

Megadoses of Ascorbate as a New Chemotherapeutic Approach in Uveal Melanoma: A Preliminary *In Vitro* Investigation

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Abstract

Background: Despite the more recent advances, there is still no effective systemic therapy for Uveal Melanoma (UM). However, a better understanding of the role of oxidative stress in cancer, has more recently led to a completely new approach to the systemic therapy of cancer, and modulators of the oxidative balance, such as sodium ascorbate (ASC) or arsenic trioxide (ATO), have already entered advanced phases of preclinical and clinical development.

Since high doses of ASC have already demonstrated a strong cytotoxic effect on different human cancer cell lines, we have undertaken the present investigation in order to test the sensitivity of OCM1 and C918 uveal melanoma (UM) cell lines to high doses of ASC, *in vitro*, as compared to ATO, a pro-oxidant drug which has already undergone extensive *in vitro* and pre-clinical investigation in UM.

Methods: Both OCM1 and C918 UM cell lines have been exposed to increasing doses of either ASC or ATO, to build a dose-response curve around the peak plasma concentrations reached by both chemicals. The assessment of cell count and viability was performed with flow cytometry.

Results: Both OCM1 and C918 UM cell lines are highly sensitive to ASC in the range of millimolar (mM) concentrations which can be usually reached by the intravenous injection of high doses of the compound. ATO at the dosages used in this study, never reached the LC₅₀. When the exposure to ASC was reduced to two hours, it still had significant effects on survival of both OCM1 and C918 UM cells.

Conclusions: This report shows that ASC is highly cytotoxic for both OCM1 and C918 UM cells, when used in high, pro-oxidant doses. To our knowledge, this is the first report showing that UM cells can be efficiently killed, *in vitro*, with high doses of ASC.

Keywords: Uveal melanoma; OCM1 cell lines; C918 cell lines; Ascorbate; Vitamin C; Megadoses of ascorbate

Abbreviations: UM: Uveal Melanoma; ASC: Sodium Ascorbate; ATO: Arsenic Trioxide; LC₅₀: "Lethal" concentration which kills 50% of the cells *in vitro*; ROS: Reactive Oxygen Species

Introduction

"Uvea" is a portion of the eye, encompassing the iris, the ciliary body and the choroid, which, because of its content in melanocytes, is at risk for the development of "uveal" melanoma (UM).

UM is a malignant cancer of the uvea, affecting around 4.3-5 individuals per million, in the United States, the vast majority of which are Caucasian males [1].

Despite its paucity, UM is the most common primary tumor of the eye in adults, with the choroid as the most commonly involved anatomical structure [2].

Up to 50% of patients develop metastatic disease in a time lapse variable from 5 to 15 years after diagnosis, with preferential involvement of the liver, and about 90% of them will ultimately succumb to metastatic spread in less than three months [3-8].

Until the late eighties the only treatment available for UM was enucleation of the affected eye, though brachytherapy, thermotherapy and radiation therapy can be used to treat small/medium size tumors with preservation of the eye [9].

Several different chemotherapeutic agents have been used, such as Dacarbazine, alone or in combination with Interferon- alpha 2b, after

primary treatment, to patients at high risk of developing metastatic disease, but they have not improved the outcome of these patients [10].

Globally, despite the improvements in the treatment of primary tumors and although very rarely patients show detectable metastasis at presentation, still half of the patients die of metastatic disease [11].

Regarding the mechanisms of malignant transformation, in UM, emphasis has been given to genetic changes, but, although both cytogenetics and genetics have enhanced prognosis, allowing the distinction of tumors with higher metastatic potential, metastatic disease remains the leading cause of death among patients with UM [12].

In recent years, Reactive Oxygen Species (ROS), the key mediators in oxidative stress, have emerged as promising targets for anticancer drug discovery and extensive research has documented a causative

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Received October 16, 2013; **Accepted** December 13, 2013; **Published** December 21, 2013

Citation: Mastrangelo D, Massai L, Valyi-Nagy K, Muscettola M, Aglianò M, et al. (2013) Megadoses of Ascorbate as a New Chemotherapeutic Approach in Uveal Melanoma: A Preliminary *In Vitro* Investigation. J Clin Exp Ophthalmol 4: 315. doi: 10.4172/2155-9570.1000315

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involvement of alterations of the redox (reduction/oxidation) balance in tumor progression, particularly for oncological indications with little treatment options, including metastatic melanoma [13-17].

