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Apoptosis induction by (+)α-tocopheryl succinate in the absence or presence of all-trans retinoic acid and arsenic trioxide in NB4, NB4-R2 and primary APL cells

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ABSTRACT

We analyzed the effect of (+)α-tocopheryl succinate (α-TOS) alone or associated with arsenic trioxide (ATO) or all-trans retinoid acid (ATRA) in acute promyelocytic leukemia (APL). α-TOS-induced apoptosis in APL clinical samples and in ATRA-sensitive (NB4) and ATRA-resistant (NB4-R2) APL cell lines. The effective dose 50% (ED-50) was calculated to be 71 and 58 μM, for NB4 and NB4-R2, respectively. α-TOS neither induced nor modified ATRA-induced differentiation of APL cells, and did not affect the proliferation and differentiation of normal $CD34^+$ hematopoietic progenitors in methylcellulose assays. α-TOS exerted a moderate antagonistic effect to ATO-induced apoptosis when treatment was done simultaneously but when α-TOS was added 24 h after ATO, an additive effect was observed. Our results support the concept of α-TOS as an anti-leukemic compound which spares normal hematopoiesis.

1. Introduction

Acute promyelocytic leukemia (APL) blasts are unique in their sensitiveness to the pro-differentiating action of all-trans retinoic acid (ATRA), and this vitamin A derivative combined with chemotherapy induces prolonged remissions in over 80% of APL patients [1]. However, relapses frequently accompanied by ATRA resistance occur in 15–30% of APL patients [2]. *De novo* and relapsed APL patients respond well to treatment with arsenic trioxide (ATO) [3,4] and the association of ATRA and ATO has proven to provide a better therapeutic outcome compared to either ATO or ATRA alone [5–7]. Nevertheless, therapy with ATO is not available to the majority of APL patients in developing countries [8,9] and may be associated with serious adverse effects, such as hepatotoxicity and even sudden cardiac death [10,11]. Therefore, the development of new strategies for APL therapeutics reducing the relapse rate and/or ATO associated toxicity would be beneficial.

(+)α-Tocopheryl succinate (α-TOS) is a succinate ester of (+)α-tocopherol (the most abundant form of vitamin E in human tissues) and has distinct functional properties compared to the latter. α-TOS has been demonstrated to induce apoptosis in multiple carcinoma cell lines [12,13] and to inhibit the growth of several tumour types in animal models [14–16]. The proapoptotic effect of α-TOS in APL has been determined in the HL-60 cell line [17,18], which was derived from an APL patient but do not harbour the *Retinoid Receptor α* (*RARα*) gene rearrangement [19]. Therefore, this is not a representative model of the molecular pathogenesis of APL [20].

α-TOS and ATO belong to the class of mitochondrially targeted anticancer drugs ("mitocans"), which exert their cytotoxic effect through the generation of reactive oxygen species (ROS) in the mitochondria [21]. Based on their common mechanism of action, we hypothesized α-TOS and ATO could have synergistic effects. We have analyzed the proapoptotic effect of α-TOS, ATO alone and in combination in NB4 [22], NB4-R2 (ATRA-resistant) cells, and APL clinical samples, and used the median-effect equation and the Combination Index (CI) theorem to characterize their interaction. According to it, CI values <1, =1 and >1 indicate synergism, addictive effect and antagonism for a given dose–effect respectively [23]. In addition, we determined if α-TOS-induced granulocytic differentiation or impaired the formation of normal hematopoietic colonies.

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2. Materials and methods

2.1. Materials

(+)α-Tocopheryl succinate, (+)α-tocopherol, all-trans retinoic acid, arsenic trioxide, 1,1,3,3-tetramethoxypropane, propidium iodide (PI), trichloroacetic acid, thiobarbituric acid, hydrochloric acid, dimethyl sulfoxide, gentamicine, sodium hydroxide and HEPES were purchased from Sigma–Aldrich (St. Louis, USA). RPMI-1640 medium, IMDM medium, fetal calf serum (FCS) and L-glutamine were purchased from GIBCO (Grand Island, USA). MethoCult H4434 was purchased from StemCell Technologies (Vancouver, Canada).

