Vitamin C Antagonizes the Cytotoxic Effects of Antineoplastic Drugs

Mark L. Heaney,[1] Jeffrey R. Gardner,[2] Nicos Karasavvas,[2] David W. Golde,[1,2] David A. Scheinberg,[1,2] Emily A. Smith,[1] and Owen A. O'Connor[3]

Departments of [1]Medicine and [2]Molecular Pharmacology and Chemistry, Memorial Sloan-Kettering Cancer Center; [3]Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, New York Presbyterian Hospital, Columbia University, New York, New York

Abstract

Vitamin C is an antioxidant vitamin that has been hypothesized to antagonize the effects of reactive oxygen species–generating antineoplastic drugs. The therapeutic efficacy of the widely used antineoplastic drugs doxorubicin, cisplatin, vincristine, methotrexate, and imatinib were compared in leukemia (K562) and lymphoma (RL) cell lines with and without pretreatment with dehydroascorbic acid, the commonly transported form of vitamin C. The effect of vitamin C on viability, clonogenicity, apoptosis, P-glycoprotein, reactive oxygen species (ROS), and mitochondrial membrane potential was determined. Pretreatment with vitamin C caused a dose-dependent attenuation of cytotoxicity, as measured by trypan blue exclusion and colony formation after treatment with all antineoplastic agents tested. Vitamin C given before doxorubicin treatment led to a substantial reduction of therapeutic efficacy in mice with RL cell–derived xenogeneic tumors. Vitamin C treatment led to a dose-dependent decrease in apoptosis in cells treated with the antineoplastic agents that was not due to up-regulation of P-glycoprotein or vitamin C retention modulated by antineoplastics. Vitamin C had only modest effects on intracellular ROS and a more general cytoprotective profile than *N*-acetylcysteine, suggesting a mechanism of action that is not mediated by ROS. All antineoplastic agents tested caused mitochondrial membrane depolarization that was inhibited by vitamin C. These findings indicate that vitamin C given before mechanistically dissimilar antineoplastic agents antagonizes therapeutic efficacy in a model of human hematopoietic cancers by preserving mitochondrial membrane potential. These results support the hypothesis that vitamin C supplementation during cancer treatment may detrimentally affect therapeutic response. [Cancer Res 2008;68(19):8031–8]

Introduction

The effects of vitamin C on cancer and its treatment are controversial (1, 2). Clinical studies suggesting a therapeutic benefit of vitamin C supplementation in patients with cancer have not been consistently reproducible (2, 3). Nonetheless, vitamin C, predominantly in the form of ascorbic acid, remains a commonly used nutritional supplement, and the influence of vitamin C on the action of agents used in treating cancer is unknown. Some antineoplastic agents, such as cisplatin and doxorubicin, lead to increases in intracellular reactive oxygen species (ROS) that may contribute to their therapeutic effect (4, 5). Vitamin C is a potent antioxidant, raising the theoretical concern that vitamin C supplementation might attenuate the antineoplastic activity of drugs that lead to increased ROS (2). Conversely, some reports have suggested that vitamin C might potentiate the effects of some antineoplastic agents, such as arsenic trioxide (6, 7). In the case of arsenic trioxide, however, the enhanced cytotoxicity by vitamin C may be due to the extracellular generation of hydrogen peroxide and increased intracellular concentrations of vitamin C seem to attenuate the cytotoxic effects of arsenic (8).

Ascorbic acid and dehydroascorbic acid are the principle physiologic chemical forms of vitamin C. Whereas ascorbic acid is present in much higher concentrations in the serum, intracellular transport is restricted to a limited number of tissues (9). In contrast, dehydroascorbic acid, the oxidized form of vitamin C, enters cells via facilitated transport through the glucose transporters, primarily GLUT1, and has a substantially wider distribution (10, 11). After transport, dehydroascorbic acid is reduced to ascorbic acid and trapped intracellularly (10, 11). This mechanism leads to rapid and durable accumulation of high concentrations of intracellular ascorbic acid. In the cell, ascorbic acid and other intracellular antioxidants, such as glutathione, serve to mitigate the effects of ROS, function as important biochemical substrates, and are the first antioxidants consumed in conditions of elevated ROS (12–14). Ascorbate is also an electron donor for eight different enzymes and thereby plays an important role in a wide variety of biochemical processes, including collagen formation (15), norepinephrine biosynthesis (16), and mitochondrial fatty acid transport (17). The mitochondria, in particular, generate ROS as byproducts of respiration, making the simpler architecture of mitochondrial DNA and mitochondrial proteins particularly susceptible to oxidative damage. Vitamin C may, thus, play an important role in protecting the mitochondria and mitochondrial translation products from ROS (18, 19).