In this perspective, arsenic trioxide (ATO) (chemical formula As_2O_3), a pro-oxidant drug, introduced a few years ago in the treatment of acute promyelocytic leukemia [18], has undergone clinical testing in the treatment of metastatic melanoma [19,20] with some results, particularly in *in vitro* studies [21,22].

More importantly, sodium ascorbate (ASC) delivered at very high doses that raise plasma concentrations to millimolar (mM) levels, not achievable by oral administration, has shown significant anticancer effects *in vitro*, due to its pro-oxidant activity, with increased production of intracellular H_2O_2 , and consequent oxidative damage [23-25].

Given all the above, we have undertaken the present *in vitro* investigation to test the effectiveness of both ATO and high doses of ASC in killing UM cells.

Materials and Methods

Cells, reagents, and equipment

OCM1 and C918 UM cell lines used in this study (Figure 1) originate from primary uveal melanomas and have undergone extensive authentication including genetic analysis as described elsewhere [26].

All reagents, including culture media, sodium ascorbate (ASC), calcium chloride, sodium citrate, sodium phosphate, sodium chloride, potassium chloride, and Trypan Blue, were purchased from Sigma-Aldrich (St. Louis MO).

ATO (Trisenox™) was kindly supplied by the dept. of Hematology of the hospital of Pescara (Italy). Automated cell count and viability was performed by using the “Muse”™ (Merck-Millipore) automated cell analyzer.

OCM1 and C918 human UM cells were cultured at 37°C in a humidified 5% $CO_2/95\%$ air in RPMI 1640 supplemented with 15% heat inactivated fetal bovine serum (FBS) and penicillin (100 UI/ml)-streptomycin (100 μ g/ml), as a monolayer in 25 cm^2 flasks.

For count and viability, the cells were detached/disaggregated by preparing a 10× solution containing 1.35 M potassium chloride, and 0.15 M sodium citrate dehydrate (citrate saline). This stock solution was diluted to 1× just before use [27]. To detach UM cells growing in monolayer from the bottom of flasks, they were incubated for 5 minutes with citric saline solution at 37°C in 5% $CO_2/95\%$ air, and then mechanically detached by gently tapping the flasks, as for the trypsinization procedure. An equal volume of PBS 2× was then added,

and the resulting cell suspension, centrifuged for 5 minutes at $500 \times g$. After a second PBS 1× washing and centrifugation step, the pellet was re-suspended in fresh culture medium, at the concentration of about $3-4 \times 10^5/ml$, before exposure to ASC or ATO.

Cell count and viability

Cell counting, before and after exposure to increasing doses of either ASC or ATO, was performed with the automated (“Muse”™) method according to the instructions supplied by the manufacturer, which encompass an in house method of nuclear staining for the assessment of cell viability.

Experimental protocol

During the phase of exponential growth, the cells, detached and collected as described, were counted with Trypan Blue, diluted to a concentration of about $3-4 \times 10^5/ml$, and plated, in flat bottom twelve-well plates, at a volume of 1 ml ($3-4 \times 10^5$ cells) per well.

Four dilutions of 2, 4, 6, and 8 μ g/ml of ATO were used, starting from a stock solution of ATO commercially available at a concentration of 1 mg/ml (Trisenox™), by adding 2, 4, 6, and 8 μ l of Trisenox™, respectively, to the four different wells, each containing 1 ml ($3-4 \times 10^5$ OCM1 or C918 cells) of cell suspension. The concentrations of ATO were chosen according to the data reported by Yediou and coll. in acute promyelocytic leukemia cell lines (HL60) [28]. ATO can be stored at room temperature for up to 36 months.

A 1 M solution of ASC was prepared, fresh each time, as described elsewhere [29], and aliquots of 1, 3, 5, and 7 μ l of this stock solution were added to the four wells, each containing 1 ml ($3-4 \times 10^5$ OCM1 or C918 cells) of cell suspension, to a final concentration of 1, 3, 5, and 7 mM, respectively.