2.2. Leukemic samples from patients and normal cord blood

Leukemic cells from twelve APL patients were obtained at diagnosis by bone marrow (BM) aspiration after informed consent. APL diagnosis was confirmed by cytogenetic analysis and/or RT-PCR for PML/RARα as described [24]. All samples contained at least 90% of blasts and were used fresh. Human cord blood cells ($n = 7$) were obtained after informed consent. The study was approved by local Ethics Committee (Process 3463/2003).

2.3. Cell cultures

APL clinical samples, NB4 and NB4-R2 cells were maintained in RPMI-1640 medium (GIBCO, Grand Island, USA) with 10% fetal calf serum, 2 mM of L-glutamine and 40 mg l^{-1} of gentamicine at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity.

2.4. Apoptosis induction by α-TOS, ATO and their association

In the experiments with cell lines, α-TOS was used at the doses of 18.84, 37.68, 75.36 and 94.20 μM, ATO at 1, 2, 4 and 5 μM and the combination α-TOS + ATO at a ratio of 18.84 μM per 1 μM respectively. Samples were incubated for 24 h. Five independent experiments were performed. In time-curve experiments using cell lines and experiments with primary cells α-TOS was used at a fixed dose of 75.36 μM.

α-TOS was diluted in DMSO (0.1% final concentration). ATO was dissolved in sodium hydroxide and diluted to desired concentration in growth medium. The sodium hydroxide and DMSO in culture had no influence on medium pH and cell growth.

Apoptosis was assessed by Annexin V-binding and propidium iodide staining. Five hundred thousand cells were harvested by centrifugation and resuspended in 100 μl of binding buffer (10 mM Hepes, 140 mM NaCl and 25 mM $CaCl_2$, pH 7.4) with 5 μl of Annexin V–FITC (BD Biosciences Pharmingen San Diego, CA, USA) and 10 μl of a 50 μg ml^{-1} PI solution Sigma–Aldrich (St. Louis, USA) for 20 min at room temperature and acquired in a FacsCalibur flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA). The FlowJo 7.1 software (Tree Star; Ashland, OR, USA) was used to analyse data. In addition, cytospin preparations stained with Leishman or with 4′,6-diamidino-2-phenylindole (DAPI) were used for morphological evaluation.

2.5. Sequential treatment of ATO and α-TOS

NB4 cells were treated with ATO at 1, 2 and 4 μM or α-TOS at 37.68, 75.36 and 150.72 μM for 48 h. Sequential treatment was performed with the same doses of ATO and α-TOS, but with α-TOS added after 24 h of ATO incubation. Apoptosis was determined as described above.

2.6. Granulocytic differentiation induction

NB4 and NB4-R2 cells were incubated with α-TOS (37.68 and 75.36 μM) and ATRA (1 μM) alone or in combination for 96 h. The cells were then washed and resuspended in 0.1 ml PBS with 1% FCS and incubated with 5 μl of anti-CD11b-PE or IgG1-PE (BD Biosciences Pharmingen, San Diego, CA, USA) for 15 min, washed and resuspended in PBS with 1% FCS. The percentage of CD11b positive cells and intensity of CD11b expression was determined in a FacsCalibur flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA). In addition, cytospin preparations stained with Leishman were used for morphological evaluation. Five independent experiments were performed.

2.7. Toxicity analysis of α-TOS to normal human cord blood progenitors

CD34 positive cells were isolated from human cord blood ($n = 7$) using the MACS direct CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as directed by the manufacturer. Five thousand cord blood $CD34^+$ cells diluted in 0.3 ml of IMDM media, with purity higher than 80%, were seeded in 3 ml of MethoCult-H4434 in presence or not of α-TOS 75.36 μM. Samples were incubated at 37 °C in 5% CO_2. Burst-forming units erythroid (BFU-E) and colony-forming units erythroid (CFU-E) were counted after 7 days and colony-forming units granulocyte–monocyte (CFU-GM) after 14 days.