To ascertain the influence of vitamin C on the cytotoxicity of antineoplastic agents, we used dehydroascorbic acid to increase intracellular concentrations of vitamin C. When intracellular vitamin C levels were increased in the myeloblastic chronic myeloid leukemia cell line K562 and the lymphoma cell line RL, as well as in mice with RL cell xenografts, the cells and tumors were more resistant to the therapeutic effects of anticancer drugs that exert their cytotoxic effects through increased ROS, as well as antineoplastic agents that do not appreciably affect ROS. Treatment with all of the antineoplastic agents tested led to mitochondrial membrane depolarization. Pretreatment with vitamin C substantially attenuated the loss of mitochondrial membrane potential, suggesting a mitochondrial site of action.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

David W. Golde died on August 9, 2004.

Requests for reprints: Mark L. Heaney, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: 212-639-2275; Fax: 646-422-0640; E-mail: heaneym@mskcc.org.

doi:10.1158/0008-5472.CAN-08-1490

Materials and Methods

Cell culture. K562 cells (American Type Culture Collection) were maintained in RPMI containing 10% fetal bovine serum (FBS; Hyclone Laboratories), 50 units/mL penicillin, 50 μg/mL streptomycin (Gemini Bio-Products), 25 mmol/L HEPES (Invitrogen), and 2 mmol/L L-glutamine (Invitrogen). RL cells (American Type Culture Collection) were maintained in RPMI containing 20% FBS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate with penicillin/streptomycin, and L-glutamine, as above.

Vitamin C loading and intracellular concentration determination. Cellular ascorbic acid uptake was determined, as described previously (20). Briefly, cells were added to incubation buffer containing 3:1 L-ascorbic acid (Sigma)/L-[^{14}C]ascorbic acid (4 mCi/mmol, PerkinElmer) and supplemented with 20 μmol/L DTT (Sigma) to achieve final concentrations of 0 to 750 μmol/L ascorbic acid and 20 μmol/L DTT. Dehydroascorbic acid was generated by preincubation of ascorbic acid for 5 min with ascorbate oxidase (Sigma) at 20 units/μmol L-ascorbate. After incubation for 1 h at 37°C, cell pellets were washed thrice by centrifugation at 600 × *g* in cold PBS and lysed, and the incorporated radioactivity was determined by liquid scintillation spectrometry. Intracellular volume was estimated by incubating cells with [^{3}H]oxy-methyl-glucose, as described previously (10, 11), applying a 30% correction for trapped extracytosolic radioactivity (21). Experiments measuring ascorbic acid retention used the same dehydroascorbic acid loading method cited above, and scintillation spectrometry was performed on cell pellets washed thrice by centrifugation at 600 × *g* in cold PBS at each time point after the addition of antineoplastic agents. Residual ascorbate oxidase does not influence dehydroascorbic acid uptake (22). Vitamin C uptake was analyzed using nonlinear regression analysis with GraphPad Prism 5.0a for Mac OS X (GraphPad Software, Inc.).

Effects of vitamin C and *N*-acetylcysteine on cell growth and viability. RL and K562 cells were incubated with 0 to 500 μmol/L dehydroascorbic acid for 1 h to achieve intracellular concentrations between 0 and 9 mmol/L and 0 and 18 mmol/L ascorbic acid, respectively. After loading, cell pellets were washed thrice in PBS and resuspended in media supplemented with antineoplastic agents at IC_{75} concentrations and cultured for up to 48 h. In experiments with *N*-acetylcysteine, 25 mmol/L *N*-acetylcysteine (American Regent) was added with the antineoplastic agents and remained in culture throughout the course of exposure. Cellular viability was determined by trypan blue exclusion. For colony assays, cells were treated with dehydroascorbic acid to achieve high intracellular vitamin C concentrations (K562 [ascorbic acid] = 18 mm, RL [ascorbic acid] = 8.5 mmol/L) and then treated for 48 h with antineoplastic agents, washed thrice in PBS, and resuspended at equal densities in MethoCult H4100 (StemCell Technologies). Plates were assessed at 14 d for colonies of 50 cells or greater. The measured IC_{75} concentrations are as follows: vincristine sulfate (GensiaSicor Pharma), 500 nmol/L; doxorubicin HCl (Bedford Labs), 600 nmol/L; methotrexate (Xanodyne Pharmacal), 100 μmol/L; cisplatin (Bristol Labs), 200 μmol/L; imatinib mesylate (kindly provided by Dr. William Bornmann), 2.25 μmol/L; actinonin (Sigma), 52 μmol/L.

