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Megadoses of Sodium Ascorbate Efficiently Kill HL60 Cells *in Vitro*: Comparison with Arsenic Trioxide

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ABSTRACT

Arsenic Trioxide (ATO) is widely acknowledged as the treatment of choice for Acute Promyelocytic Leukemia (APL). It is a "two-sided" drug since it can induce differentiation or kill APL and other tumor cells according to the dosage. Part of the cytotoxic effects of ATO on APL cells is due to its pro-oxidant activity, a characteristic which ATO shares with a number of other compounds, including high doses of ascorbate (ASC). In a comparative investigation on the cytotoxic effects of both ATO and ASC on HL60 (APL) cell lines, *in vitro*, we have been able to confirm the known cytotoxic effects of ATO, but, more importantly, we have demonstrated that ASC is significantly more effective than ATO, in killing these cancer cells *in vitro*, when the concentrations are maintained within the millimolar (mM) range, *i.e.* the range of plasma concentrations at which ASC induces oxidative damage to tumor cells. Since these plasma levels can be reached only by the intravenous administration of high doses of ASC, we propose that intravenous high doses of ASC may represent a potentially revolutionary new approach in the management of APL.

Keywords: HL60; Acute Promyelocytic Leukemia; High Doses of Ascorbate; Cell Count and Viability Assays

1. Introduction

Acute Promyelocytic Leukemia (APL) is a rare, though extremely malignant subtype of acute myeloid leukemia (AML), which, only a few decades ago, Hillestad described as characterized by "a very rapid fatal course of only a few week's duration" with a white blood cell (WBC) picture dominated by promyelocytes and a severe bleeding tendency [1,2].

The use of all-trans retinoic acid (ATRA) as a differentiating agent is well established, in the treatment of APL [3], but in the last two decades or more, Arsenic Trioxide (ATO), initially proposed by Chinese authors as the first line treatment of APL [4], has been largely acknowledged as the "most effective single agent" [5], "the most biologically active single drug" [6], 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 [7], for its efficacy, cost effectiveness, and low toxicity [8,9].

Studies on HL60 (human promyelocytic leukemia) cell line, have shown that ATO can be viewed as a two-sided drug, inducing either differentiation or apoptosis on these cell lines [10], according to the dosage employed. These data have been confirmed by studies on retinoblastoma cell lines, showing that in low doses (≤1 μM) it induces differentiation, while in high doses (≥2 μM) it behaves as a pro-apoptotic drug and effectively inhibit tumor formation *in vitro* [11].

As reported by different Authors [12-14], 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 [15]. Interestingly, ATO shares this pro-oxidant activity with a number of other compounds, including sodium ascorbate (ASC) administered in high doses by intravenous injection [16-20].

Given the above mentioned similarities underlying the anticancer activity of ATO and ASC, and the high cytotoxic potential shown by ASC in different cancer cell lines *in vitro* [16-21], we have undertaken the present investigation in order to assess the cytotoxic effect of ASC on HL60 cells *in vitro* in comparison with ATO, which is the drug of choice in the treatment of APL.

2. Materials and Methods

HL60 (Human Promyelocytic Leukemia) cell lines were kindly supplied by the Department of Molecular and Developmental Medicine of the University of Siena. All reagents, including culture media, Sodium Ascorbate (ASC), Trypan Blue, Hoechst 33342, and Propidium Iodide (PI), were purchased from Sigma-Aldrich. Arsenic trioxide (ATO chemical formula: As_2O_3) vials (Trisenox) were kindly supplied by the Department of Haematology of the Pescara main hospital (Pescara, Italy).

Automated cell count and viability was performed by using the "Muse"™ (Merck-Millipore) automated cell analyzer. A Zeiss Axioplan2 microscope was used for fluorescence microscopy and a Shandon cytocentrifuge for microscopic/morphologic analysis of cell suspensions.

Cell counting, before and after exposure to increasing doses of either ATO and ASC was performed with both the manual (Trypan Blue Exclusion Test) and automated ("Muse"™) methods. The automated method, using the "Muse"™, was carried out according to the instructions supplied by the manufacturer which encompass an in house method of nuclear staining for the assessment of cell viability. The Trypan Blue Exclusion Test was performed according to the standard procedures [22,23]. At the end of each count and viability test HL60 cell suspensions were stained with Hoechst/PI as described elsewhere [24] and aliquots of about 1×10^5 cells were deposited onto alcohol-washed glass microscope slides by using a cytocentrifuge, at 1000 r.p.m. for 5 minutes, and then observed under fluorescence microscopy.

