Ascorbic acid and 6-deoxy-6-chloro-ascorbic acid: Potential anticancer drugs*

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Received July 24, 1996

The role of ascorbic acid (AA) in prevention and suppression of carcinogenesis has been known for a long time. It was also found that AA may inhibit the growth of some tumor cells in vitro and in vivo. We examined the influence of ascorbic acid and 6-chloro-6-deoxy ascorbic acid (6-Cl-AA) on the growth of various human cell lines: lung fibroblasts (Hef), ovarian adenocarcinoma (OVAR), colon adenocarcinoma (HT-29), laryngeal carcinoma (HEp2) cells, HEp2 cells resistant to vincristine (HEp2VA3), cervical carcinoma (HeLa) cells, HeLa cells resistant to cisplatin (HeLacis), breast adenocarcinoma (SK-BR-3) cells, and SK-BR-3 resistant to doxorubicin (SK-BR-3-Dox), as well as mouse fibroblasts L929, mouse melanoma B16 (Mel B16) cells and Chinese hamster fibroblasts (V79). Both drugs arrested the growth of: HeLa, SK-BR-3, SK-BR-3-Dox, L929, and Mel B16 cells, but did not influence the growth of others: Hef, OVAR, HEp2, HEp2VA3 and V79. 6-Cl-AA suppressed more the proliferation of HeLacis, SK-BR-3-Dox and Mel B16 cells than AA, while AA was active only against HT-29 cells. Inhibitory effect of 6-Cl-AA was confirmed by the in vivo experiments on solid melanoma B16 tumors. Our results indicate that AA and 6-Cl-AA could serve as potential antitumor agents, especially against some tumor cells resistant to chemotherapy.

Key words: Ascorbic acid, anticancer drugs, cell cultures, drug-resistance.

The role of L-ascorbic acid in prevention and suppression of carcinogenesis has been a subject of longstanding interest and controversy [11]. Ascorbic acid (AA) can be involved in each step of carcinogenesis. The best known example of inhibition of carcinogen formation by ascorbic acid is prevention of the formation of endogenous nitrosamines by scavenging nitrite in the gut [2]. The suppression of tumor formation by AA was observed in mouse skin [24, 25]. AA, as the most important scavenger of peroxide radicals in plasma [9], also inhibited estrogen induced renal cancer in Syrian hamsters even if it was only administered during the last three months of the experiment [14]. Inhibition of neoplastic transformation was also documented in vitro [3, 23].

In clinical treatment, AA was used both alone or in combination with chemotherapy. The published data, however, offer no unique conclusion: results of these studies varied from the total negation of any positive effect of AA [15] to the claims of significant improvement in cancer therapy [7, 11, 16].

It was also found that AA can be cytotoxic toward several types of normal and tumor cells in vitro [1, 6, 12, 21, 22]. The extent of inhibition was dependent on the concentration of the amount of ascorbate absorbed by a given cell and on the sensitivity of the cell line examined.

It should be mentioned, however, that there is increasing evidence that cytotoxicity is not confined only to the vitamin C itself, but also to some of its oxidation products and side-chain substituted derivatives. For instance, it was found that replacement of the terminal-chain (C-6) hydroxyl group by bromine led to the enhanced antitumor activity towards human cervical carcinoma HeLa cells as well as towards mouse melanoma B16 and leukemia L1210 cell lines [17, 26]. Similarly, 6-deoxy-6-amino AA inhibited the growth of several human tumor cell lines, such as cervical carcinoma HeLa cells, pancreas MiaPaca 2 and laryngeal HEp2 cells [10].

*This study was supported by Ministry of Science and Technology of the Republic of Croatia (Projects No. 1-8-210 and 1-7-193).
In this work we extended our earlier studies on the cytotoxicity of L-ascorbic acid to the cytotoxic effect of 6-chloro-6-deoxy-ascorbic acid (6-Cl-AA) towards cell lines, especially those resistant to chemotherapy. For this purpose we have used three sublines which have been developed during the treatments with three different cytostatics (cisplatin, doxorubicin and vincristine), which exert their cytotoxicity in different ways. For comparison, the same cell lines were also treated with parental vitamin C. Finally, in vivo activity of 6-Cl-AA was examined on mouse melanoma B16 tumors and compared to the results obtained previously with AA.