Animals. Fox Chase ICR SCID mice (Taconic) were grafted with 1×10^7 RL cells via s.c. injection to the right flank. After the development of palpable tumor (2 × 2 mm minimum 14 d postengraftment), animals were divided into cohorts (n = 5) and treated on days 0, 2, 4, 6, 14, 16, 26, and 28 with vehicle, vitamin C (250 mg/kg dehydroascorbic acid) by tail vein, doxorubicin (1 mg/kg) i.p. or vitamin C and doxorubicin by the same route of administration and at the same doses. Dehydroascorbic acid for injection was generated by incubation of ascorbic acid with ascorbate oxidase (23, 24). Animals with tumors >2000 mm^3 were euthanized, so few animals treated with vehicle or vitamin C alone were carried beyond 18 d after treatment induction. Any animals that showed signs of toxicity, either through visual inspection or as measured by weight loss (>10%), were treated with 100 μL normal saline s.c. until weight returned to pretreatment levels. All animal procedures were done in accordance with the guidelines of the Memorial Sloan Kettering Cancer Center's Institutional Animal Care and Use Committee. After final tumor dimension measurements, intratumor vitamin C concentrations were determined 2 h after injection of dehydroascorbic acid on day 32 by HPLC-ECD with an ESA CoulArray system (ESA) equipped with a modified C18 column (Phenomenex). Doxorubicin levels were determined on the tumors of three mice taken from each cohort on day 32, 4 d after the last doxorubicin administration. Tumor samples for vitamin C and doxorubicin levels were stored at −80°C.

Fluorometric assays. TUNEL reaction mixture (TUNEL enzyme and TUNEL label, Roche) was added to cells according to the manufacturer's

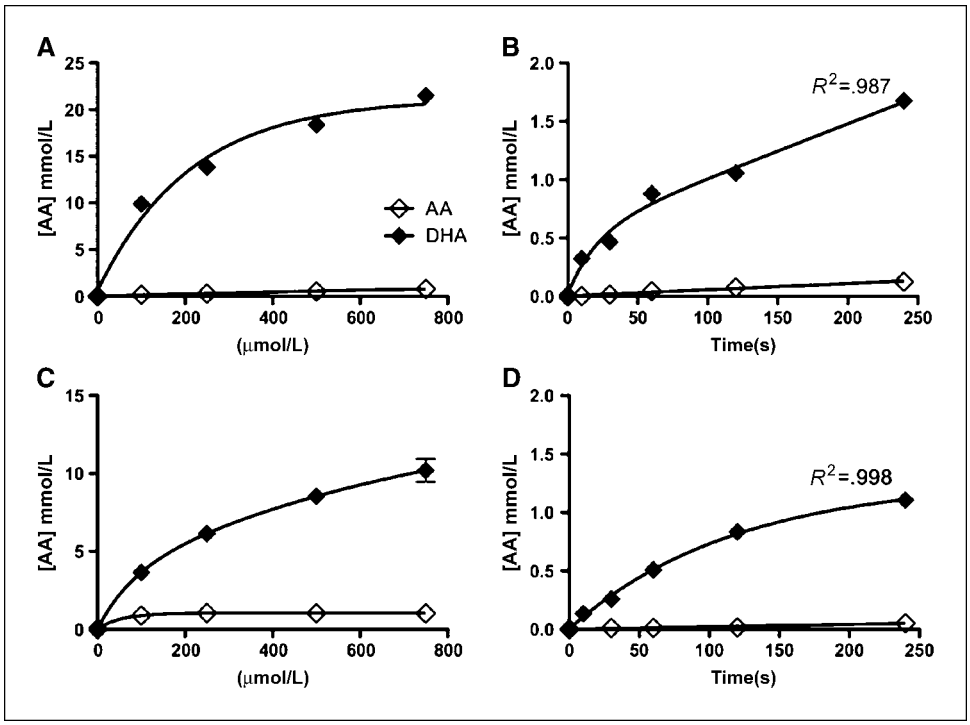

Figure 1. Dehydroascorbic acid, but not ascorbic acid, is taken up in malignant hematopoietic cells. K562 cells (*A, B*) and RL cells (*C, D*) were exposed to either ascorbic acid (*AA*) or dehydroascorbic acid (*DHA*). Dose-dependent uptake after 1 h of exposure to varying concentrations of either ascorbic acid or dehydroascorbic acid (0–750 μmol/L; *A* and *C*). Time-dependent uptake after exposure to either ascorbic acid or dehydroascorbic acid (500 μmol/L) for 0 to 240 s (*B* and *D*). Three separate experiments were conducted in triplicate. *Points,* mean values of a single representative experiment; *bars,* SD.