Both OCM1 and C918 cells were exposed for 18-24 hours. One control (no treatment) sample was also included in each experiment. At the end of the incubation period the cells were collected in vials and mixed with the Muse™ Count & Viability Reagent, according to the procedure supplied by the manufacturer, for automated counting and viability analysis. Namely, 10 μ l of each sample were added to 190 μ l of the Muse™ Count & Viability Reagent, which differentially stains viable and non-viable cells based on their permeability to the two DNA binding dyes present in the reagent. A specific software module then performs calculations automatically and displays data in two different types of dot plots, one of which is reported in Figure 2.

Each count and viability test was repeated at least twice, and a total of 96 tests were carried out, with the automated cell counter (“Muse”™), with and without (control sample) scalar doses of ATO and ASC, as reported.

A second group of tests was also performed, but this time by exposing both OCM1 and C918 cell lines to both ASC and ATO, for only two hours. Moreover, in order to explore potential synergisms between both ASC and ATO, part of these tests was carried out by mixing the lowest concentrations of both ATO (2 and 4 μ g/ml) and ASC (1, 3, and 5 mM/ml).

After exposure, the cells were washed twice with PBS 1×, re-suspended in fresh medium, and further incubated for 18-24 hours. Even in this case, each count and viability test was repeated at least twice and a total of 72 tests were performed with the automated cell counter (“Muse”™).

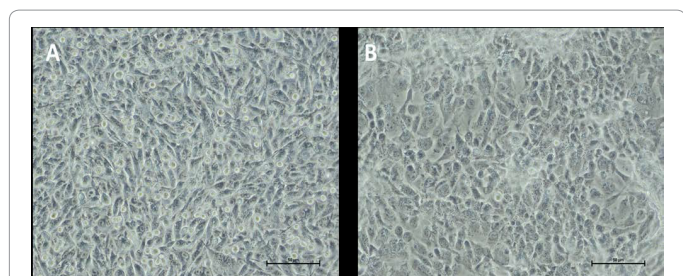


Figure 1: Phase-contrast photomicrograph of OCM1 (A) and C918 (B) UM cell lines, growing as a monolayer in 25 cm^2 flasks (see text). Magnification: 200×. Details of cell morphology showing a larger mean size with prevalence of spherical elements in C918 and spindle-like elements in OCM1.

