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# High Doses of Ascorbate Kill Y79 Retinoblastoma Cells *In vitro*

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## Abstract

**Objectives:** To tests the sensitivity of Y79 retinoblastoma cell lines to high doses of ascorbate, *in vitro*, and compare its effects with those of some chemotherapeutic agents routinely employed in the treatment of retinoblastoma.

**Methods:** Y79 retinoblastoma cells have been exposed to increasing doses of either sodium ascorbate (SA) or Melphalan (MEL), to define a dose-response curve around the peak plasma concentrations reached by both chemicals when administered according to the existing therapeutic procedures and protocols. The assessment of cell number and viability was performed, before and after exposure, with both the manual (Trypan Blue Exclusion Test) and automated (flow cytometry) methods. Fluorescence microscopy and direct observation of cells in culture, with inverted microscope, were also performed.

**Results:** Y79 cells are highly sensitive to the cytotoxic effect of SA, with cell viability reduced of over 90% in some experiments. As reported in the literature, this effect is directly cytotoxic and most probably mediated by acute oxidative stress on different cellular components. The same does not apply to Melphalan which, at the doses commonly used for therapeutic purposes, did not show any significant effect on cell viability, *in vitro*.

**Conclusion:** To our knowledge, this is the first report showing that high doses of SA can actively kill retinoblastoma cells *in vitro*. While it is not surprising for SA, to show direct cytotoxic effect on tumor cells, the data reported herein represent the first evidence in favor of the possible clinical use of high doses of intravenous SA, to treat children affected by retinoblastoma. Given the many advantages of SA over the chemotherapeutic agents commonly employed to treat cancer (including its almost total absence of toxic or side effects, and its exclusive specificity for cancer cells), it is reasonable to assume, from the data reported herein, that the high doses of intravenous ascorbate, have the potential to represent a real revolution in the treatment of retinoblastoma.

## Introduction

Retinoblastoma is a rare intraocular tumor affecting the retina of young children and infants [1]. Chemotherapeutic agents such as carboplatin and etoposide, have been shown to effectively reduce the volume of intraocular tumors in children affected by retinoblastoma [2,3]. However, the toxicity of systemic chemotherapy, still represents an issue which deserve further investigation [4,5], particularly when genomic instability is involved, as in the case of retinoblastoma [6,7].

In an effort to improve drug delivery to the tumor, and simultaneously reduce systemic toxicity, Superselective Ophthalmic Artery Infusion (SOAI) of chemotherapeutic agents [8], has been more recently developed and become a well established, though still controversial [9,10] treatment for more advanced retinoblastoma, leading to a dramatic increase in the preservation rate of affected eyes [11-15]. More specifically, the SOAI of Melphalan (SOAIM) has become one of the preferred therapeutic procedures in the local treatment of advanced retinoblastoma, given its low systemic toxicity and good tolerability [16-19], although SOAI can be used to deliver other chemotherapeutic and also non chemotherapeutic agents to the tumor.

However, even if the dose of Melphalan (MEL) used in each therapeutic procedure is relatively low, doses greater than 0.48 mg/kg, such as those given for bilateral tandem infusion, are still associated with an increased risk of neutropenia, and some authors suggest that SOAI combination chemotherapy be used rather than MEL alone [13,14,20].

Furthermore, the evidence behind the use of MEL in retinoblastoma, is limited to studies which tested only a few chemotherapeutic agents [21,22], using the clonogenic assay, which has been largely criticized, in the past [23,24] and almost completely abandoned nowadays, given its low reliability [25].

Moreover, there is sufficient evidence, in both human and experimental models, that Melphalan is carcinogenic and causes acute leukemia in humans [26,27] and this strongly argues against its use in children with retinoblastoma.

Interestingly, with the advent of Pharmacogenomics, it has become increasingly clear that drug therapy, including chemotherapy, should be tailored to the individual patient, in order to limit toxic effects and maximize the therapeutic ones [28-31].

Also and more importantly, the effectiveness of high intravenous doses of ascorbic acid (AA) or its sodium salt, sodium ascorbate (SA), in the treatment of cancer, has been recently demonstrated both *in vitro* and *in vivo*, and this has fueled new research in the field of cancer therapy, given the almost total lack of toxicity and the reported tumor

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specificity of SA, which, at plasma levels below the 20 mM, selectively kills cancer cells without affecting the normal ones [32-42].