HL60 human promyelocytic leukemia cells were grown in RPMI supplemented with antibiotics, glutamine, and 20% FBS, at 37˚C in 5% CO_2/95% air. During the phase of exponential growth, the cells were harvested and counted with the "Muse"™ automated cell counter/analyzer and then diluted to a concentration of about $(2 - 5) \times 10^5$/ml.

Four dilutions of 2, 4, 6, and 8 μg/ml of ATO were used, starting from a stock solution of ATO commercially available at a concentration of 1mg/ml (Trisnox™), by simply adding 2, 4, 6, and 8 μl, respectively, of Trisenox™, to 1 ml of culture medium containing $(2 - 5) \times 10^5$ HL60 cells. These concentrations were chosen according to the data reported by Yediou and coll. [10].

A 1 M solution of ASC was prepared fresh each time as described elsewhere [21], and aliquots of 1, 3, 5, and 7 μl of this stock solution were added to four different wells containing 1ml each of culture medium in which $(2 - 5) \times 10^5$ HL60 cells were suspended, to a final concentration of 1, 3, 5, and 7 mM, respectively. ATO can be stored at room temperature for up to 36 months.

Aliquots of 1 ml of culture medium containing $(2 - 5) \times 10^5$ HL60 cells were loaded into each of 12 well plate, and added with the four different concentrations of ATO (2, 4, 6, and 8 μg/ml); the same procedure was used for ASC (1, 3, 5, and 7 mM). The cells were exposed for a total of 18 - 24 hours. One control (no treatment) sample was also included. 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 μl of each sample were added to 190 μl 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 dot plots as shown in **Figure 1**.

Each experiment was repeated at least twice, and a total of six experiments was carried out, with the automated ("Muse"™) cell counting and viability tests, with and without (control sample) increasing doses of ATO and ASC, as reported. The results have been cumulatively analyzed by calculating the mean, standard error, standard deviation, and 95% confidence intervals (95% CI) of the percentages of living cells for each experiment. The statistics was performed by calculating the two-tailed "p" value of the difference between the mean percentage of viable cells in each of the four doses of ASC (1, 3, 5, and 7 mM) as compared to ATO (2, 4, 6, and 8 μg/ml).

Aliquots of cells were also stained with Hoechst/PI, according to the standard protocols, deposited onto glass microscope slides with a cytocentrifuge, and finally observed under fluorescence microscopy [25]. For statistical analysis, the mean values and related 95% confidence intervals were calculated for each set of repeated measurement by using the SPSS statistical package, version 10.

3. Results

The results of this experiment are reported in **Table 1** and summarized in the diagram of **Figure 2**. As shown in the graph, the mean percentages of live HL60 cells (the "y" axis) proportionally decrease by increasing the concentrations of both ASC and ATO (the "x" axis). However, at any given concentration, ASC always kills more HL60 cells than ATO. The two tailed "p" value of the difference in the percentage of live cells between ASC and ATO suggests that ASC 1, and 3 mM is more effective than ATO 2, and 4 μg/ml, respectively, in reducing the survival of HL60 cells in culture even though the difference does not appear statistically significant. However, at 5 mM, ASC is significantly more effective ($p = 0.0038$) than ATO (6 μg/ml) and the difference becomes highly statistically significant ($p = 0.0001$) in favor of

