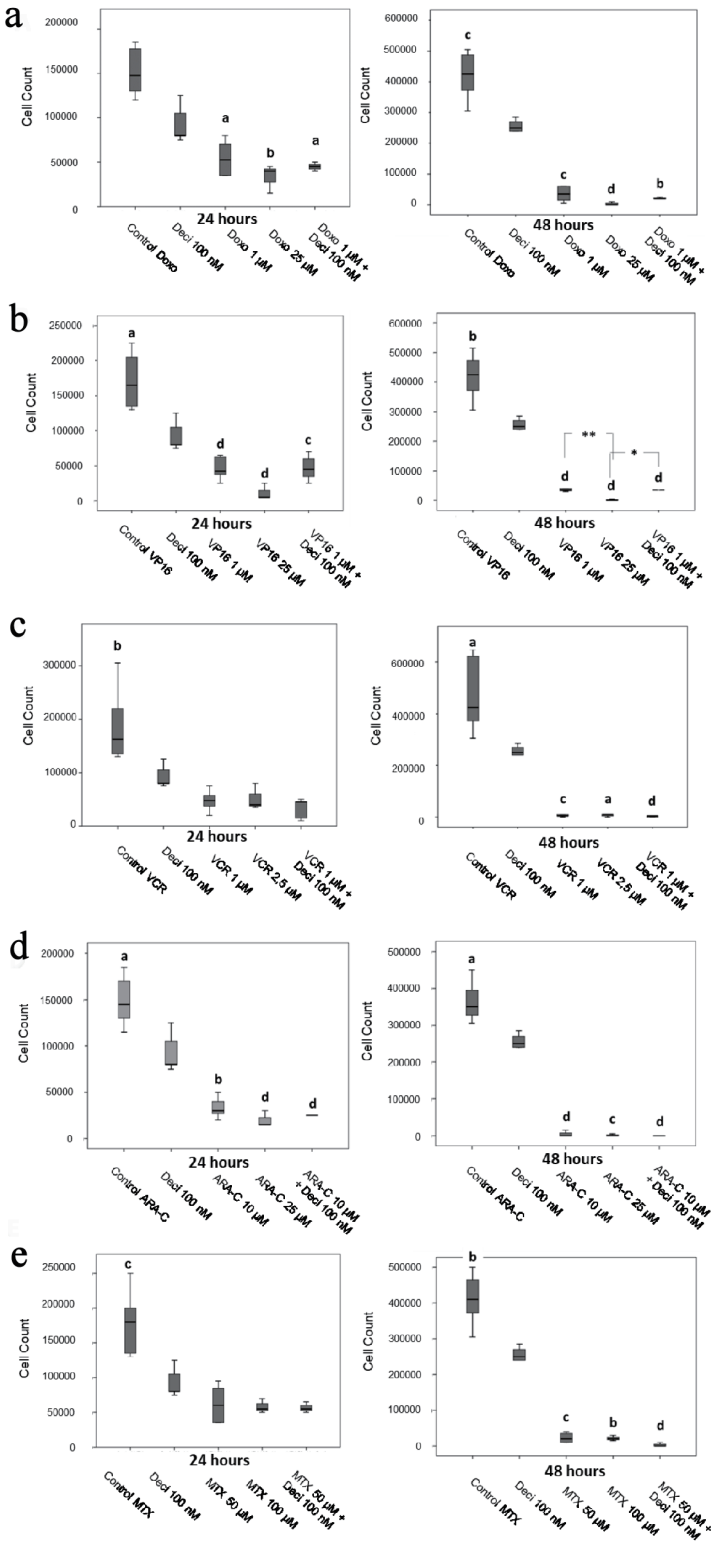

Figure 2. Cell counts after 24 and 48 h of treatment with doxorubicin (a), etoposide (b), vincristine (c), cytarabine (d) and methotrexate (e) in the KASUMI-1 cell line alone or in association with decitabine. The differences shown are in relation to treatment with decitabine. Values were determined using one-way ANOVA, followed by the Tukey *post-hoc* test (parametric), or Kruskal-Wallis test, followed by Dunn's multiple correlations test (non-parametric), where $P < 0.05$ values determined statistically significant differences. a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$, d: $P < 0.0001$. Results are expressed as absolute cell counts.