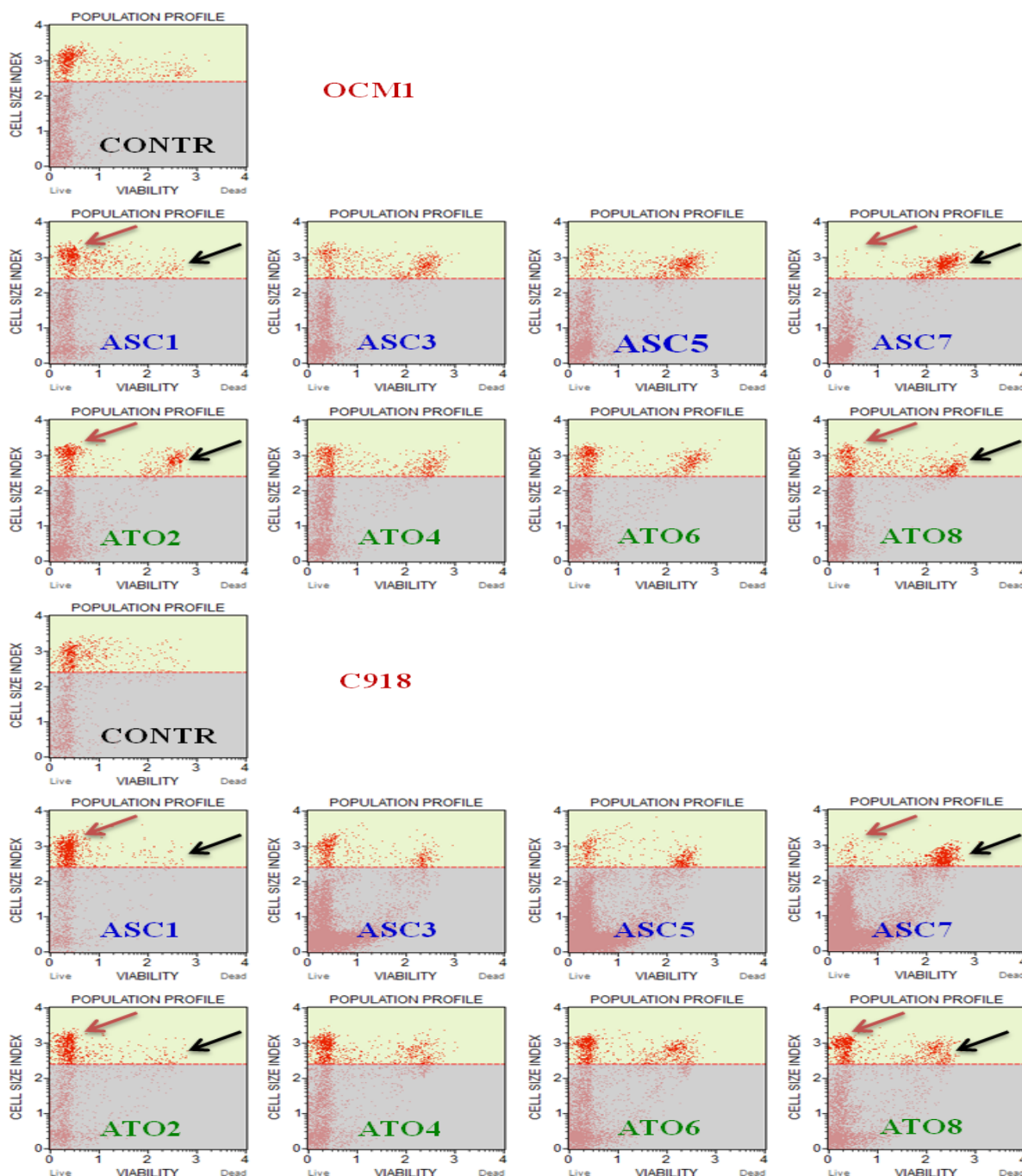


Figure 2: Typical plot obtained with the Muse™ automated cell counter/analyzer (Merk - Millipore), by analyzing OCM1 and C918 UM cells after exposure to ASC 1, 3, 5, and 7 mM/ml, and ATO, 2, 4, 6, and 8 µg/ml. The red and black arrows in the plots indicate live and dead cells respectively. As shown in diagrams, a “cloud shift” can be appreciated, going from ASC1 (1 mM ASC) to ASC7 (7 mM ASC), for both OCM1 and C918 cell lines, thus indicating a progressive decrease of cell survival by increasing the concentration of ASC. The same does not apply to ATO.

Statistical analysis

The statistical analysis was performed, for each independent sample (Table 1), by comparing the proportion of viable cells at any given dosage (of either ASC or ATO), with the effect of the chosen dose on cell viability (response). The Chi Square (ChiSq) statistics with three degrees of freedom (DF) was used to summarize the information for each experiment at a suitable scale level of cell count.

Results

After exposure to increasing concentrations of ASC (1, 3, 5, and 7 mM), both OCM1 and C918 UM cell lines showed a consistent decrease in cell viability, with an inverse relationship between the concentration of ASC (in mM/ml) and the number of live cells in culture. The same did not apply to ATO in which, after an initial decrease of cell viability, at the concentration of 2 µg/ml, the percentage of live cells in culture

did not vary significantly by increasing the concentrations of the drug up to 8 µg/ml. These data are exemplified in the diagrams of Figure 2, which shows the distribution of live/dead cells in a representative sample analyzed by flow cytometry, and in Table 1, reporting the percentages of live cells after exposure to any given concentration of either ASC or ATO.

As shown in Table 2, when the number of viable cells in each independent sample, at any given dosage of either ASC or ATO, were