2.8. Measurement of lipid peroxidation

Lipid peroxidation of NB4 cells treated with 75.36 μM of α-TOS and ATO 1 μM alone or in combination for 6, 12 and 24 h was evaluated by measuring the concentration of thiobarbituric acid reactive substances (TBARS), according to the method described by Buege and Aust [25]. Cell suspension samples were thawed and after warm up until they reached a temperature of 37 °C. One ml of each cell suspension was mixed with 2 ml of 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25N hydrochloric acid, and then samples were heated in a boiling-water bath for 15 min. After cooling, the precipitate was centrifuged at $1000 \times g$ for 10 min. The absorbance of the supernatant was measured at 535 nm wavelength using a Minton Roy Spectronic 601 spectrophotometer (Minton Roy, Ivyland, USA). The concentrations of thiobarbituric acid reactive substances was determined by considering the molar absorption coefficient of the product (1.56×10^5 M^{-1} cm^{-1}) and the results reported as nM of MDA. A standard curve was constructed by using a stock solution of 10 mM MDA prepared from 1,1,3,3-tetramethoxypropane, and the concentrations detected in the samples were within this curve, showing good linearity with the standard. The assays were performed in triplicate.

2.9. HPLC analysis

The concentration of α-tocopherol in the supernatant of untreated NB4 cells and NB4 cells treated with α-TOS 75.36 μM for 6, 12 and 24 h was determined by high performance liquid chromatography assay as described previously [26]. Briefly, a 0.5-ml sample of cell culture suspension was homogenized in 2 ml of ethanol, placed in 1 ml *n*-hexane, and shaken for 2 min. A 0.5-ml aliquot of the supernatant was dried under nitrogen. The evaporated organic layer was reconstituted in 0.5 ml of the mobile phase containing 70% acetonitrile, 20% dicloromethan, and 10% methanol and was filtered. A 0.1-ml amount was injected onto a Shimadzu LC-9^A high performance liquid chromatography apparatus (Shimadzu Corporation, São Paulo, Brazil) under the following working conditions: UV detector at 292 nm, flow rate 2.0 ml/min, 4.0 mm × 1.0 cm precolumn, 4.6 cm × 25 cm (Simpack CLC-ODS) C18 column. Calibration of the equipment with standard (+)α-tocopherol solution was performed at 10, 20, and 200 μM. The standard solution of 20 μM was injected before each reading. The assays were performed in triplicate.

2.10. Dose–effect, drug combination and statistical analysis

Dose–effect analysis was performed using the median-effect equation and the CI equation [23]. Conformity of the data with the mass-action law principle was evaluated by the linear correlation coefficient (r value) of the median-effect plot. These analyses were calculated by the Calcusyn software (Biosoft®, Great Shelford, UK). Statistical differences between populations from control and α-TOS treated samples were evaluated by the nonparametric Mann–Whitney test. A p-value <0.05 was defined as significant.

3. Results

3.1. Apoptosis induction by α-TOS, ATO and their combination

Fig. 1A shows the percentage of apoptotic NB4 cells as a function of time. α-TOS- (75.36 μM) induced apoptosis with an evident effect detected at 24 h, so this time-point was selected for the dose–effect analysis. A similar effect was detected in APL clinical samples (Fig. 1B). The proapoptotic was dose dependent with an effective dose 50% (ED-50) of 71 μM with a confidence interval 95% (CI 95%) of 64.18–78.58 μM for NB4 and 58 μM (44.70–75.17 μM) for NB4-R2 cells (Fig. 2A and D). As expected, ATO was a potent inducer of apoptosis with a ED-50 of 1.73 μM (1.14–2.62 μM) for NB4 and 1.48 μM (0.91–2.40 μM) for NB4-R2 cells (Fig. 2B and E). The analysis of the interaction between ATO and α-TOS demonstrated a moderate antagonism with a Combination Index at ED-50 of 1.84 for NB4 and 3.08 for NB4-R2. The antagonism was present at all dose ranges as showed by the CI *versus* Fractional Effective plot (Fig. 2C and F). Morphologic evaluation corroborated the flow data (Fig. 3).