Based on the above evidences, we have compared the effects of both MEL and SA, *in vitro*, on Y79 retinoblastoma cell lines by exposing tumor cells to increasing doses of both chemicals, chosen according to the peak plasma levels reported, respectively, for SOAIM and pharmacologic (high) doses of SA, administered by intravenous injection.

## Materials and Methods

### Cells, reagents, and equipments

Y79 human retinoblastoma cell lines were supplied by European Collection of Cell Cultures (ECACC). All reagents, including culture media, Sodium Ascorbate (SA), Melphalan (MEL), Trypan Blue, Hoechst 33342, and Propidium Iodide (PI), were purchased from Sigma-Aldrich. 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 morphologic analysis of cell suspensions.

### Cell count and viability

Cell counting, before and after exposure to increasing doses of either MEL or SA, 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 [43,44].

At the end of each counting and viability test, Y79 cell suspensions were stained with Hoechst/PI as described elsewhere [35,45], and aliquots of about $1 \times 10^4$ cells were deposited onto alcohol-washed glass microscope slides by using a cytocentrifuge, at 1,000 r.p.m. for 5 minutes, and then observed under fluorescence microscopy.

### Experimental protocol

Y79 human retinoblastoma cells were grown in RPMI supplemented with antibiotics, glutamine, and 10% FBS, at 37°C in 5% $CO_2$/95% air. During the phase of exponential growth, the cells were collected and counted with the "Muse"™ automated cell counter/analyzer and then diluted to a concentration of about $1 \times 10^6$/ml.

MEL is not or only partially soluble in water. According to Miller et al. [46], it must be prepared immediately before use, by dissolving it in acid alcohol (1 ml of 12 N HCl in 120 ml 95% ethan ol). Other authors suggest the use of both acid ethanol and propylene glycol, to store MEL as a stock solution at -70°C for several weeks [47]. For our purposes, we used the method proposed by Miller et al.. A 1mM stock solution of MEL, dissolved in acid ethanol and then diluted to the final concentration of 1mM with Phosphate Buffered Saline (PBS), was prepared and four aliquots, of 1, 3, 5, and 7 μl, respectively (corresponding to 1, 3, 5, and 7 μM in the final medium containing the cells), were added to each of the four wells of a twelve well plate containing 1 ml of medium with Y79 cells in the phase of exponential growth. Cells were exposed to MEL at 37°C in 5% $CO_2$/95% air, for one hour. The doses of MEL (1, 3, 5, and 7 μM) were chosen according to the pharmacokinetic profile of the drug after SOAIM, as reported by Shaiquevichet al. [20].

The same procedure (including the incubation for one hour at 37°C in 5% $CO_2$/95% air) was used for SA, starting from a 1M stock solution, to a final concentration of 1, 3, 5, and 7 mM in each well containing Y79 cells. These doses of ascorbate were chosen according to the pharmacokinetic profile of SA, when administered in high doses by i.v. injection [32,33,35,41].

At the end of the incubation time, the cells were collected, centrifuged for 5 min at 500 x g, re-suspended ("washed") in fresh medium and incubated for other 18 - 24 hours at 37°C in 5% $CO_2$/95% air, before the analysis.

Four control (no treatment) samples were also included in each experiment. A cell viability assay with Trypan Blue, was performed after exposure to both MEL and SA, at the end of the incubation period, according to the reported procedures [43,44]. Each "manual" (Trypan Blue) counting was performed by three of us, in triplicate, and the mean percentage of live/dead cells calculated for each sample.

At the end of the incubation period (18 - 24 hours after exposure to MEL and SA), 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, 20 μl of each sample were added to 380 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 twelve experiments was carried out, encompassing four "manual" (Trypan Blue) and eight automated ("Muse"™) cell counting and viability tests, before and after exposure to MEL and SA. The results have been cumulatively analyzed by calculating the mean, standard error, standard deviation, and confidence intervals (CI) of the percentages of living cells for each experiment (Tables 1 and 2).

| SAMP. | %L | %L | %L | %L |
|---|---|---|---|---|
| C | 88 | 87 | 92 | 88 |
| A1 | 79 | 88 | 95 | 87 |
| A3 | 55 | 28 | 47 | 18 |
| A5 | 1 | 7 | 21 | 10 |
| A7 | 0 | 2 | 5 | 0 |
| M1 | 85 | 81 | 98 | 93 |
| M3 | 87 | 85 | 96 | 90 |
| M5 | 91 | 82 | 96 | 90 |
| M7 | 80 | 95 | 91 | 88 |