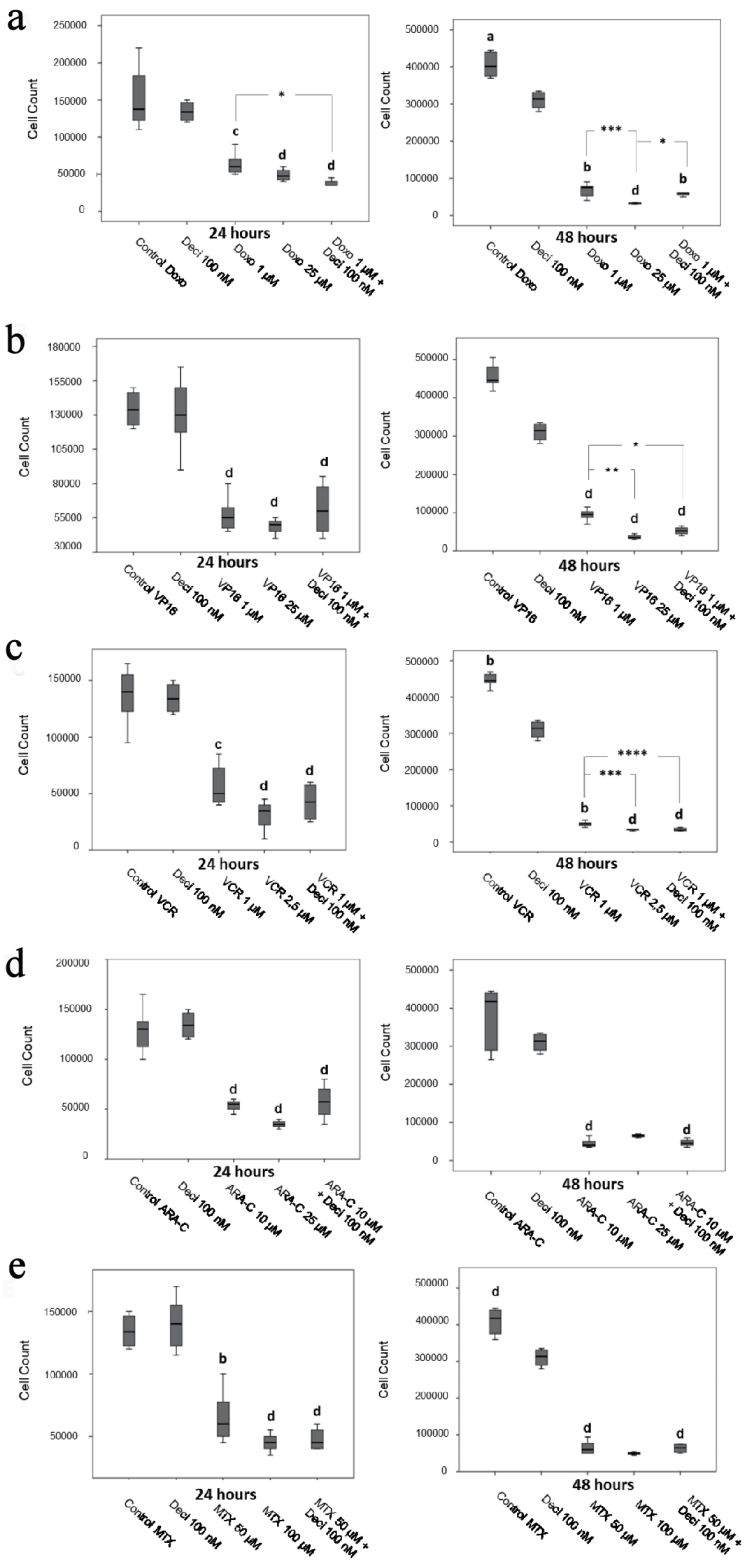

Figure 3. Cell counts after 24 and 48 h of treatment with doxorubicin (a), etoposide (b), vincristine (c), cytarabine (d) and methotrexate (e) in the K-562 cell line. The differences shown are in relation to treatment with decitabine. P values were determined using the Kruskal-Wallis test, followed by Dunn's multiple correlation test, using $P < 0.05$ to determine statistically significant differences. */a: $P < 0.05$, b: $P < 0.01$, c/***: $P < 0.001$, d: $P < 0.0001$; values of P represented in letters are relative to decitabine; values of P represented in * are relative to the isolated chemotherapeutic treatment. Results are expressed as absolute cell counts.