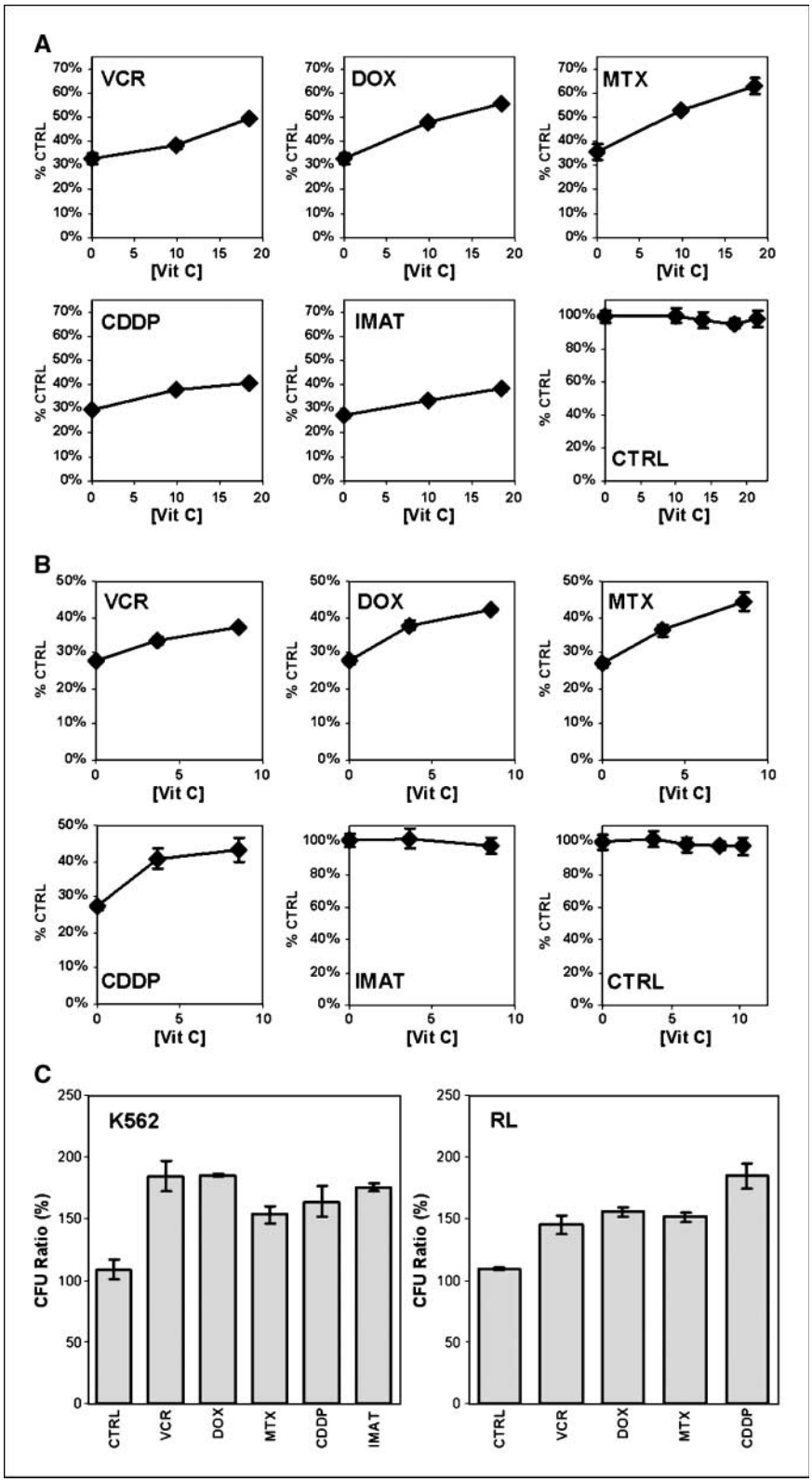

Figure 2. Vitamin C attenuates the cytotoxicity of antineoplastic agents in K562 and RL cells without inhibiting normal cell growth. K562 (*A*) and RL (*B*) cells were loaded with dehydroascorbic acid to varying concentrations of intracellular vitamin C. The cells were washed and treated with either vehicle or antineoplastic agents for 48 h. Cell viability was measured by Trypan Blue exclusion. The results are plotted as the percentage of viable cells compared with cells not treated with either vitamin C or antineoplastic agents. The effect of vitamin C on colony formation after treatment with antineoplastic agents was determined (*C*). K562 cells, either treated with vehicle or with vitamin C to an internal concentration of 18 mmol/L vitamin C, and RL cells, either treated with vehicle or with vitamin C to an internal concentration of 8.5 mmol/L vitamin C, were washed and then treated with either vehicle (*CTRL*) or antineoplastic agents for 48 h. The cells were plated in methylcellulose, and colony numbers were compared with and expressed pair-wise, as a percentage of CFUs resulting from treatment with antineoplastic agents without vitamin C pretreatment. Three separate experiments were conducted in triplicate, and the results shown represent the mean values and SDs of a single representative experiment.

protocol. Cells were preloaded with vitamin C as above and treated for 48 h with antineoplastic agents at IC_{75} concentrations. P-glycoprotein detection was performed by blocking nonspecific binding with heat-inactivated human serum and subsequent treatment of 1×10^6 cells with U1C2-A488 (Alexa Fluor 488–conjugated hu-P-glycoprotein antibody, MSKCC Monoclonal Antibody Core Facility) at 100 μg/mL in PBS for 1 h on ice and in the dark. Cells were then washed and resuspended in PBS + 0.5% paraformaldehyde for data acquisition. To determine intracellular ROS, cells were washed in Krebs Ringer solution, incubated with 0.1 μg/mL CM-H2DCFDA (ref. 16; Invitrogen) for 15 min at 37°C, and immediately placed