Material and methods

Drugs. 6-deoxy-6-chloro-ascorbic acid was prepared by reacting L-ascorbic acid with HCl-CH₃COOH following the procedure developed by Bock et al. [4] and Kiss et al. [13]. For in vitro and in vivo experiments, AA and 6-Cl-AA were dissolved in distilled water and subsequently diluted with growth medium. The pH of medium was adjusted to 7.2 using 0.1 mol NaOH.

Cell cultures. Following human cell lines were used for the in vitro experiments: lung fibroblasts (Hef), ovarian adenocarcinoma (OVCAR), colon adenocarcinoma (HT-29) and laryngeal carcinoma (HEp2) cells incubated for 18 (1) or 72 (2) h. The data show the ratio (in %) between the number of cells treated with AA or 6-Cl-AA and control cells. *Values statistically different from the control.

Fig. 1. The influence of AA and 6-Cl-AA on the proliferation of exponentially growing human cells: normal lung fibroblasts (Hef), ovarian adenocarcinoma (OVCAR), colon adenocarcinoma (HT-29) and laryngeal carcinoma (HEp2) cells incubated for 18 (1) or 72 (2) h. The data show the ratio (in %) between the number of cells treated with AA or 6-Cl-AA and control cells. *Values statistically different from the control.
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Fig. 2. The influence of AA and 6-Cl-AA on the proliferation of exponentially growing human cell lines: cervical carcinoma (HeLa) cells, HeLa cells resistant to cisplatin (HeLa cis), breast adenocarcinoma (SK-BR-3) cells and SK-BR-3 resistant to doxorubicin (SK-BR-3-Dox) cells incubated for 18 (1) or 72 (2) h. The data show the ratio (in %) between the number of cells treated with AA or 6-Cl-AA and control cells. *Values statistically different from the control.

...cinoma (OVCAR), colon adenocarcinoma (HT-29), laryngeal carcinoma (HEp2) cells, HEp2 cells resistant to vincristine (HEp2VA3; 18), cervical carcinoma (HeLa) cells, HeLa cells resistant to cisplatin (Helacis; 19), breast adenocarcinoma (SK-BR-3), and SK-BR-3 resistant to doxorubicin (SK-BR-3-Dox; 20), as well as mouse fibroblasts L929, mouse melanoma B16 (Mel B16) cells and Chinese hamster fibroblasts (V79). They were grown at 37°C as monolayer cultures in DMEM medium supplemented with 10% of fetal calf serum and antibiotics in humid mixture of 95% air and 5% CO₂.

**In vitro growth assay.** In order to examine the effect of AA or 6-Cl-AA on the proliferative capacity, 3 x 10⁴ cells were seeded in 1.2 ml of growth medium in 24-wells plates in quadruplicates. Following overnight incubation, AA or 6-Cl-AA were added in final concentrations 10⁻³–10⁻⁵ mol and incubated for 18 or 72 h. 96 h after plating, the cells were harvested by trypsinization and counted. Number of cells in AA and 6-Cl-AA treated samples were compared with the corresponding number of control samples. Each experiment was done at least two times.
**In vivo experiments.** Three months old inbred male C57BL mice were used in this study. The animals were housed in plastic cages and fed standard pellets and tap water ad libitum. Melanoma B16 was maintained and passed into C57BL mice. In all experiments, the tumor was induced by subcutaneous transplantation of $2 \times 10^6$ cells. The tumors developed as almost prolate spheroids. Two opposite tumor diameters were measured by a caliper. The tumor volumes were calculated by the formula $V = AB^2 \pi / 6$, where A is the larger and B the smaller diameter. Each experimental group consisted of 11 animals.