OCM1	TOT (E+05)	Viability%	C918	TOT (E+05)	Viability%
Run 1			Run1		
Control	1.98	75.74	Control	1.74	76.92
ASC3	1.92	38.95	ASC3	1.68	61.06
ASC5	1.93	20.81	ASC5	1.62	41.97
ASC7	1.91	3.66	ASC7	2.32	11.91
ATO2	2.28	61.10	ATO2	2.27	60.17
ATO4	2.03	54.10	ATO4	2.70	54.10
ATO6	2.23	52.70	ATO6	3.61	53.00
ATO8	2.25	46.00	ATO8	3.96	55.70
Run 2			Run2		
Control	2.07	77.51	Control	1.85	93.19
ASC1	2.17	67.74	ASC1	1.66	39.02
ASC3	2.24	12.86	ASC3	1.85	9.50
ASC5	2.41	4.76	ASC5	2.29	2.56
ASC7	2.11	2.78	ASC7	1.91	2.49
ATO2	2.01	61.00	ATO2	1.59	71.05
ATO4	2.62	65.46	ATO4	1.71	61.80
ATO6	2.70	64.01	ATO6	2.13	61.18
ATO8	3.18	65.50	ATO8	1.96	64.39
Run 3			Run 3		
Control	1.78	91.97	Control	1.48	89.62
ASC1	1.44	92.04	ASC1	1.40	93.59
ASC3	1.30	70.17	ASC3	.995	21.35
ASC5	1.15	38.93	ASC5	1.47	4.19
ASC7	1.35	18.84	ASC7	1.53	2.37
ATO2	1.45	69.69	ATO2	1.13	73.27
ATO4	1.51	61.11	ATO4	1.51	57.04
ATO6	1.58	62.06	ATO6	1.56	56.91
ATO8	1.63	65.01	ATO8	1.60	53.77

Table 1: Cell viability of OCM1 and C918 UM cell lines after exposure to increasing doses of both ASC (sodium ascorbate) and ATO (arsenic trioxide). ASC 1, 3, 5, and 7 correspond to concentrations of 1, 3, 5, and 7 mM/ml of ASC. ATO 2, 4, 6, and 8, correspond to doses of 2, 4, 6, and 8 µg/ml of ATO. Run 1, 2, and 3=experiment no. 1, 2, and 3. TOT (E+05)=total number of cells × 100,000. Viability%=percentage of live cells. Control=untreated samples.

	OCM1-1		OCM1-2		OCM1-3	
	ChiSq (*)	p	ChiSq (*)	p	ChiSq (*)	p
ASC	226.33	<<0.01	367.05	<<0.01	175.84	<<0.01
ATO	9.97	>0.01	1,31	>0.50	2,89	>0.25
	C918-1		C918-2		C918-3	
	ChiSq	p	ChiSq	p	ChiSq	p
ASC	244.09	<<0.01	150.91	<<0.01	375.10	<<0.01
ATO	3,13	>0.25	4,53	>0.10	11,85	<0.01

Table 2: Statistical analysis of the data reported in Table 1. ASC treatment shows cytotoxic effects, across the different dose levels, which are extremely significant, compared to ATO, in all runs (experiments). This means highly significant dose-effect relationship of ASC treatment far superior to ATO in both OCM1 and C918 UM cell lines processed.

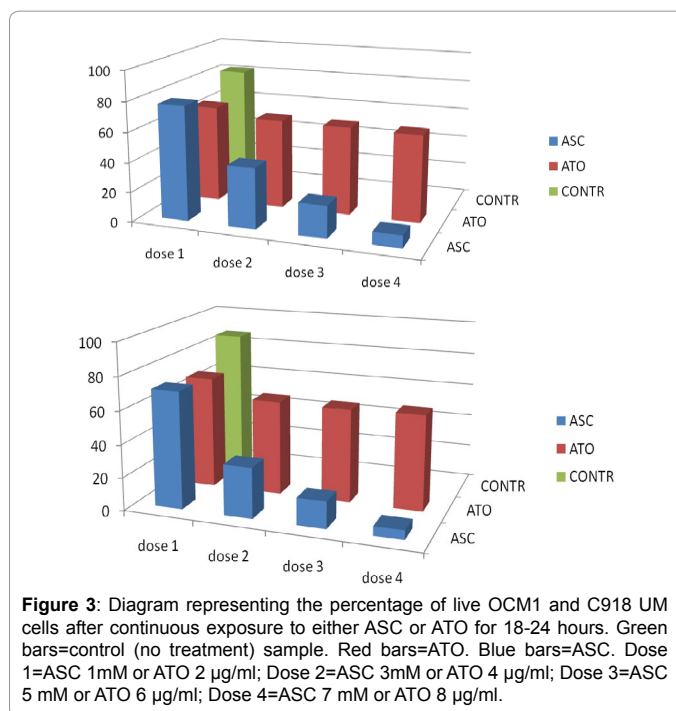


Figure 3: Diagram representing the percentage of live OCM1 and C918 UM cells after continuous exposure to either ASC or ATO for 18-24 hours. Green bars=control (no treatment) sample. Red bars=ATO. Blue bars=ASC. Dose 1=ASC 1mM or ATO 2 µg/ml; Dose 2=ASC 3mM or ATO 4 µg/ml; Dose 3=ASC 5 mM or ATO 6 µg/ml; Dose 4=ASC 7 mM or ATO 8 µg/ml.