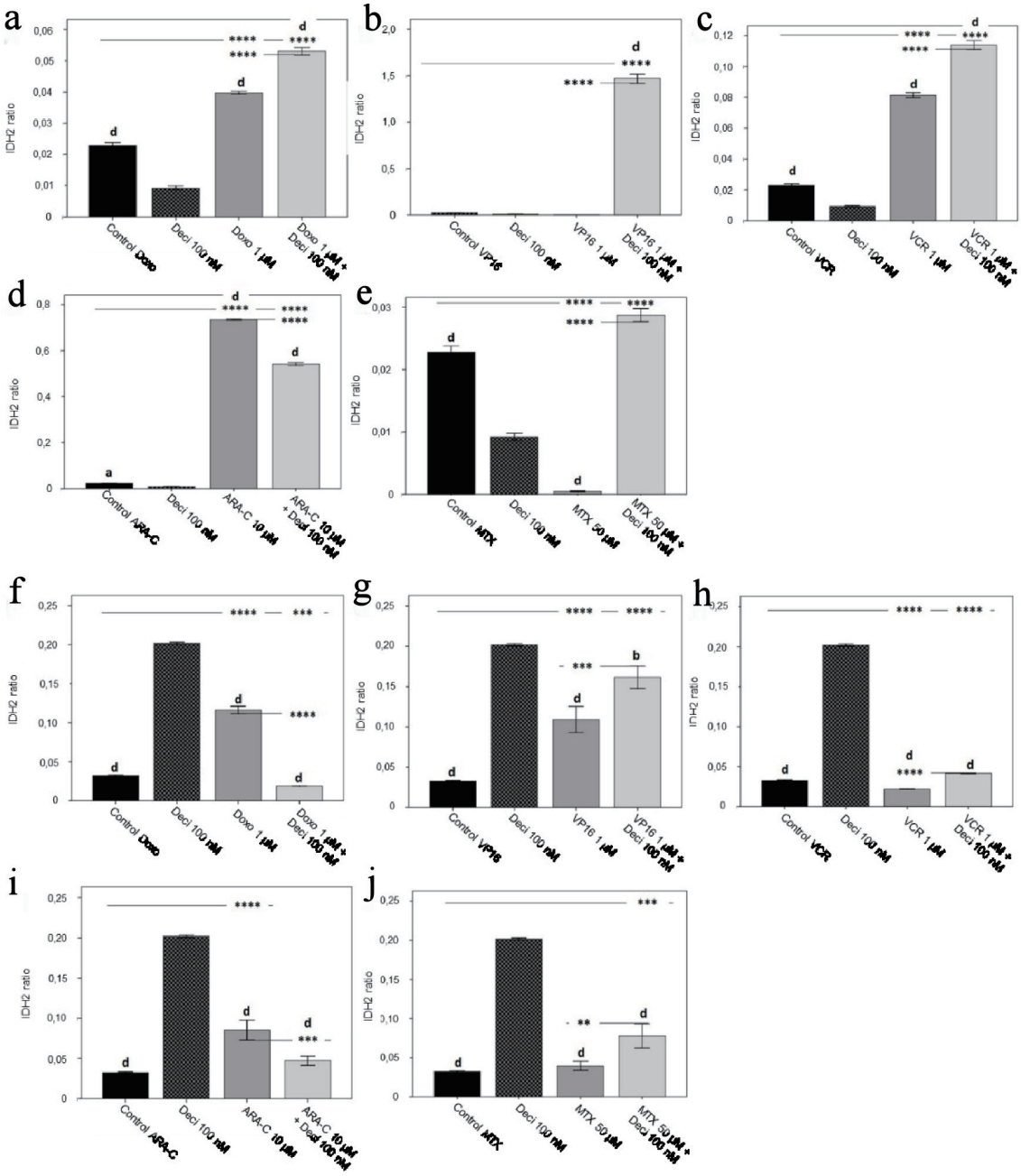

Figure 4. *IDH2* gene expression levels in the KASUMI-1 cell line after treatment with (a) doxorubicin, (b) etoposide, (c) vincristine, (d) cytarabine, and (e) methotrexate, and in the K-562 cell line after treatment with (f) doxorubicin, (g) etoposide, (h) vincristine, (i) cytarabine, and (j) methotrexate. The differences shown are in relation to treatment with decitabine. P values were determined using one-way ANOVA, followed by the Tukey *post-hoc* test, using $P < 0.05$ to determine statistically significant differences. a/*: $P < 0.05$, b/**: $P < 0.01$, c/***: $P < 0.001$, d/****: $P < 0.0001$; values of P represented in letters are relative to decitabine; values of P represented in * are relative to the isolated control or chemotherapeutic agent.

liferation. High-dose decitabine can decrease natural killer cell proliferation and reduce viability through inhibition of mRNA transcription. This data corroborate that of Issa and collaborators (2004) [25], and suggests that decitabine's optimal immunomodulation occurs at low doses, whereas high doses are associated with inhibition of proliferation and direct toxicity to cells. Low doses of decitabine also induced differentiation and maturation of myelodysplastic megakaryocytes in MDS patients, which is useful in the clinical setting [27].

A multicenter analysis performed in patients with intermediate- or high-risk MDS exploring decitabine in very low doses showed reasonable efficacy and tolerability, as well as lower medical costs [28]. Taking into consideration the costs involved in the treatment of oncological diseases, studies dem-