3.2. Evaluation of lipid peroxidation induced by ATO, α-TOS and their combination

ATO, but not α-TOS, induced a time dependant increase in lipid peroxidation in NB4 cells, which was clearly inhibited by co-treatment with α-TOS suggesting a free radical scavenger

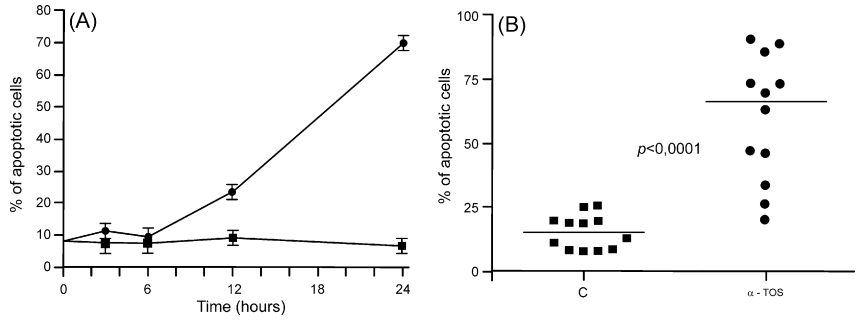

Fig. 1. Analysis of the proapoptotic effect of α-TOS in NB4 cells and APL clinical samples. (A) NB4 cells were cultivated in the presence of α-TOS 75.36 μM (●) or vehicle (■) for the indicated periods of time. (B) Leukemic cells obtained from the bone marrow of 12 patients with APL were cultured in the presence of 75.36 μM α-TOS (●) or DMSO (control, C) (■) for 24 h. Horizontal bars represent the median percentage of apoptotic cells ($p < 0.0001$ Mann–Whitney test). The number of apoptotic cells in both was determined by flow cytometry (Annexin V-FITC and propidium iodide).