**Table 1:** Mean percentages of live/dead cells after exposure to SA 1, 3, 5, and 7 mM (A1, A3, A5, A7), and MEL 1, 3, 5, and 7μM (M1, M3, M5, and M7), as calculated by Trypan Blue Exclusion Test.

| SAMP. | %L | %L | %L | %L | %L | %L | %L | %L |
|---|---|---|---|---|---|---|---|---|
| C | 64.4 | 64.4 | 79.5 | 79.5 | 73.5 | 73.5 | 65.5 | 66.1 |
| A1 | 66.30 | 67.30 | 63.10 | 64.20 | 65.10 | 65.7 | 68.8 | 70.4 |
| A3 | 40.90 | 39.70 | 5.30 | 3.50 | 40.80 | 46.5 | 29.2 | 31.7 |
| A5 | 10.70 | 7.90 | 3.10 | 2.00 | 14 | 14.2 | 8.7 | 8.7 |
| A7 | 8.70 | 7.90 | 2.80 | 1.50 | 6.20 | 6.6 | 6.6 | 6.6 |
| M1 | 69.00 | 69.20 | 66.40 | 62.90 | 68.4 | 68.4 | 66.7 | 68.9 |
| M3 | 67.60 | 67.50 | 66.50 | 65.90 | 67.1 | 70.3 | 67.6 | 71.5 |
| M5 | 74.10 | 72.00 | 67.50 | 65.80 | 68.9 | 68.9 | 70.8 | 72.5 |
| M7 | 68.60 | 67.90 | 67.90 | 67.60 | 69.4 | 71.2 | 59.7 | 65.7 |

**Table 2:** Mean percentages of live/dead cells after exposure to SA 1, 3, 5, and 7 mM (A1, A3, A5, A7), and MEL 1, 3, 5, and 7μM (M1, M3, M5, and M7), as reported by the Muse™.
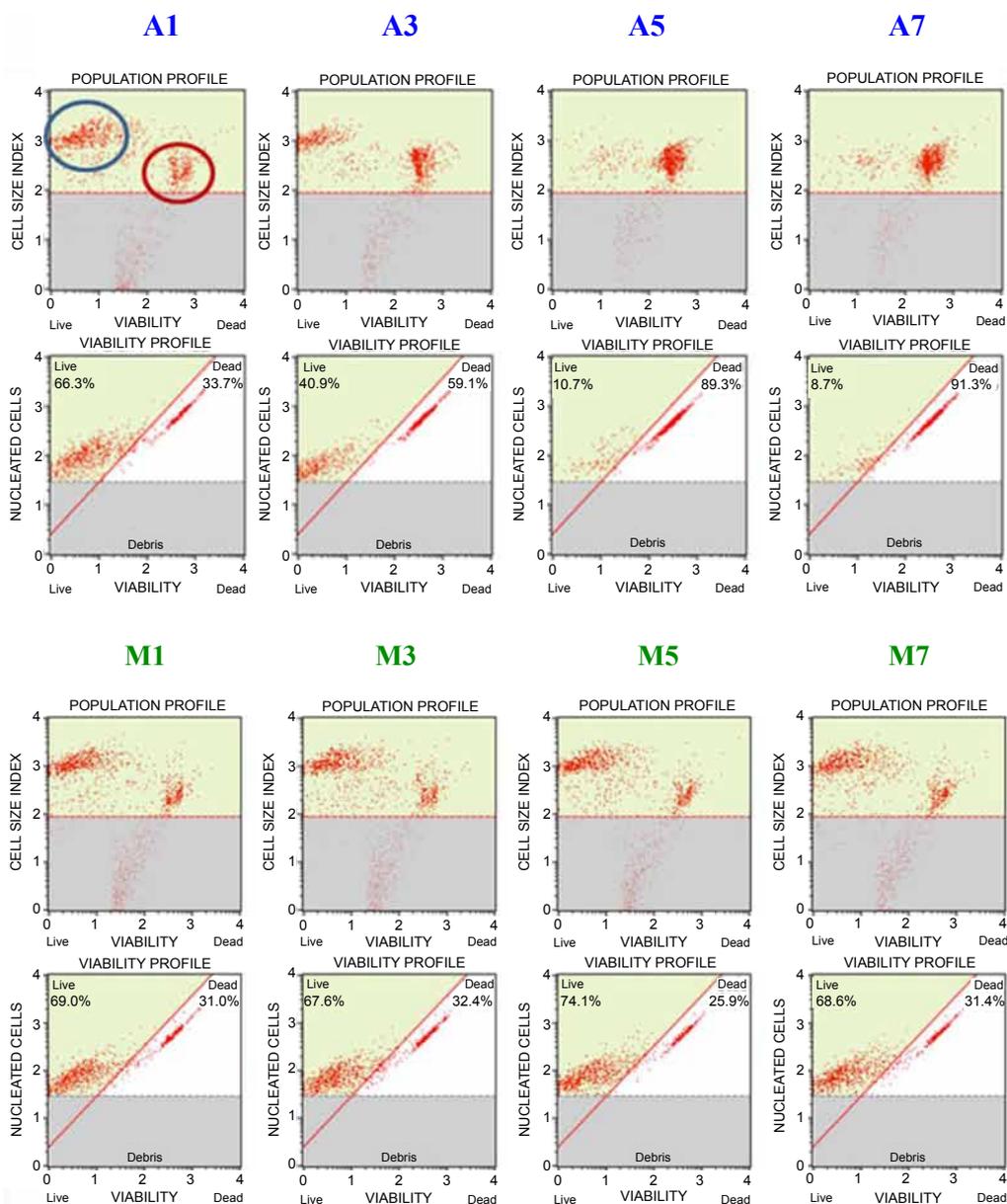
**Figure 1:** Plots obtained by analyzing Y79 cell after exposure to SA 1, 3, 5, and 7 mM (A1, A3, A5, A7, respectively), and MEL 1, 3, 5, and 7µM (M1, M3, M5, and M7) with the Muse™ automated cell counter/analyzer (Merk - Millipore). In the upper left plot, the black circle indicate the live cells while the red circle indicates the dead cells. As it can be seen, the "cloud" of live cells decreases in intensity, going from A1 (1mM SA) to A7 (7mM SA). The same does not apply to Melphalan, thus indicating that doses 3, 5, and 7 mM ascorbate (SA) kill a progressively increasing number of Y79 cells. The lower group of plots, reporting the percentage of live/dead cells, confirms this result.