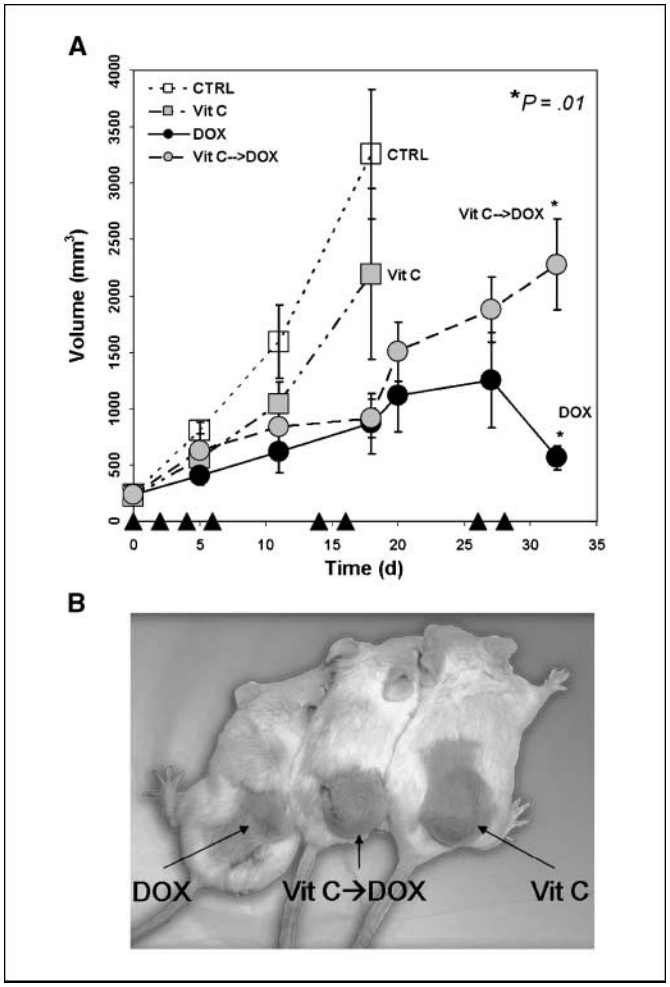

Figure 3. Vitamin C attenuates the cytotoxicity of antineoplastic agents *in vivo*. ICR SCID mice were xenografted with RL cells in the right flank. Cohorts of five mice were treated with vehicle control, 1 mg/kg doxorubicin, 250 mg/kg dehydroascorbic acid, or dehydroascorbic acid 2 h before doxorubicin on days as noted (▲), and tumor volumes were measured (*A*). Statistics were single-factor ANOVA. Representative animals on day 28 from the left are doxorubicin, dehydroascorbic acid before doxorubicin, and dehydroascorbic acid. The background color was slightly modified to enhance contrast, and the image was converted from color to black and white. *B,* two separate experiments were conducted in quintuplicate, and the results shown represent the mean values and SDs of a single representative experiment.

on ice until assayed. For the detection of mitochondrial membrane potential ($\Delta\psi_m$), cells were incubated with 10 μg/mL JC-1 (Invitrogen) for 5 min at 37°C. Cells were washed thrice in PBS, resuspended in normal growth media, and incubated under normal growth conditions for 1 h before being loaded with vitamin C. After loading, cells were exposed to antineoplastic agents at IC_{75} concentrations in media for 6 h at 37°C. All flow cytometric acquisitions were performed on a BD FACSCalibur system (Becton Dickinson) with data analysis performed using FlowJo analysis software, version 6.0 (Tree Star).

Results

RL and K562 cells take up only dehydroascorbic acid. Two cell lines [RL, derived from a patient with transformed B-cell follicular lymphoma, and K562, derived from a patient with myeloblastic chronic myelogenous leukemia (CML)] were treated with dehydroascorbic acid and ascorbic acid (Fig. 1). As has been noted in other hematopoietic cell lines (8, 11), only dehydroascorbic acid, and not ascorbic acid, was taken up by K562 and RL cells to any appreciable extent. In each case, the cells accumulated substantial intracellular concentrations of ascorbic acid in a dose-dependent manner, achieving intracellular ascorbic acid concentrations up to 8.5 mmol/L in RL and 18 mmol/L in K562 cells (Fig. 1*A* and *C*). The kinetics of dehydroascorbic acid uptake was typical of other cell lines. Kinetic analysis supported a two-phase uptake model of dehydroascorbic acid with the initial rate of uptake thought to correspond to transport through the glucose transporters, and the slower second rate thought to correspond to the intracellular conversion of dehydroascorbic acid to ascorbic acid (Fig. 1*B* and *D*; ref. 11).