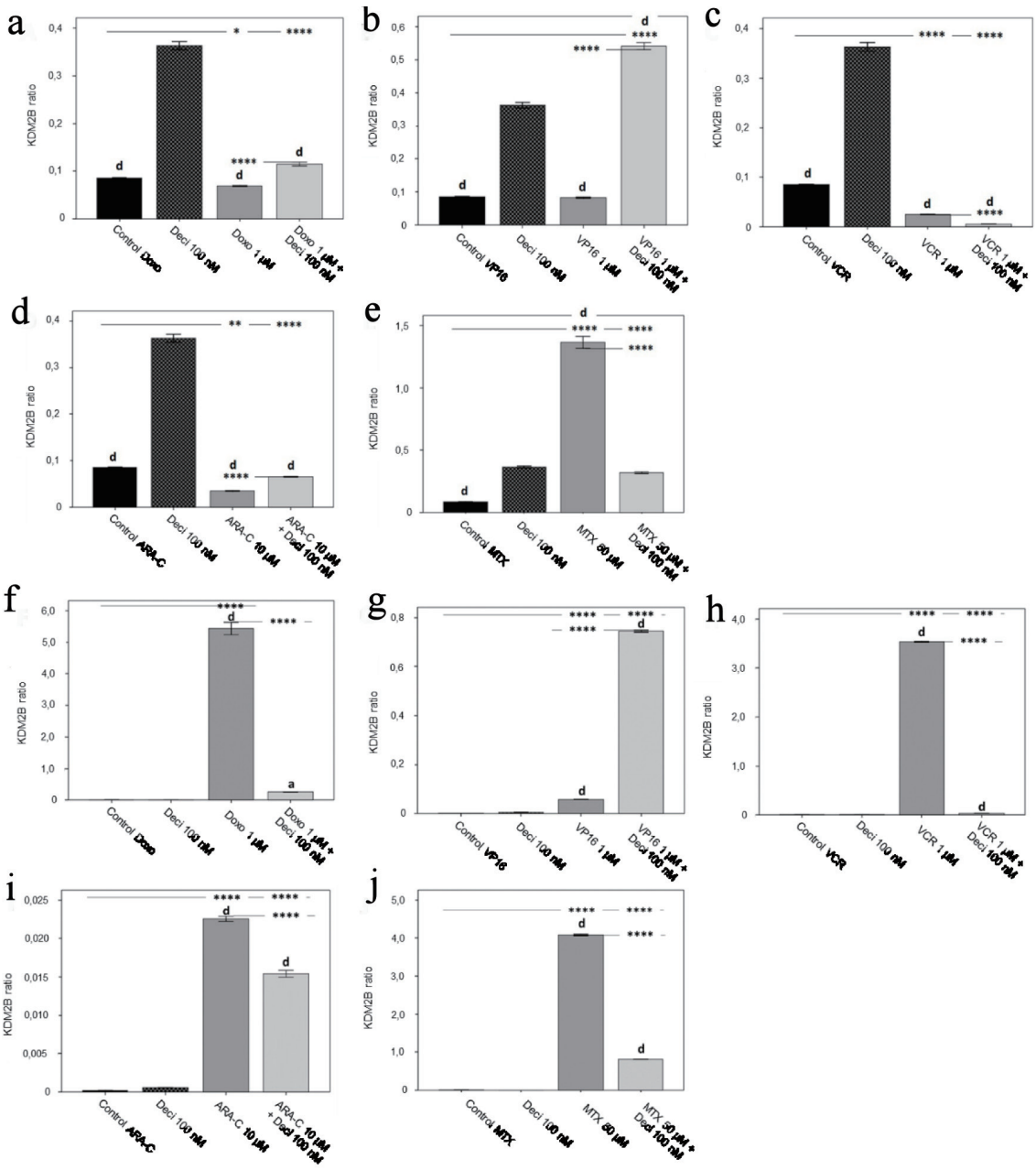

Figure 5. *KDM2B* gene expression levels in the KASUMI-1 cell line after treatment with (a) doxorubicin, (b) etoposide, (c) vincristine, (d) cytarabine, and (e) methotrexate, and in the K-562 cell line after treatment with (f) doxorubicin, (g) etoposide, (h) vincristine, (i) cytarabine, and (j) methotrexate. The differences shown are in relation to treatment with decitabine. P values were determined using one-way ANOVA, followed by the Tukey *post-hoc* test, using $P < 0.05$ to determine statistically significant differences. a/*: $P < 0.05$, b/**: $P < 0.01$, c/***: $P < 0.001$, d/****: $P < 0.0001$; values of P represented in letters are relative to decitabine; values of P represented in * are relative to the isolated control or chemotherapeutic agent.

onstrating that low doses of decitabine are equally efficient as higher doses are welcome. This also explains why the association of hypomethylating agents, such as decitabine, with other pharmacological treatments is widely used in the search for more efficient and cost-effective therapies [29-31].

Low doses of decitabine were associated with clinical improvement and hematological remission in MDS/MPN patients [32], disease remission and hematologic improvement in AML and MDS patients [33], and reduction of cell viability and induction of apoptosis in ALL patients [34]. The use of low-dose hypomethylating agents is safe and effective in patients with low-risk MDS and MDS/MPN [35] as well as in high-risk MDS [28]. Moreover, there are favorable data with regards to decitabine's effects on overall survival [2], toxicity profile and clinical efficacy [36]. These data are in accordance with what was observed in this study: decitabine can greatly