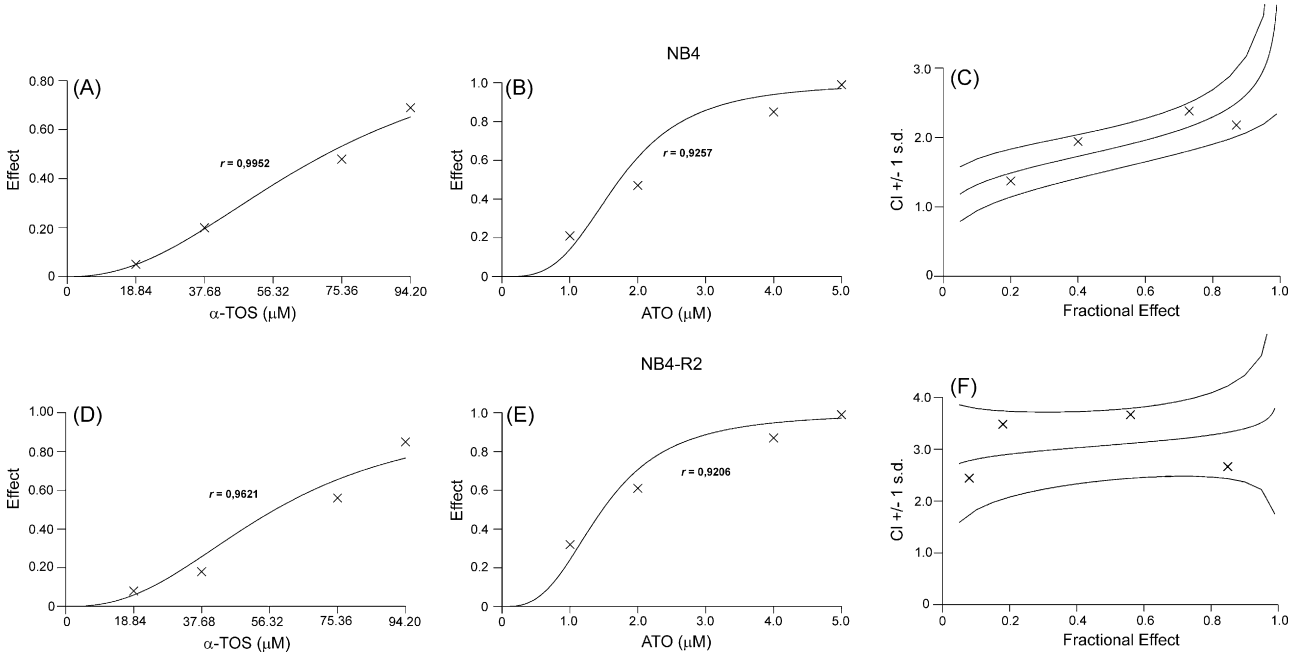

Fig. 2. Dose–effect curves (A–E) of a representative experiment using α-TOS, ATO and their association in APL cell lines. NB4 (upper row–A and B) and NB4-R2 (lower row–D and E) cells were cultured for 24 h in the presence of α-TOS (A and D) 18.84, 37.68, 75.36 and 94.20 μM or ATO (B and E) 1, 2, 4 and 5 μM. *r* Values represent the goodness of fit of the experimental data to the median-effect principle. C and F show the Combination Index *versus* Fractional Effect plot of α-TOS + ATO treatment at 18.84 μM per 1 μM ratio of the five experiments performed. Samples were stained with Annexin V-FITC and propidium iodide, and the number of apoptotic cells was determined by flow cytometry. S.D.: standard deviation.

effect (Table 1). Since α-TOS lacks antioxidant activity [21] and NB4 was demonstrated to present esterase activity [27] a possible explanation for the antagonism could be the cleavage of α-TOS into α-tocopherol, an antioxidant, and succinic acid by lysossomal esterases. In order to test this hypothesis, we determined α-tocopherol (median, 25th–75th percentiles) concentration in the cell extracts of untreated NB4 cells or NB4 cells treated with α-TOS 75.36 μM. The concentration was significantly higher in samples incubated for 6 h (13.51; 10.60–17.57 μM), 12 h (14.61; 13.84–17.39 μM) and 24 h (16.37; 13.45–17.53 μM) with α-TOS compared to control samples (0.83; 0.35–1.19 μM) ($p < 0.0001$).

Table 1
Thiobarbituric reactive substance (TBARS) concentrations in NB4 cell extracts treated with α-TOS, ATO or their association at different time-points.

Time-point (h)	TBARS concentration (μM)			
	Control	α-TOS (75.36 μM)	ATO (1 μM)	ATO + α-TOS (1 μM + 75.36 μM)
0	0.19 (0.11–0.26)	0.19 (0.11–0.26)	0.21 (0.13–0.27)	0.18 (0.9–0.26)
6	0.17 (0.05–0.29)	0.16 (0.04–0.29)	0.31 (0.2–0.41)[a]	0.22 (0.1–0.28)
12	0.12 (0.02–0.22)	0.11 (0.02–0.22)	0.53 (0.35–0.68)[a]	0.25 (0.15–0.29)[b]
24	0.14 (0.03–0.29)	0.13 (0.03–0.22)	0.61 (0.31–0.7)[a]	0.24 (0.1–0.32)[b]

Values represent median (25th–75th percentiles).
[a] Indicate $p < 0.05$ compared to control.
[b] Indicate $p < 0.05$ compared to ATO.