Vitamin C protects RL and K562 cells from the cytotoxic effects of antineoplastic agents. To test whether vitamin C exposure could protect cells from chemotherapeutic agents in which the generation of ROS is considered to contribute to cytotoxicity, we used chemotherapeutic agents that act through various mechanisms: vincristine is a *Vinca* alkaloid that binds tubulin and inhibits cell division; doxorubicin is an anthracycline that intercalates into DNA and also inhibits topoisomerase II; methotrexate (MTX) inhibits dihydrofolate reductase, leading to inhibition of DNA synthesis; cisplatin forms DNA cross-link adducts; imatinib mesylate is a selective tyrosine kinase inhibitor that inhibits bcr-abl, the constitutively activated tryosine kinase that plays a central pathophysiologic role in CML (25, 26). Although ROS have been reported to play a role in mediating the therapeutic cytotoxic effects of cisplatin and doxorubicin (4, 5), they are not considered to play an important mechanistic role in the efficacy of the other agents tested.

When cells were exposed to dehydroascorbic acid to increase intracellular ascorbic acid levels, washed, and subsequently treated with antineoplastic agents at concentrations corresponding to an IC_{75}, we found that the cytotoxicity of all agents tested, regardless of mechanism of action, was reduced (Fig. 2*A*). The absolute magnitude of the reductions ranged from 11% (VCR) to 27% (MTX) and corresponded to relative reduction in cytotoxicity of 30% to 70%. Cytotoxicity was reduced more in cells that had higher intracellular concentrations of vitamin C, indicating a dose-dependent effect. High intracellular concentrations of vitamin C, however, had no effect on either the viability or proliferation of untreated cells. RL cells pretreated with vitamin C showed a similar reduction in chemotherapy-mediated cytotoxicity, with the exception that imatinib was not cytotoxic (Fig. 2*B*). The lack of imatinib cytotoxicity was expected because RL cells are not transformed by the bcr-abl kinase. These findings were broadened to show that increased intracellular vitamin C also antagonizes the effects of chemotherapeutic agents, as measured by the ability of drug-treated cells to form colonies in methylcellulose (Fig. 2*C*). This indicates that the protection conferred by vitamin C treatment extends to cells with *in vitro* clonogenic potential. These results also show that vitamin C can attenuate the cytotoxicity of antineoplastic drugs in cells of both myeloid and lymphoid lineage and reduces the effectiveness of agents that act through a broad range of mechanisms.

Vitamin C antagonizes doxorubicin cytotoxicity in murine RL xenografts. We used RL cells to generate xenograft tumors in ICR SCID mice to determine whether vitamin C attenuates the effects of chemotherapy *in vivo*. As expected, doxorubicin treatment reduced tumor growth compared with untreated mice (Fig. 3*A*).

Whereas treatment with dehydroascorbic acid alone did not appreciably alter tumor growth compared with untreated mice

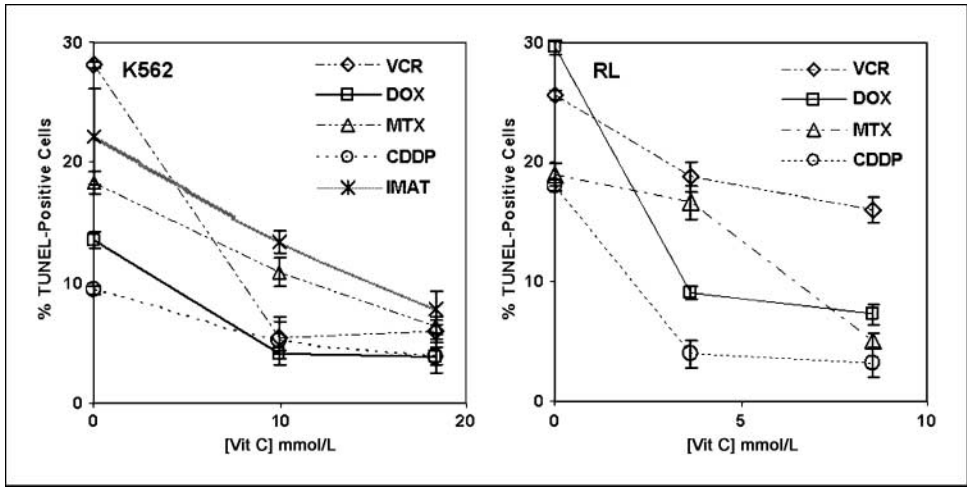

Figure 4. Vitamin C reduces the percentage of apoptotic cells. K562 and RL cells were treated with dehydroascorbic acid to achieve varying concentrations of intracellular vitamin C. The cells were washed and treated with antineoplastic agents for up to 48 h. Apoptosis was measured by TUNEL after 48 h of treatment. Results are expressed as a percentage of TUNEL-positive cells. Three separate experiments were conducted in triplicate. *Points,* mean values of a single representative experiment; *bars,* SD.