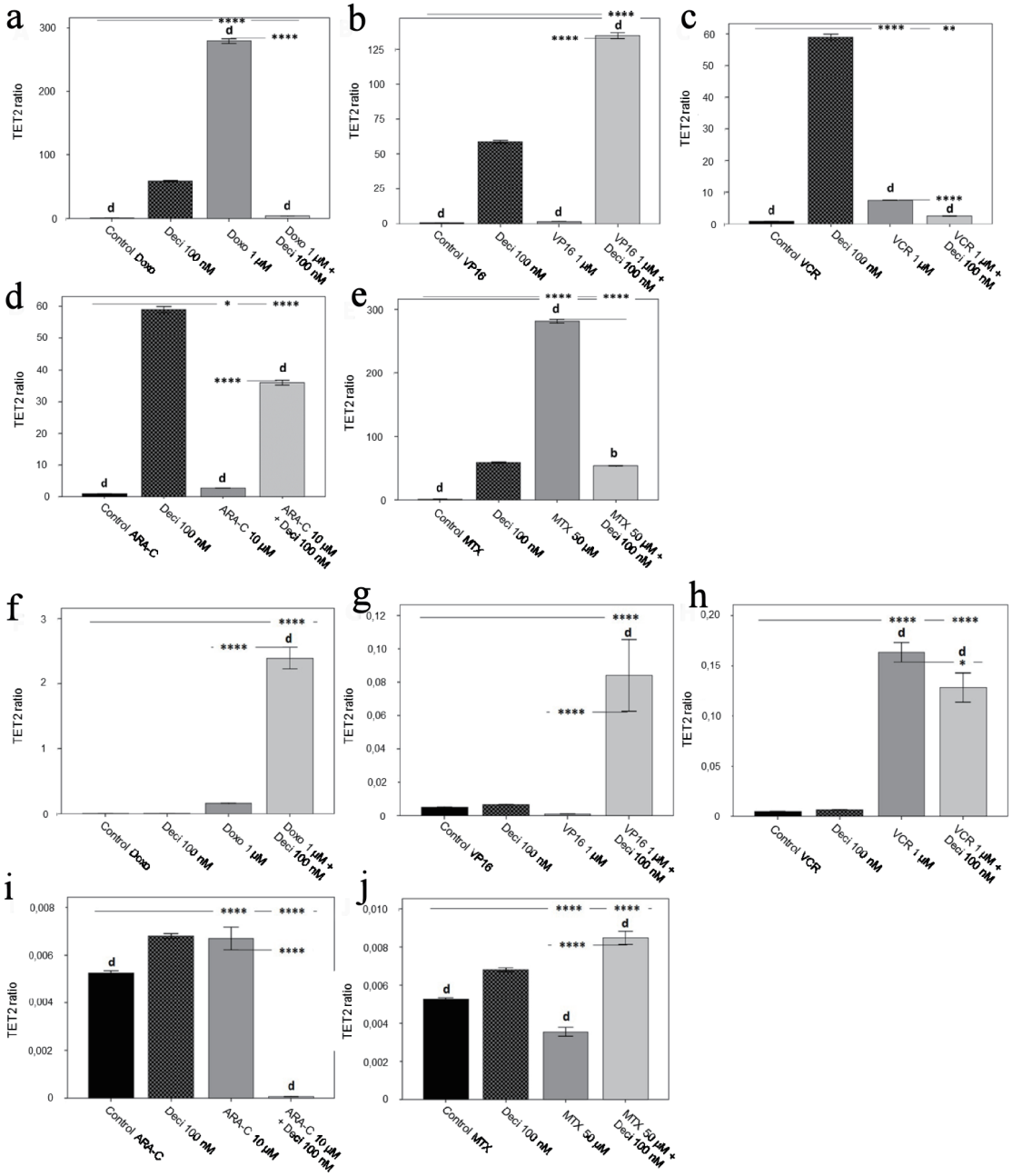

Figure 6. *TET2* gene expression level in the KASUMI-1 cell line after treatment with (a) doxorubicin, (b) etoposide, (c) vincristine, (d) cytarabine, and (e) methotrexate, and in the K-562 cell line after treatment with (f) doxorubicin, (g) etoposide, (h) vincristine, (i) cytarabine, and (j) methotrexate. The differences shown are in relation to treatment with decitabine. P values were determined using one-way ANOVA, followed by the Tukey *post-hoc* test using $P < 0.05$ to determine statistically significant differences. a/*: $P < 0.05$, b/**: $P < 0.01$, c/***: $P < 0.001$, d/****: $P < 0.0001$; values of P represented in letters are relative to decitabine; values of P represented in * are relative to the isolated control or chemotherapeutic agent.

modulate gene expression differently than chemotherapeutic agents by themselves. Here, even though changes in gene expression as a result of decitabine treatment did not necessarily translate to changes in cell behavior *in vitro*, the fact that low doses of decitabine in combination with chemotherapeutic agents modulated the expression of *IDH2*, *TET2* and *KDM2B*, more so than with each treatment alone, strengthens the argument that decitabine should be considered for combination therapy with the chemotherapeutic agents currently employed in the clinical setting.

IDH2 plays a role in energy production and a crucial role in cellular proliferation [37]. Mutations in *IDH2* can lead to decreased myeloid differentiation, and has already been associated with the development of MDS and AML [17, 38, 39].

Its downregulation was found to exacerbate the malignant progression of osteosarcoma cells via NF-kB and MMP9 pathway activation [40]. Leukemic cells showed decreased expression of *IDH2* when compared to untransformed B cells; lower *IDH2* expression accelerates cell cycle progression and increases the tumor's invasive capacity, all of which are associated with malignant progression [41]. Here we demonstrate that in the KASUMI-1 cell line the association of decitabine with doxorubicin, etoposide, cytarabine and methotrexate increased *IDH2* gene expression. The same increase in *IDH2* expression was observed in the K-562 cell line when decitabine was associated with etoposide, vincristine and methotrexate. This suggests that the association of low-dose decitabine with certain chemotherapeutic agents may be beneficial, for it modulates expression of a gene that is crucial for the leukemogenic process.