Each animal was injected with 90 mg/kg of body mass of AA or with 95 mg/kg of body mass of 6-Cl-AA (equimolar concentration) i. p. in three daily doses for 16 days. The tumors were observed daily, and the results are presented as the mean values obtained with 11 animals ± standard deviation.

**Statistics.** The significance of the differences in the cell number between control and treated cells, or the differences between the tumor volume for control and treated groups of animals were assessed by Student’s t-test. The differences were considered as significant with $p < 0.05$.

**Results**

The effect of ascorbic acid and 6-chloro-deoxy-ascorbic acid on the proliferation of different cell lines in vitro are presented in Figs 1–3. As shown in Fig. 1, both agents did not influence the growth of human normal fibroblasts (Hef), ovarian adenocarcinoma and laryngeal carcinoma (HEp2) cells. Evenmore, a small, but statistically insignificant stimulation of growth was observed for Hef and HEp2 cells. Similarly, for HEp2 cells resistant to vincristine no effect of AA and 6-Cl-AA was observed (data not shown). AA inhibited the growth of colon adenocarcinoma, if given in concentrations of $10^{-3}$ mol for 18 and 72 h, or $10^{-4}$ mol for 18 h, while 6-Cl-AA had no effect on the growth of these cells.

Figure 2 shows the results obtained with AA and 6-Cl-AA on parental and cisplatin resistant human cervical carcinoma cells, as well as on parental and doxorubicin resistant breast adenocarcinoma cells. Both substances if given at their highest concentrations, similarly inhibited the growth of HeLa cells, reducing the growth to 20% of control. However,
HeLa cells were insensitive to AA. They were sensitive to 6-Cl-AA but less than parental HeLa cells.

Similarly to HeLa cells, both agents at their highest concentrations inhibited the growth of human breast adenocarcinoma cells. These cells were more sensitive to AA than to 6-Cl-AA. SK-BR-3-Dox cells were more sensitive to examined substances than parental SK-BR-3 cells. The concentration of $10^{-3}$ AA reduced the cell growth to 42 and 50% of SK-BR-3 cells after incubation of 18 and 72 hours, respectively, while for the same experimental conditions the proliferation of SK-BR-3 cells resistant to doxorubicin was reduced to 30 and 10%. If 6-Cl-AA was used, the growth-inhibition was observed at lower concentrations of this drug ($10^{-4}$ mol and $10^{-5}$ mol, 18 h incubation).

Figure 3 shows the results obtained with cells of animal origin. AA and 6-Cl-AA given at highest concentrations inhibited the growth of mouse fibroblasts, while lower concentrations had no effect. L929 cells were more sensitive to AA than to 6-Cl-AA.

Mouse melanoma B16 cells were sensitive to both substances examined: all three examined concentrations of AA if given for 18 h inhibited the growth of Mel B16 (60–80% of control). The strongest inhibition was observed for AA when incubated for 72 h at $10^{-3}$ mol concentration (22% of control). Mel B16 cells were even more sensitive to 6-Cl-AA if incubated for 72 h: this substance inhibited the cell growth to 12 and 41% if given in concentrations of $10^{-3}$ and $10^{-4}$ mol, respectively. Finally, Chinese hamster cells were insensitive to both drugs.

The influence of AA and 6-Cl-AA on the growth of solid melanoma tumors is presented in Fig. 4. It is shown that AA reduced the tumor volume only slightly. On the other hand, the inhibition of tumor growth due to addition of 6-Cl-AA became obvious from the 6th day after the tumor transplantation, and was more pronounced with the increasing time after transplantation. On the 16 day, the volume of treated tumors were 2 times smaller than in control animals.

Discussion

In this study we have examined the influence of AA and 6-Cl-AA on the growth of different cell lines in vitro and on the growth of solid melanoma tumors in vivo. We have found that growth-inhibitory effect of these substances will depend on the nature of the cell line examined. No effect was observed for human cells: normal fibroblasts (HeF), ovarian carcinoma (OVCAR), laryngeal carcinoma (HEp2), and Chinese hamster fibroblasts (V79), while human cervical carcinoma (HeLa), breast adenocarcinoma (SK-BR-3), mouse fibroblasts (L929) and melanoma B16 cells were sensitive to these drugs. Human colon adenocarcinoma HT-29 cells were also sensitive to AA, but not to 6-Cl-AA.