(P = 0.114), mice treated with dehydroascorbic acid 2 hours before doxorubicin administration had tumors that were about four times larger than tumors in mice treated with doxorubicin alone at day 32 (P = 0.01; Fig. 3*A* and *B*). At day 32, because mice treated with dehydroascorbic acid before doxorubicin administration had average tumor volumes of >2,000 mm^3, the animals were sacrificed to assess intratumor doxorubicin and ascorbic acid levels coincident with animals treated only with doxorubicin. The intratumor concentration of vitamin C, in dehydroascorbic acid–treated animals was 5.5 ± 0.9 mmol/L compared with 0.65 ± 0.08 mmol/L in vehicle-treated mice, indicating that dehydroascorbic acid treatment effectively increased the intratumor concentration of vitamin C. The doxorubicin concentration in the tumors of vitamin C–treated mice was 9.8 ± 1.1 $\mu g_{dox}/mg_{tumor}$. In the tumors of mice that did not receive vitamin C, the doxorubicin concentration was 9.9 ± 2.0 $\mu g_{dox}/mg_{tumor}$. This shows that dehydroascorbic acid treatment did not alter tumor cell uptake of doxorubicin. Therefore, vitamin C given before doxorubicin

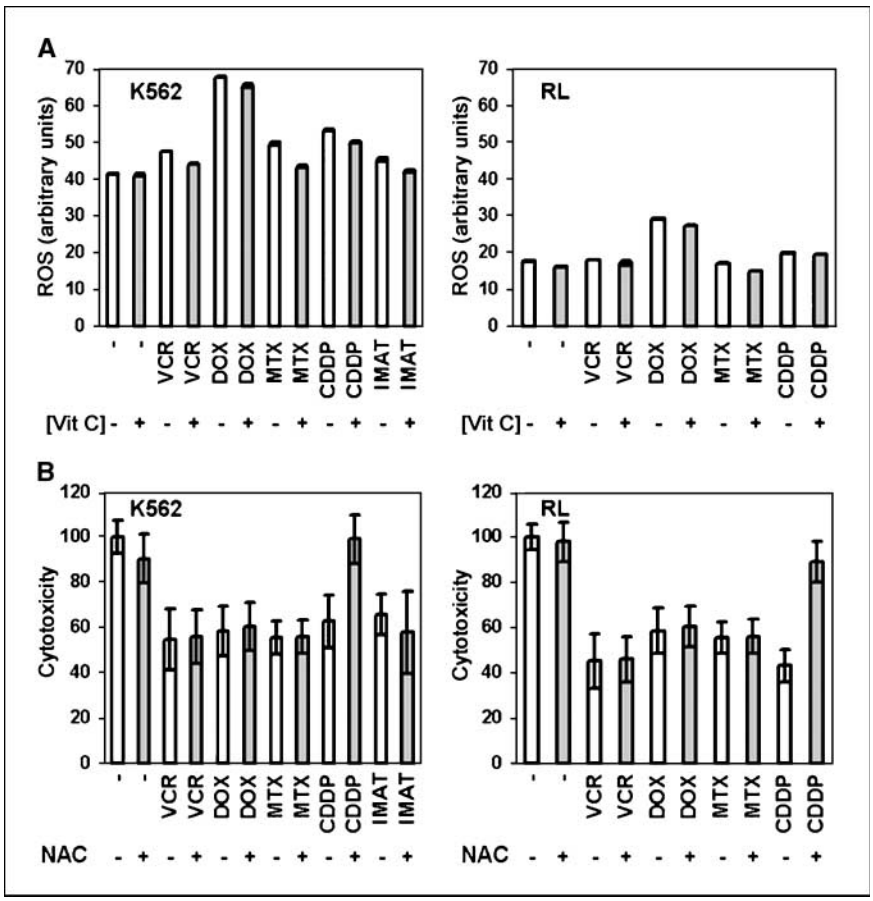

Figure 5. Vitamin C has minimal effects on intracellular ROS and has a different effect on cytotoxicity than *N*-acetyl cysteine. K562 and RL cells were either untreated (−) or treated (+) to internal vitamin C (*Vit C*) concentrations of 18 and 8.5 mmol/L, respectively, and treated with antineoplastic agents for 6 h. ROS was measured by incubation with 0.1 μg/mL CM-H2DCFDA (*A*). Results are expressed as median fluorescence intensity (arbitrary units). K562 and RL cells were treated with antineoplastic agents for 48 h in the presence (+) or absence (−) of 25 mmol/L *N*-acetylcysteine (*NAC; B*). Cell viability was measured by Trypan Blue exclusion, and results are expressed as a percentage of cells not concomitantly treated with *N*-acetylcysteine. Three separate experiments were conducted in triplicate. *Columns,* mean values of a representative experiment; *bars,* SD.

substantially reduced the effectiveness of treatment and suggests, in a preclinical model, that vitamin C might interfere with cancer chemotherapy inside the cell rather than affecting drug clearance or tumor uptake.