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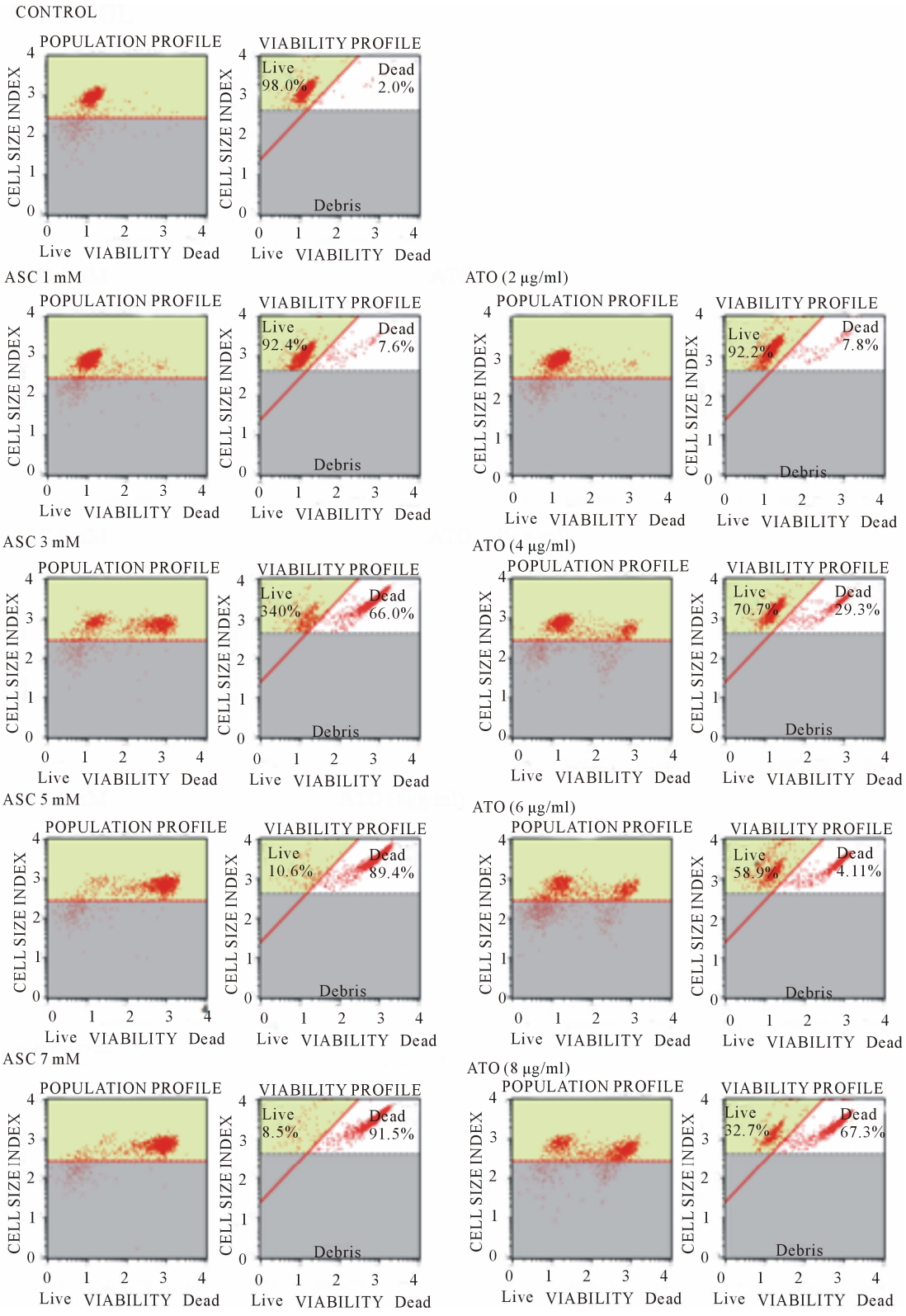

Figure 1. A representative sample of the cell viability profile obtained by treating HL60 cells with ASC (left column of diagrams) and ATO (right column) according to the "MUSE"™ cell analyzer. The upper left set of diagrams is referred to the control (untreated) sample. As shown in the diagram, both ASC and ATO efficiently kill HL60 cells *in vitro*, but, at any given concentration, ASC always kills more cells than ATO, and this difference is statistically significant in favor of ASC, particularly at 5 and 7 mM (dose 3 and 4).

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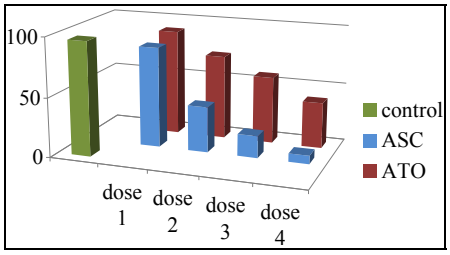

Figure 2. Comparison of the mean percentages of viable HL60 cells ("y" axis) after treatment with ASC (blue bars) and ATO (red bars). The green bar indicates the untreated (control) cells. The statistical analysis of these data reveals that at "dose 3" (ASC 5 mM and ATO 6 μg/ml) and "dose 4" (ASC 7 mM and ATO 8 μg/ml) ASC is significantly more efficient than ATO in killing HL60 cells *in vitro*.

Table 1. Mean percentages of viable cells in the untreated (control) and treated (ASC and ATO) HL60 cells, at different dosages, in six consecutive experiments.