Recent studies involving the*TET2* gene suggests that its loss may be a key to leukemic transformation. It has been shown that this gene is important in normal myelopoiesis and that its dysregulation favors myeloid tumorigenesis [42]. Mice with loss of *TET2* showed increased hematopoietic stem cell (HSC) turnover and myeloproliferation, associated with splenomegaly, monocytosis, and extramedullary hematopoiesis [43]. Other studies have also shown that the inactivation of *TET2* leads to a decrease of 5-hC in HSCs of the myeloid lineage [44, 45]. Changes in the *TET2* gene have already been described as an important event in the pathogenesis of different myeloid malignancies including SMD, SMP and AML [46]. Moreover, *TET2* gene expression levels are also significantly reduced in breast, liver, lung, pancreas and prostate tumors [47]. In the present study, we demonstrate that, in the KASUMI-1 cell line, the association of decitabine with etoposide or cytarabine significantly increased *TET2* gene expression. The same was seen in the K-562 cell line when decitabine was associated with doxorubicine, etoposide, or methotrexate. Thus, associating low-dose decitabine to an already established therapeutic regimen may increase *TET2* expression, which could contribute to a more efficient and long-lasting therapeutic response.

The *KDM2B* gene exhibits opposing roles in hematological malignancies, as it can promote or antagonize tumor progression depending on the cellular context. This gene is highly expressed in leukemias exhibiting protoncogenic activity via repression of tumor suppressor pathways and is highly expressed in murine and human HSPCs compared to other tissues [18, 48]. *KDM2B* is required for the maintenance of human leukemia cell lines via the regulation of cell fate and lymphocyte-specific signaling pathways. Analysis of gene expression changes in human leukemia cell lines revealed a pleiotropic role for *KDM2B* in differentiation, quiescence, and lymphoid lineage specification through modulation of cell signaling; *KDM2B* also plays an important role in lymphoid commitment and T cell maturation [49, 50]. Here we demonstrate that the association of decitabine with vincristine or methotrexate in the KASUMI-1 cell line, and with doxorubicin, vincristine, cytarabine or methotrexate in the K-562 cell line decreased *KDM2B* gene expression. This suggests that the association of decitabine with other cytotoxic drugs may improve treatment response via epigenetic modulation. Further studies need to be undertaken in order to better explore how restoring cellular differentiation in leukemia may be employed as a viable therapeutic approach. The investigation of *KDM2B* modulation may provide further information regarding this matter.

Conclusions

Considering the information already presented with regards to decitabine's effects on cellular differentiation and epigenetic modulation, it is imperative to investigate how this drug may be employed in a safe and economically viable manner to current treatments that are already in order. Here we demonstrate that decitabine at low doses, when combined with certain chemotherapeutic agents already employed in the clinical setting, can modulate three genes that are key to the leukemogenic process: *IDH2*, *TET2*, and *KDM2B*. Moreover, these changes in gene expression were observed before any changes in cellular proliferation were seen, strengthening the rationale for investigating these genes as potential biomarkers for tumor regression and/or disease relapse. Epigenetic modulation has proven to be a key event in disease remission, and may present other benefits besides potentiation of cellular toxicity, such as cellular differentiation, reduction of adverse events and toxicity to treatment, stability of disease via avoidance of gene switching, and an increase in disease-free survival. This report strengthens the rationale for the use of combined, gene-modulating treatments. Further studies are necessary to fully elucidate the exact mechanisms by which this gene modulation occur, and its long-term effects post-treatment.

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Conflict of Interest

The authors hereby declare that there is no conflict of interest pertaining to the work described here.

Informed Consent

Not applicable.

Author Contributions

Study concept and design: ALA. Experiments: JA, GMD and LR. Data analysis and statistics: MG, ALA and VB. Figures: JA and GMD. Manuscript preparation and submission: ALA, JA and GMD.

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