compared with the effect of that dose on cell viability, a high statistically significant ($p < 0.01$) dose-response relationship could be found for ASC in all (6/6) experiments, while this was true only in two out of six experiments performed with ATO. This was a clear indication that at the chosen dosages, ASC killed a significantly higher number of UM cells than ATO, *in vitro*, and that this (cytotoxic) effect was strictly proportional to the dose of ASC (but not of ATO) employed.

To further confirm the statistical analysis, the data reported in Table 1 clearly show that while ATO never reaches the LC_{50} , ASC reaches its LC_{50} at around 3 mM/ml, i.e. less than a half of the highest concentration (7 mM) used in this experiment.

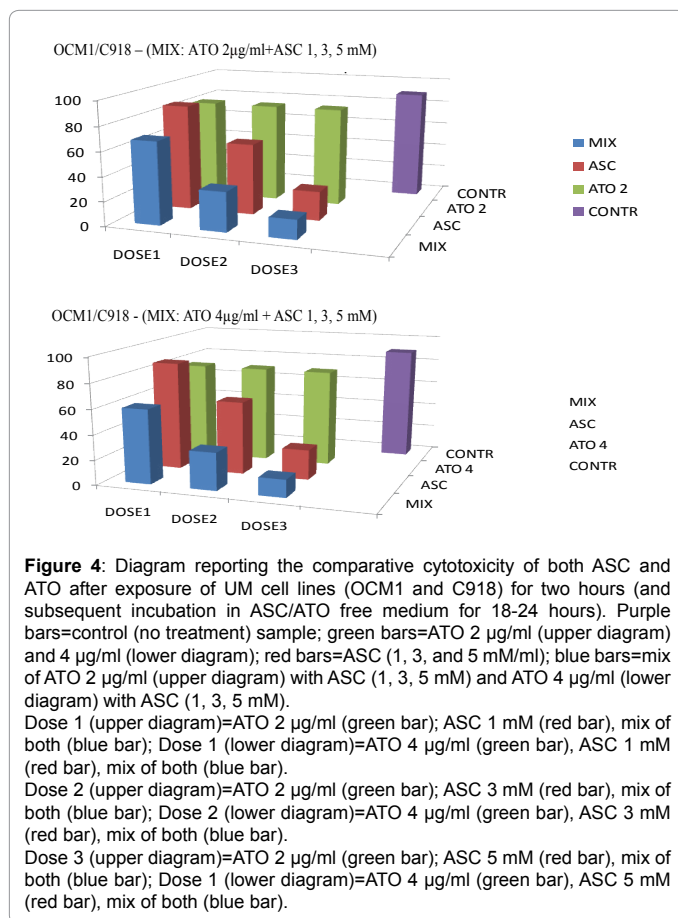
When the responses of both OCM1 and C918 to ASC and ATO were compared (Figure 3), a rather similar pattern was observed, with both cell lines; i.e.: ATO showed some effects on cell viability at the dosage of 2 and 4 µg/ml, but no effect at all at higher doses, while ASC produced a constant and progressive decrease in cell survival at any of the chosen concentrations.

By reducing the exposure of both UM cell lines to two hours, an evident decrease in the effectiveness of both ATO and ASC could be appreciated, even if ASC still killed a considerably higher number of UM cells than ATO. This led us to conclude that ASC is rapidly internalized, after exposure, and remains inside the cells even after washing it out from the culture medium.

More importantly, when the lowest doses of ATO and ASC are mixed together, they kill significantly more UM cells than if taken alone, thus indicating a clear cytotoxic synergism of both ATO and ASC, when administered together to either OCM1 or C918 UM cells (Figure 4).

Discussion

The levels of ROS, and particularly of H_2O_2 are tightly controlled under physiological conditions, to maintain redox homeostasis in the melanosome and regulate the melanogenesis process [30].



The human melanocyte is continually exposed to ROS, and the oxidative balance is maintained by intracellular antioxidants, but this control is usually lost during melanoma development [16] and the disruption of this balance seems to be an early event in the etiology of melanoma, leading to increased oxidative stress and mutation [31].