EXP.N.	Control (% vital)	DOSE	ASC (% vital)	ATO (% vital)
1	96.5	1	91.9	93.2
		2	61.5	74.8
		3	19.9	59.7
		4	15.4	44.1
2	98	1	92.4	92.2
		2	34	70.7
		3	10.6	58.9
		4	8.5	32.7
3	97.4	1	89.3	73.2
		2	48.3	28.5
		3	30.6	21.7
		4	2.2	21.9
4	97.2	1	82	93.1
		2	13	81.8
		3	4.3	62.5
		4	2.2	45.4
5	97.2	1	84.3	95.2
		2	59.3	86.5
		3	34.4	69.8
		4	8.6	39.2
6	91.2	1	70.3	94.3
		2	15.5	86.8
		3	11.7	65.8
		4	5.8	50.1

ASC when ASC 7 mM is compared to ATO 8 μg/ml. The LC_{50} for ATO is above 6 μg/ml, as reported by Yediou and coll.10, while for ASC it is in between 3 and 5 mM, as reported by different Authors [16-21].

The Trypan Blue analysis, even if not informative regarding the apoptotic condition of treated HL60 cells, confirmed the data obtained with "Muse"™.

Regarding the Hoechst/PI staining, as shown in **Figure 3** the cellular changes induced by ATO seems to be substantially different from those obtained with the exposure to millimolar (mM) doses of ASC. Namely, while ATO, at the maximum dosage tested herein, clearly shows signs of apoptotic cell damage, encompassing, nuclear fragmentation and chromatin condensation, ASC treated HL60 cells are clearly necrotic, as shown by the red color

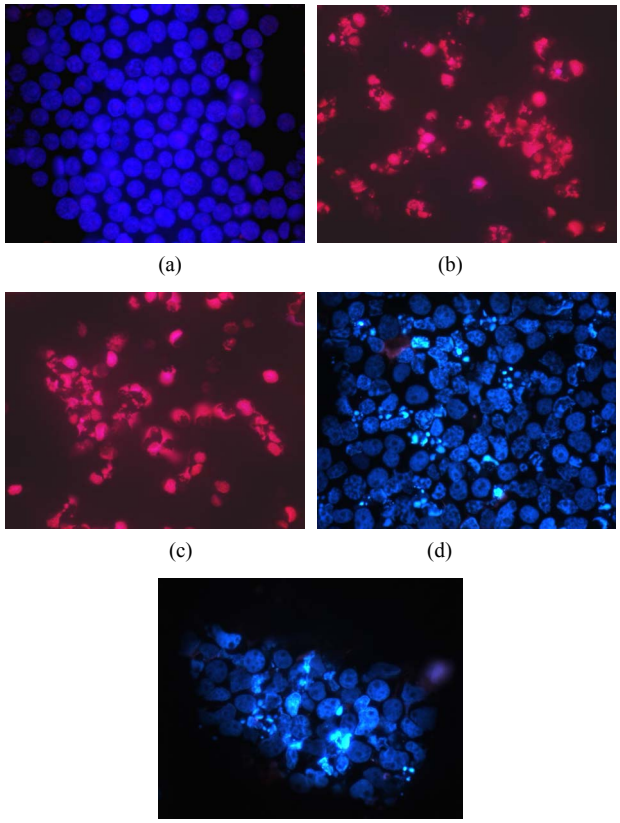

Figure 3. Hoechst/PI fluorescent microscopy staining of untreated (controls) and treated (ASC and ATO) HL60 cells. 400× magnification. Untreated (control) cells (a) appear uniformly blue with nuclei showing a loose chromatin, and no signs of necrosis or pyknosis. This feature is compatible with a well preserved viability, as also confirmed by the "MUSE"™. The same cells, treated with 3 and 5 mM ASC ((b) and (c) respectively), appear uniformly stained in red (PI) thus indicating an advanced necrotic condition. On the contrary, HL60 cells treated with ATO at 4 and 6 μg/ml ((d) and (e) respectively), are almost uniformly stained in blue, but the condensed chromatin and nuclear fragmentation are indicative of early/late stage apoptotic condition.

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of the nuclei which were stained by PI, but not by Heochst 33342. These results confirm our previous observation of the effects of mM doses of ASC on Y79 retinoblastoma cells *in vitro* [21].

4. Discussion

Historically, four periods have been identified, in the treatment of APL, which have marked a progressive improvement in the cure of this disease, namely: 1) chemotherapy (CT) alone (with antracyclines and Ara-C), from 1957 to 1985; 2) all-trans retinoic acid (ATRA), introduced in 1985; 3) Arsenic Trioxide (As_2O_3-ATO) introduced in the mid 1990s; 4) Combination of ATRA and ATO, in 1998. With all these achievements, APL has passed from a 5-year disease-free survival (5yDFS) of about 35% - 45%, to a 6 year disease-free survival (6yDFS) of 86% (±10%) [26].