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 [35,45].

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.

## Results

The results of this experiment are summarized in the diagrams of figures 2 and 3, reporting the mean percentage of live cells on the y-axis and the doses of drugs on the x-axis, for the manual (Trypan Blue) and the automated (Muse™) procedures, respectively.
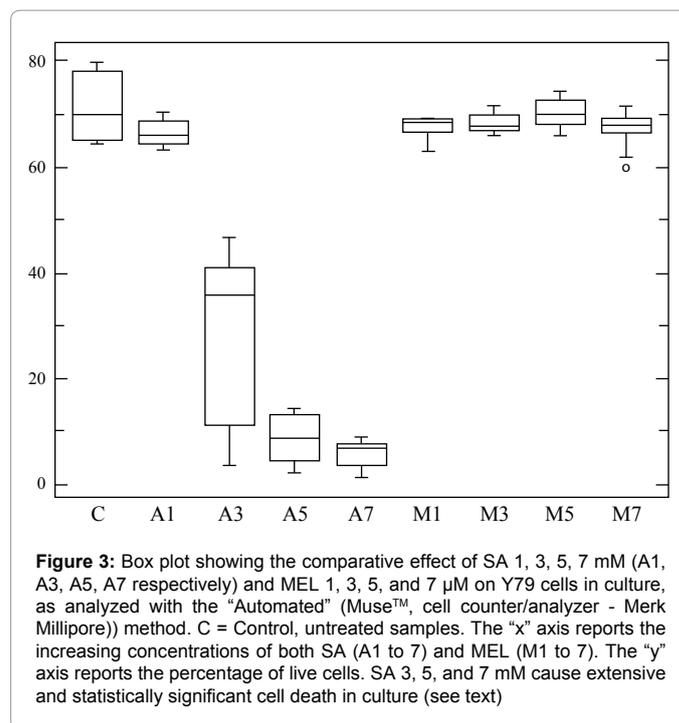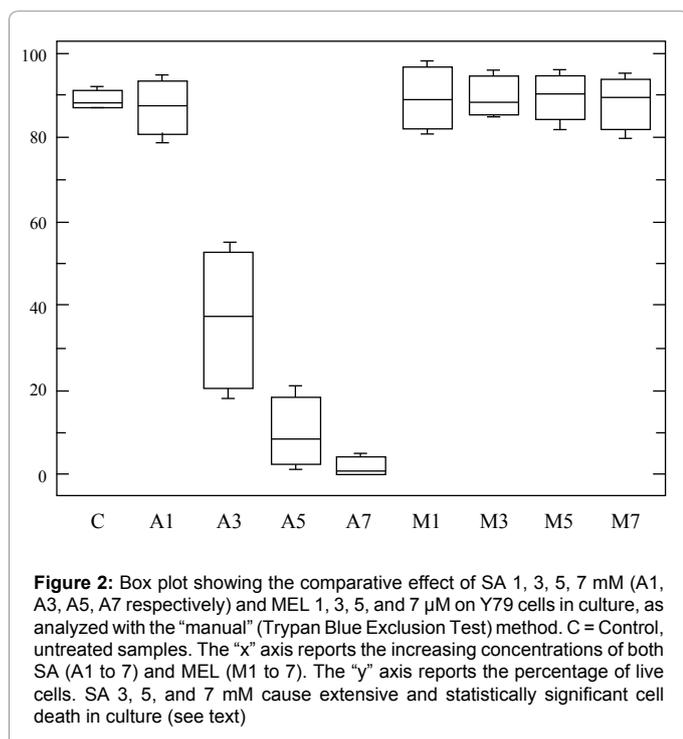
It is very well known that Trypan Blue Exclusion Test, is a robust direct test to measure cell viability, in which dead cells can be easily detected, because, by incorporating the blue dye, their cytoplasm stains blue, when observed under microscopy. The only limit of this procedure is represented by its inability to detect both early or late apoptotic cells which still possess an intact cytoplasmic membrane, and therefore, do not stain with the dye [43,44].

Methods based on the use of fluorescent dyes and microscopic observation, on the other hand, are laborious and not completely reliable, particularly when chromatin morphology is investigated [45].

The automated cell counting and viability analysis used herein (MuseTM, Merk-Millipore) utilizes a proprietary mix of two DNA intercalating fluorescent dyes in a single reagent. One of the dyes is membrane permeant and stains all cells with a nucleus. The second dye only stains cells whose membranes have been compromised and are dying or dead. This combination allows for the discrimination of nucleated cells from those without a nucleus or debris, and live cells from dead or dying resulting in both accurate cell concentration and viability. The use of dual fluorescent probes that clearly identify all nucleated cells, live and dead, allows for greater sensitivity and accuracy compared to colorimetric methods. Using multiparametric fluorescent detection of individual cells via microcapillary flow technology, the system enables highly sensitive and rapid detection of cellular samples using minimal cell numbers [48].