The high sensitivity of melanoma B16 cells to AA and 6-Cl-AA was confirmed in vivo. If given three times daily in equimolar doses, AA inhibited the growth of solid melanoma tumors only slightly, while the reduction of tumor growth for 6-Cl-AA was obvious (Fig. 4).

The major obstacle to the ultimate success in cancer treatment is the ability of malignant cells to develop resistance to cancer therapy or their intrinsic resistance to cytostatics. Several molecular mechanisms may be involved in this phenomenon: increased drug efflux mediated by plasma membrane glycoproteins, decreased drug uptake, increased intracellular detoxification by glutathione with related enzymes and metallothioneins, amplification of the target genes, alteration in the nuclear enzymes like topoisomerases, and/or increased DNA damage tolerance and repair. In several cases drug resistance has been found to be multifactorial [5, 8].

As resistant cells are the major cause of failure in successful treatment of cancer patients, we examined in our study also the effect of ascorbic acid and its chloro derivative on such cells. We selected three sublines that we developed by the treatment with three cytostatics that exert their cytotoxicity through different mechanisms: cisplatin, doxorubicin and vin-
Human laryngeal carcinoma HEp2VA3 cells were obtained due to the treatment with vincristine. They are cross-resistant to doxorubicin, 5-fluorouracil, methotrexate, equally sensitive to vinblastine, etoposide and mitomycin C, and sensitive to cisplatin, as compared to parental cells [18]. Parental HEp2 cells were resistant to AA, as well as HEp2VA3 cells.

Human cervical carcinoma HeLacis cells were obtained by repeated treatments with escalating doses of cisplatin. These cells are resistant to cisplatin, cross-resistant to vincristine, etoposide, doxorubicin, 5-fluorouracil and methotrexate, and are equally sensitive to mitomycin C as parental cells [19]. While HeLa cells are sensitive to highest concentrations of AA and 6-Cl-AA, the resistance to cisplatin in HeLacis cells was accompanied with resistance to AA and reduced sensitivity to 6-Cl-AA (Fig. 2).

Human breast adenocarcinoma SK-BR-3-Dox cells were obtained due to treatment with doxorubicin. They are cross-resistant to cisplatin, carboplatin, mitomycin C, vincristine, equally sensitive to etoposide, and slightly resistant to vinblastine and 5-fluorouracil [20]. Parental SK-BR-3 cells are sensitive to highest concentrations of both AA and 6-Cl-AA, but the sensitivity to AA was more expressed. From all examined cells, SK-BR-3 cells resistant to doxorubicin are mostly sensitive to AA, if given in concentration of 10^{-3} mol. They are less sensitive to this concentration of 6-Cl-AA, but are also sensitive to lower concentrations of this drug (Fig. 2).

We like also to point out our findings achieved with human colon adenocarcinoma HT-29 cells (Fig. 1). Namely these cells exhibit the intrinsic resistance to different cytostatics, causing the failure of chemotherapy. We have shown that HT-29 cells are sensitive to ascorbic acid (but not to 6-Cl-AA), suggesting again that this drug may be active against some cells resistant to cytostatics.

To our knowledge, it is the first time that the cytostatic effects of ascorbic acid and its derivatives are studied in cells resistant to cytostatics.

Unsuccessful treatment of cancer is the cause of death of many people. Therefore, new drugs that could increase the efficacy of tumor treatment are desirable. Vitamin C is involved in normal physiological processes and exert beneficial effects in several ways. Our finding that ascorbic acid and its chloro-derivative inhibit the growth of some tumor cells suggest that AA and 6-Cl-AA could be used in combined therapy of certain tumors, and could be a promising additional drug for the treatment of tumors resistant to chemotherapy.

We are very grateful to Dr. D. Petrović for his helpful suggestions. Also we thank Mrs. Lj. Krajac for her excellent technical assistance.

References