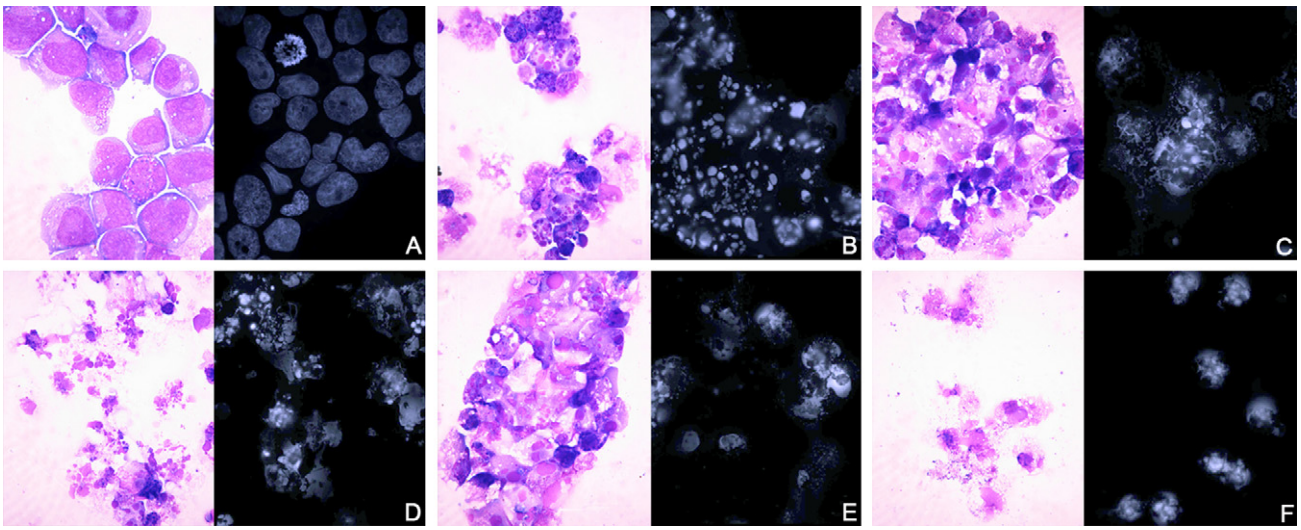

Fig. 3. Leishman and DAPI stained cytospins of NB4-R2 cells treated for 24 h with vehicle (A) α-TOS 75.36 μM (B), ATO 4 μM (C) and α-TOS 75.36 μM + ATO 4 μM (D). Alternatively, cells were treated with ATO 4 μM for 48 h without (E) or with (F) the addiction of α-TOS 75.36 μM after 24 h exposure to ATO.

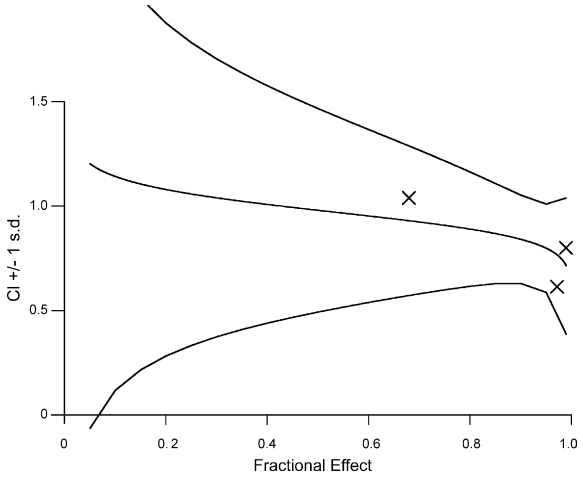

Fig. 4. Combination Index *versus* Fractional Effect plot of the sequential treatment with ATO followed by α-TOS of the five experiments performed. NB4 cells were treated with ATO (1, 2 and 4 μM) for 24 h followed by α-TOS (37.68, 75.36 and 150.72 μM) treatment for another 24 h. Apoptosis was determined by Annexin V-FITC binding and propidium iodide by flow cytometry. S.D.: standard deviation.

3.3. Sequential treatment with ATO followed by α-TOS

To confirm that the antagonistic interaction between α-TOS and ATO could be caused by the inhibition of ATO-induced lipid peroxidation, we repeated the same experiment except that α-TOS was added to the NB4 cell suspension 24 h after ATO. Under this condition, the CI at ED-50 was 0.98 thus indicating an addictive effect [23] (Fig. 4).

3.4. Evaluation of α-TOS toxicity to normal human cord blood progenitors

Fig. 5A shows the fold change of the number of CFU-E, BFU-E and CFU-GM colonies in the methylcellulose assays using isolated $CD34^+$ cells from cord blood treated or not with α-TOS (75.36 μM). No significant difference ($p = 0.65$) was detected.

3.5. Evaluation of α-TOS effect and its interaction with ATRA on granulocytic differentiation of NB4 and NB4-R2 cells

Compared to controls, no significant change in the percentage of CD11b positive cells (Fig. 5B and C) or in the intensity of