The role of ROS, H_2O_2, redox potential, and oxidative stress in the pro-apoptotic, anticancer activity of ATO, further illustrated in detail by de Thé & coll. [27], Selvaraj & coll. [28], Sanchez & coll. [29], among others, suggests that drugs with oxidizing potential may represent the new frontier in cancer treatment [30], especially if we consider that cancer cells have more commonly exhausted their antioxidant machinery [31] and therefore are more vulnerable to oxidizing compounds.

ASC (Vitamin C) was first isolated in 1928 by the Hungarian biochemist Dr. Szent-Gyorgyi who received the Nobel Prize for this discovery in 1937 [32].

The history of ASC as an anticancer molecule is very controversial [33]. McCormik, nearly 60 years ago, suggested that ASC protects against cancer by increasing collagen synthesis [34,35] while Ewan Cameron hypothesized that ASC could have anti-cancer effects by inhibiting hyaluronidase and thereby preventing cancer spread [36]. Although successful clinical trials had been reported, by Cameron and the twofold Nobel laureate Linus Pauling, on terminal cancer patients, Charles Moertel, at Mayo Clinic, reported negative results, and his trials were credited as the definitive proof of the inefficacy of ASC in treating cancer [36,37]. In Moertel's trial, however, ASC was given orally, while Cameron and Pauling had used both the intravenous and oral route of administration simultaneously. Report survey data on intravenous ASC show that ASC is used in doses up to 200 g per infusion, for a variety of pathological conditions, with very few adverse effects [38], and that, when taken orally, plasma ASC concentrations never raise beyond the level of 100 μmol/l, due to the limited bioavailability of the molecule and renal excretion [39]. On the contrary, when administered by intravenous infusion, ASC reaches plasmatic concentrations in the order of millimolar range, which would never be obtained by the administration of oral doses [18-20,40,41], and behaves as a very powerful oxidant, suitable for the therapeutic use in cancer [42].

The anticancer effects of ASC have been widely demonstrated in a number of different tumor cell lines [17-20], and phase I clinical trials have been already performed, in different cancers, showing that, even in very high doses, it is well tolerated [43] and has the potential to selectively kill cancer cells due to its capacity of producing intracellular H_2O_2, with oxidation of different cellular components, and cell death [17-20,31,40].

Although the use of ASC in the treatment of APL has been already proposed by both Yedjiou & coll. [10] and Bachleitner-Hofmann and coll. [44], in combination with ATO, ASC has never entered in the routine treatment of APL; this can be explained by the substantial difference in the dosage proposed by both Yedjiou and Bachleitner, when compared to our protocol. In fact, while it has been observed that adding ASC in the treatment of APL may slightly increase the cytotoxic potential of ATO, the dose of ASC recommended by both Authors, never overcomes 150 μM, which is within the range of plasma concentrations that can be reached by oral administration of ASC. More importantly, at plasma concentrations in the range of micromoles (μM), ASC behaves as an "antioxidant", and, as such, it has also been reported to protect HL60 cells from arsenic toxicity [45] and antagonize the toxic effects of chemotherapeutic agents [46]. On the contrary, at concentrations in the order of magnitude of the millimoles (mM), used in the present investigation, it clearly shows pro-oxidant effects, on tumor cells, which lead to an increased production of H_2O_2 and other ROS and consequent oxidative damage of different cell structures, as reported by several Authors [17-20,31,40].

The data reported herein substantially confirm the effectiveness of ATO in the treatment of APL, but add new relevant information, by showing that ASC, when used in mM concentration, as those that can be reached by intravenous injection of pharmacologic doses, is significantly more effective than ATO in killing HL60 cells *in vitro*.

The advantages of ASC, as compared to ATO, in the treatment of APL are clear:

1) In phase I clinical trials on metastatic pancreatic cancer [43], ASC has been administered at doses varying from 50 to 100 grams per infusion, three times a week for eight weeks, with plasma levels up to 30 mM and no major side effects and;

2) Due to its peculiar "metabolic" effect, ASC has been widely reported to selectively kill cancer cells, being substantially harmless for normal cells.

Given all the above, the Authors strongly believe that high ("pharmacologic") doses of ASC may represent a second revolution in the treatment of APL, and suggest to start a phase I-IV clinical investigation on ASC in the treatment of selected cases of APL, in order to verify its effectiveness in the treatment of this leukemia.

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