Epidemiological investigations implicate UVB in non-melanoma and UVA in melanoma skin cancer, and while UVB are known to mediate their action through photoproducts, such as pyrimidine dimers, it is well known that UVA acts mainly through the induction of ROS [28] and unbalance of the cellular redox steady state.

On the other hand, redox deregulation due to metabolic alterations, and the role of ROS in signal transduction, represent a specific vulnerability of malignant cells, which can be selectively targeted by redox chemotherapeutics [18,32] aimed at either increasing the levels of intracellular ROS [32] or decreasing the amount of intracellular antioxidants in cancer cells [18,33].

Among the candidate molecules in redox directed cancer chemotherapeutics, ATO (As_2O_3) and high doses of ASC have more recently received the attention of the scientific community [18].

ATO is studied as an anticancer agent whose use originated in traditional Chinese Medicine. It is an established cell growth inhibitor and apoptosis inducer [34,35] and, in patients with Acute Promyelocytic Leukemia (APL) it has been variously defined as the “most effective single agent” [36], “the most biologically active single drug” [37], and more recently, “a drug from a poison” to be used not only in the treatment of this leukemia, but also in other types of cancer [38], for its efficacy, cost effectiveness, and low toxicity [39,40].

As reported by different authors [18,41-43] oxidative stress is critical to the ATO-induced apoptotic process, with generation of ROS, including, among others, H_2O_2 , which is considered a metabolite of the drug [44].

Interestingly, ATO shares this pro-oxidant activity with a number of other compounds [18], including ASC when administered in high doses by intravenous injection; at these “pharmacological” concentrations, ASC is a powerful pro-oxidant and is considered a pro-drug of H_2O_2 [23,25,45-47].

In the experiments reported herein, ASC is significantly more efficient than ATO in killing both OCM1 and C918 UM cells *in vitro*, when used at doses commonly employed in clinical settings.

Although ASC has shown its selective cytotoxicity for different cancer cell types [23-25] and ATO has been already used to treat UM cell lines *in vitro* [21,22], this is, to our knowledge, the first report ever, concerning the effects of ASC alone or in combination with ATO on UM cells lines.

In this study, ASC has been shown significantly more efficient than ATO in killing UM cells, with an LC_{50} of less than 3 mM/ml, a plasma concentration which can be easily reached by intravenous administration of this vitamin.

Interestingly, by reducing the exposure of UM cell lines to two hours, we have been able to see the same effects obtained after 18-24 of continuous exposure of UM cells to ASC, thus indicating that ASC is captured by UM cells, as also reported in the literature on this subject [18].

Regarding the comparison between the doses of ASC (in mM/ml) and ATO (in µg/ml), it may be argued that is not appropriate, given the difference in the dosage used, but this is not the case since the doses of both compounds used herein, are those commonly employed for routine therapeutic purposes [28]. With specific reference to ASC, it should be emphasized that in a recent clinical trial on metastatic pancreatic cancer, ASC has been administered by intravenous injection in doses ranging from 50 to 100 grams per infusion, three days a week for eight weeks, without major side effects, and plasma concentrations of up to 30 mM/ml [48], i.e. more than fourfold the maximal dose (7 mM) of ASC used in the present investigation.

More importantly, when used in combination, both ASC and ATO show a clear synergism of action as illustrated in the diagrams of Figure 4, showing the cytotoxic effects of ATO and ASC alone and in comparison with the combination of both (“mix”).

When considering that:

1. ASC in high intravenous doses is a powerful pro-oxidant with cytotoxic effects on a number of different human tumor cell lines [18,23-25] (including retinoblastoma[28], and promyelocytic leukemia [49]) and no harmful effects on normal cells [23-25,47];
2. When administered simultaneously, both ASC and ATO show a marked synergistic cytotoxic effect on UM cell lines such as OCM1 and C918;
3. Both ASC and ATO are currently commercially available for intravenous administration;
4. There is no effective chemotherapy for UM;

It should be concluded that, although more experimental studies

will be necessary to confirm our data, ASC in high doses alone or in combination with ATO at conventional doses, has the potential to kill UM cells and should, therefore, rapidly gain the rank of new candidate chemotherapeutic agents in the pharmacological treatment of UM.

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