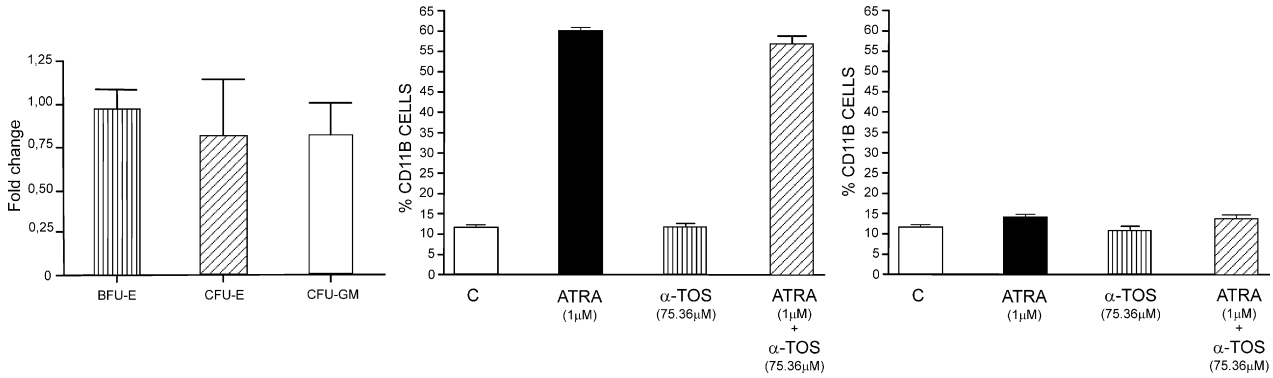

Fig. 5. (A) Analysis of the cytotoxic effect of α-TOS in normal hematopoetic progenitors. $CD34^+$ cells were isolated from normal human cord blood and seeded in methylcellulose media in the presence or absence of 75.36 μM of α-TOS. Results are presented as median fold change of the number of burst-forming units erythroid (BFU-E), colony-forming units erythroid (CFU-E) and colony-forming units granulocyte–monocyte (CFU-GM) relative to control. Seven independent experiments were performed. (B) Analysis of differentiating effect of ATRA and α-TOS in APL cell lines. NB4 cells were cultured in the presence of α-TOS 75.3 μM associated or not with ATRA 1 μM for 96 h, and stained with an anti-CD11b-PE. The percentage of CD11b positive cells was determined by flow cytometry. Bars represent the median percentage of CD11b positive cells according to treatment. Five independent experiments were performed. (C) As in (B) for NB4-R2 cells.

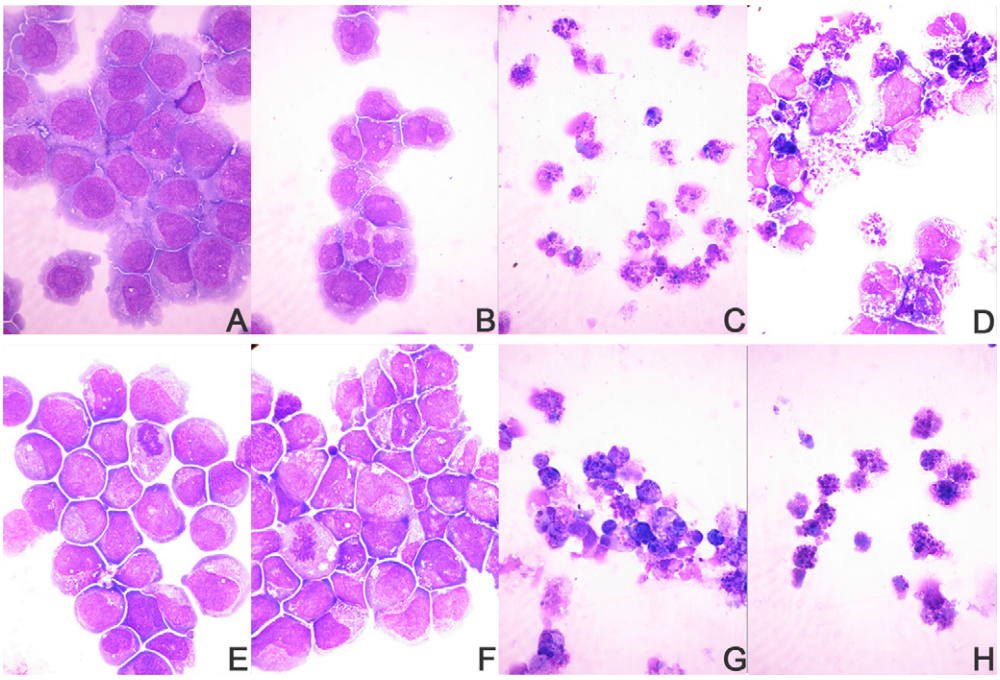

Fig. 6. Leishman stained cytospins of NB4 cells (upper row A–D) and NB4-R2 cells (lower row E–H) after 96 h culture treated with: vehicle (A and E), ATRA 1 μM (B and F), α-TOS 75.3 μM (C and G) and α-TOS 75.3 μM + ATRA 1 μM (D and H).