The cumulative data concerning the mean percentages (Tables 1 and 2) of live cells, with both the manual (Trypan Blue) (Figure 2) and the automated (Muse™) (Figure 3) methods, reveals that SA is highly efficient in killing Y79 retinoblastoma cells in culture, starting from a 3 mM concentration in the culture medium. The number of viable cell is further reduced, from about 70%, to around 10%, and less than 10%, at 5 and 7 mM SA, respectively. The same does not apply to MEL, which, at the concentrations used in the present investigation, did not show any significant effect on cells viability in culture.

The morphologic analysis, with Hoechst/PI (Figure 4), fully confirmed the data reported with both the manual and automated cell counting and viability procedures. Interestingly, after exposure for one hour to high doses (3, 5, and 7 mM) of SA, on direct observation, Y79 cells clearly showed fragmentation and "oncosis" [49,50], as if cell membrane functions were definitively compromised (Figure 5).



**Figure 3:** Box plot showing the comparative effect of SA 1, 3, 5, 7 mM (A1, A3, A5, A7 respectively) and MEL 1, 3, 5, and 7 μM on Y79 cells in culture, as analyzed with the "Automated" (Muse™, cell counter/analyzer - Merk Millipore)) method. C = Control, untreated samples. The "x" axis reports the increasing concentrations of both SA (A1 to 7) and MEL (M1 to 7). The "y" axis reports the percentage of live cells. SA 3, 5, and 7 mM cause extensive and statistically significant cell death in culture (see text)

The calculation of the mean values and related 95% confidence intervals (CIs) revealed statistically significant difference ($p<0.005$) in the mean percentages of dead cell between the group encompassing the control sample (C), A1, M1 to 7, and the group including A3, A5 and A7, i.e. the higher concentrations of SA in the culture medium.