CD11b expression (data not shown) was induced by α-TOS in NB4 or NB4-R2 cell lines. Moreover, α-TOS did not alter ATRA-induced differentiation of NB4 cells, nor reversed resistance to ATRA in NB4-R2 cells. The cytomorphological analyses were in agreement with the results of CD11b expression (Fig. 6).

4. Discussion

To the best of our knowledge, this is the first study to demonstrate that α-TOS is an inducer of apoptosis in ATRA-sensitive and ATRA-resistant leukemic cells harbouring t(15;17)/PML/RARα. This finding suggests that α-TOS may be an alternative to ATO in the treatment of relapsed cases of APL harbouring mutations in the retinoid binding domain of PML/RARα that render leukemic cells insensitive to ATRA. Importantly, the proapoptotic effect was selective to leukemic hematopoietic progenitors, since the incubation of $CD34^+$ cells with α-TOS did not change the number of CFU-E, BFU-E and CFU-GM colonies obtained. Our data corroborate those by Weber et al. [28], reporting the lack of tissue damage in heart, liver, kidney and colon samples from nude mice injected i.p. with 50 μl of a 200-mM solution of α-TOS every third day for 15 days, thus demonstrating the differential sensitivity of normal and malignant tissues to α-TOS.

Despite the proposed common mechanism of action, we detected an antagonistic interaction between ATO and α-TOS. This antagonistic effect was associated with an inhibition of ATO-induced LP and probably was mediated by α-TOS degradation and formation of α-tocopherol (a potent antioxidant) by the action of cell esterases. Indeed, the cleavage of α-TOS by intestinal esterases could represent a serious limitation for oral administration of this drug. The use of intravenous liposomal preparations of α-TOS or another derivative of α-tocopherol for oral administration (like an ether or amide instead of an ester one) may represent alternatives to circumvent this limitation.

Our results reinforce the importance of reative oxygen species in ATO-induced apoptosis. Similarly, Karasavvas et al. [29] have demonstrated that the dehydroascorbic acid, an antioxidant molecule derived from vitamin C, conferred dose-dependent protection from ATO toxicity in HL60 and U266 cells as measured by viability, colony formation, and apoptosis assays, suggesting that inhibition of LP may be a strategy for reducing ATO toxicity. In addition, glutathione depletion enhanced cell sensitivity to the effects of ATO by enhancement of ROS generation [30].

It should be pointed out that Trolox, a hydrophilic derivative of vitamin E, chemically unrelated to α-TOS, potentiated ATO-induced ROS generation and enhanced apoptosis in APL and other malignant cell lines [31]; nonetheless Trolox do not have anti-leukemic action *per se*.

As already described for other drugs [32], variations in schedule could reveal different outcomes for combination drug therapies. Indeed, when we treated NB4 cells first with ATO and added 24 h later α-TOS, we did not observed any antagonistic interaction. Therefore, it is conceivable that by giving first ATO and then α-TOS, a maximal effect could be obtained with minimal toxicity to normal tissues, but this assumption needs careful evaluation by means of an *in vivo* study.

The lack of association between the effects of ATRA and α-TOS in granulocyte differentiation is not surprising since the first acts through a ligand-dependent manner and has a negligible proapoptotic effect. Moreover, α-TOS does not have any negative interaction with ATRA, the current standard treatment for APL.

In conclusion, our results support the idea that α-TOS has anti-leukemic activity and may improve the clinical outcome of a subset of APL patients in whom co-morbidities or advanced age limit the use of anthracyclines. The efficacy and safety of new strategies based on the use of combined ATRA and α-TOS should be compared to those reported for combined ATRA and ATO therapy [33]. The same applies to our results obtained with the sequential treatment ATO followed by α-TOS, thus allowing for reduced ATO concentration and ATO-induced toxicities. We believe that these strategies should be carefully tested in future clinical trials.

Conflict of interest

None.

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