## Discussion

The history of ascorbic acid (AA) as an anticancer molecule is very controversial [36]. McCormik, nearly 60 years ago, suggested that ascorbate protects against cancer by increasing collagen synthesis [51,52], while Ewan Cameron hypothesized that ascorbate could have anti-cancer effects by inhibiting hyaluronidase and thereby preventing cancer spread [53]. Although successful clinical trials had been reported, by Cameron and the twofold Nobel laureate Linus Pauling, on terminal cancer patients [54-59], Charles Moertel, at Mayo Clinic, reported negative results, and his trials were credited as the definitive proof of the inefficacy of AA in treating cancer [60,61]. In Moertel's trial, however, ascorbate was given orally, while Cameron and Pauling had used both the intravenous and oral route of administration simultaneously.

Interestingly, from the reported survey data, we know that intravenous ascorbate (either AA or SA) is used in doses up to 200 g per infusion, to treat a variety of pathological conditions, including cancer, is well tolerated, and remarkably safe, with very few adverse effects [62]. As a matter of fact, when taken orally, plasma ascorbate concentrations never raise beyond the level of 100 μmol/l, due to the limited bioavailability of the molecule and renal excretion [63], while, by intravenous infusion, concentrations in the millimolar range can be achieved, which would never be obtained with the maximum dose administered by oral route [35,42,64].

Both AA (Vitamin C) and/or its sodium salt SA have been reported to selectively kill cancer cells, being, at the same time, totally harmless for normal cells, when peak plasma levels are maintained below the
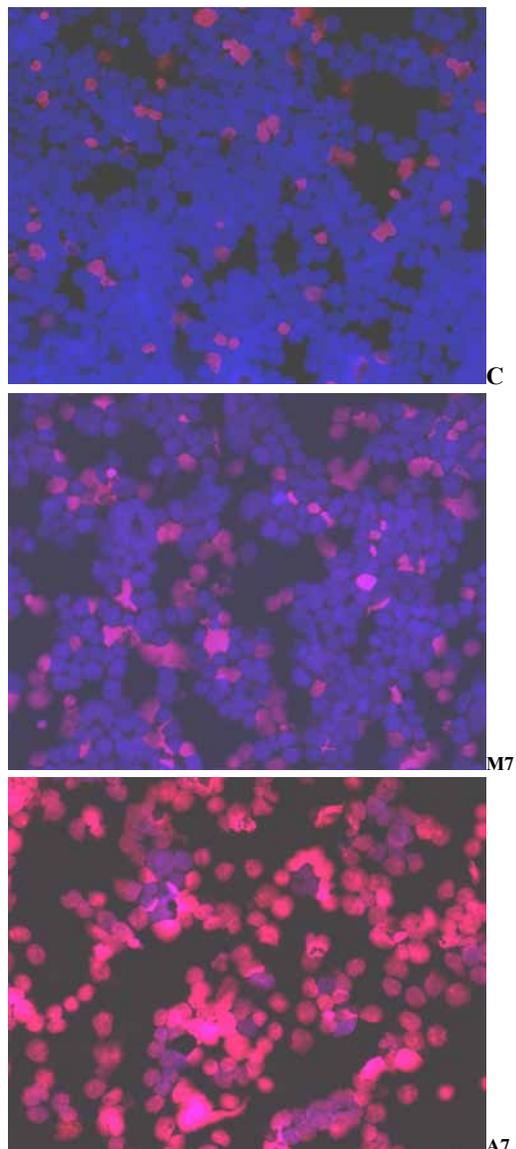


**Figure 2:** Box plot showing the comparative effect of SA 1, 3, 5, 7 mM (A1, A3, A5, A7 respectively) and MEL 1, 3, 5, and 7 μM on Y79 cells in culture, as analyzed with the "manual" (Trypan Blue Exclusion Test) method. C = Control, untreated samples. The "x" axis reports the increasing concentrations of both SA (A1 to 7) and MEL (M1 to 7). The "y" axis reports the percentage of live cells. SA 3, 5, and 7 mM cause extensive and statistically significant cell death in culture (see text)

**Figure 4:** Morphology of Y79 cells treated with MEL (M7), and SA (A7) and stained with Hoechst/PI. Cells were treated for 1 hour, harvested, washed, stained with Hoechst/PI, and examined by fluorescence microscopy, as described under "Material and Methods" (Original magnification: 200 x) Blue nuclei (Hoechst 33342) indicate live cells, while red nuclei (Propidium Iodide) indicate dead cells. C = control (untreated) cells. 7 mM SA kills more than 90% Y79 cells in culture, as denoted by the red nuclei. Cell killing with MEL (M7) is not significantly different from the control (C).

threshold of 20mM/ml [37,38]. In our experience, AA seems more efficient than SA in killing Y79 cells *in vitro* (data not shown), but SA was preferred since, when dissolved in PBS, at 1M concentration, it gives a slightly basic solution (pH 7.3) which doesn't need to be buffered, as is the case for AA (which, instead, at 1M concentration, turns the pH of the solution to 2.2 – 2.4).

Regarding the mechanisms through which SA is cytotoxic / cytocidal for cancer cells, wide agreement exists on the view that SA (and/or AA) is a pro-drug for $H_2O_2$ generation, since for pharmacologic SA to promote cancer cell death, $H_2O_2$ must be formed [32,33,35,41].

Downstream targets of $H_2O_2$ are different and almost certainly involve the actions of reactive oxygen species (ROS) [65]. Moreover, given the inherent promiscuity of the action of both $H_2O_2$ and ROS, no single target exists, among cancer cells, for cytotoxic response to SA [66]. As a matter of fact, a number of different effects of SA on cancer cells have been reported, *in vitro*, depending on the cell line investigated [67], with cytotoxicity being the main outcome with 9 L rat glioblastoma (10), SK-N-SH human neuroblastoma, and Kelly human neuroblastoma [68], human chronic lymphocytic leukemia [69], Pan02 mouse pancreatic cancer, and MIA PaCa-2 human pancreatic carcinoma [40].

In 1993, Medina and Schweigerer reported the cytotoxic effect of AA on Y79 cells in long-term incubations in the presence of limited amounts of serum in the medium [70] and, to our knowledge, the data reported herein are the only literature report, investigating the effects of high doses of AA on retinoblastoma cell lines. More recently, a nutrient mixture containing AA, lysine, proline, and green tea extract
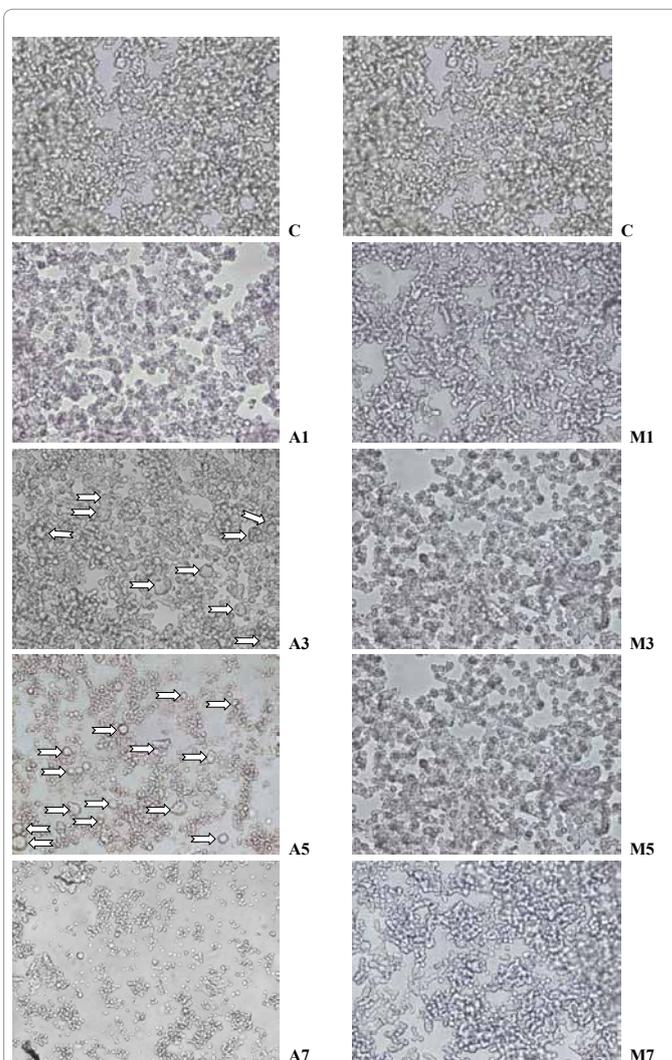


**Figure 5:** Microphotographs of y79 retinoblastoma cells incubated with sodium ascorbate (SA) m 1, 3, 5, and 7 mM (A1, A3, A5, A7), and Melphalan (MEL) 1, 3, 5, and 7µM (M1, M3, M5, and M7). C = Control (untreated). Original magnification … 200 x). Cell treated with increasing doses of SA, show a progressive decrease in the total number of cells, especially evident in A7, and an increasing number of swollen cells (white arrows in A3, and A5) indicative of the phenomenon of "oncosis".

has shown the ability to limit cell proliferation and induce apoptosis in Y79 cells [71].

In our investigation, we have compared the effects of high (mM) doses of SA on Y79 human retinoblastoma cell lines, with those produced, on the same cells, by MEL. In recent years, MEL injected in the ophthalmic artery, has become the treatment of choice for some advanced, but also localized retinoblastoma, yielding encouraging results in terms of both reduced toxicity and increasing local efficacy [72]. However, MEL is also a potentially carcinogenic drug, carrying an increased risk of leukemia, after prolonged or repeated treatments [26,27] and this aspect should not be overlooked, particularly when, as in the case of retinoblastoma, genomic instability is involved [6,73].

The present investigation was carried out in order to compare the *in vitro* effects of MEL, which is one of the chemotherapeutic drugs of choice in retinoblastoma, with those of SA which, in a number of different reports, has shown cytotoxic effects on tumor cell lines.

To our knowledge, in only one case the cytotoxic effects of SA on human retinoblastoma cell line (Y79) has been reported, although this case refers to long term exposures and particular growth conditions. In the experience reported herein, Y79 cells have shown a particular sensitivity to SA 3, 5, and 7 mM, after only one hour exposure, with a linear proportion between dose and percentage of dead cells. The same result was not achieved with increasing doses of MEL.

Y79 in culture, showed, after exposure, morphological aspects of cell death, including fragmentation and oncosis [49,50]. All data (Trypan Blue, automated cell counting and viability, cellular morphology in culture, Hoechst/PI nuclear staining, before and after exposure to SA) were concordant in showing a marked cytotoxicity of SA to Y79 cells.

The calculation of the Confidence Intervals (CI) at 95% of the two treatments (MEL and SA) and the controls (C) revealed no separation ($p > 0.05$) at 1mM SA (A1), but were clearly well separated ($p < 0.005$), starting from 3mM SA (A3) which, in this experience, represents the minimal effective concentration of SA, against Y79 cells. The difference is still statistically significant at 5 mM and 7 mM SA. These data are in agreement with the current literature on high intravenous doses of SA, which indicate the 5mM plasma level as the more effective concentration to efficiently and selectively kill cancer cells [32,33].

## Conclusions

Given all the above, it is reasonable to infer that SA in high doses, such as those achieved after intravenous injection, are highly toxic for Y79 cell lines, while MEL, at the doses commonly used for SOAIM, does not significantly affect cell viability *in vitro*. This is most probably due to the different mechanisms through which both MEL and SA kill cancer cells. More importantly, SA opposite to MEL, is almost totally harmless for normal human cells.

SA also enhances the cytotoxicity of several chemotherapeutic agents, *in vitro* [74,75], and, in our experience (data not shown) it shows a synergistic cytotoxic effect with Melphalan, on Y79 cells. Therefore, our experimental data, suggest that SA can be usefully added to MEL, in the treatment of retinoblastoma.

More importantly, our data on the effects of SA on Y79 cells, strongly argues in favor of the treatment of retinoblastoma with high doses of intravenous SA alone, repeated according to the established protocols [76-78]. Finally, given the more recent therapeutic progresses, in the treatment of retinoblastoma, with the intra arterial injection of

chemotherapeutic agents, SA represents, to the author's opinion, the ideal candidate for this new therapeutic approach, in the near future.

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