Vitamin C decreases apoptosis but does not affect P-glycoprotein expression. We next investigated the mechanism by which vitamin C attenuates the cytotoxic effects of antineoplastic agents. Pretreatment of cells with vitamin C led to a dose-dependent decrease in apoptosis with all agents tested. At the highest concentrations of intracellular vitamin C, apoptosis was reduced between 37% and 82%, as measured by TUNEL (Fig. 4). The possibility that vitamin C treatment alters the effectiveness of chemotherapeutic agents by up-regulating the P-glycoprotein (Pgp) efflux pump was excluded by the finding that Pgp expression on the cell surface was unaltered by vitamin C treatment (Supplementary Fig. S1*A*). This result is consistent with our finding that intratumor doxorubicin concentrations are similar between untreated mice and mice treated with vitamin C.

Vitamin C elimination kinetics is not affected by treatment with antineoplastic agents. To determine whether antineoplastic agents altered the kinetics of vitamin C elimination, K562 and RL were loaded with L-[^{14}C]ascorbic acid by treatment with L-[^{14}C]dehydroascorbic acid. The intracellular concentration of [^{14}C] in cell pellets was measured over 24 h (Supplementary Fig. S1*B*). There was no significant difference in [^{14}C] elimination with any of the agents tested and untreated control cells. These findings are consistent with the observation that there was no difference in vitamin C concentration within the xenogenic tumors of mice treated with doxorubicin. These results suggest that the protective effect of vitamin C is not a result of improved antioxidant retention.

Vitamin C treatment results in small reductions in ROS. When K562 and RL cells were treated with antineoplastic agents, we found that doxorubicin and, to a lesser extent, cisplatin increased the intracellular levels of ROS, as has been previously reported (4, 5), whereas other agents had little discernible effect on ROS levels (Fig. 5*A*). In cells treated with chemotherapeutic drugs, as well as in untreated cells, pretreatment of cells with dehydroascorbic acid caused a small reduction in intracellular levels of ROS. Although the reduction in intracellular ROS levels was statistically significant ($P \leq 0.02$), the magnitude of the reduction in ROS seemed to be minor compared with the reduction in cytotoxicity. To further investigate the role of ROS in attenuating cytotoxicity, we treated K562 and RL cells with *N*-acetylcysteine. *N*-acetylcysteine and vitamin C both help to replenish important ROS-quenching thiols, such as glutathione, that play an important role in neutralizing ROS and are both considered important antioxidants (27). When cells were treated with *N*-acetylcysteine before and during exposure to chemotherapeutic agents, only cytotoxicity mediated by cisplatin was reduced (Fig. 5*B*). Although the ability of thiols to protect cells from cisplatin has been described previously (28), these results show that vitamin C has a more general cytoprotective profile, suggesting a different mechanism of action that could be unrelated to the antioxidant effects of vitamin C.

Vitamin C treatment helps to preserve mitochondrial membrane potential. Vitamin C is taken up by the mitochondria and is able to preserve mitochondrial membrane potential after exposure to apoptotic stimuli, such as fas ligand and γ irradiation (18, 29, 30). We assessed mitochondrial membrane potential in RL and K562 cells treated with antineoplastic agents and found that all agents tested led to a rapid reduction in mitochondrial membrane potential (Fig. 6). Pretreatment with dehydroascorbic acid prevented early chemotherapy-induced mitochondrial membrane depolarization with all agents tested. Mitochondrial membrane potentials in either RL or K562 cells 6 hours after treatment with chemotherapeutic drugs were similar to untreated control cells after treatment with vitamin C. These results were consistent with the notion that vitamin C exerts its inhibitory effects against antineoplastic drugs by protecting cells from mitochondrial membrane depolarization. To evaluate this possibility, we studied the effects of vitamin C on actinonin, an antibiotic that targets peptide deformylase (31), an enzyme that processes proteins encoded by mitochondrial DNA in eukaryotic cells. Actinonin has broad antineoplastic activity *in vitro*, and previous investigation suggested that mitochondrial membrane depolarization plays a central role in mediating differential activity in normal and transformed cells (31). As with other cell lines, actinonin exposure led to mitochondrial membrane depolarization in K562 and RL cells that was inhibited by pretreatment with vitamin C (Fig. 6). Although the approximate 15% maximal reduction of $\Delta\psi_m$ observed with antineoplastic treatment at 6 hours may seem to be a mild effect, we have recently shown in a lymphoma model that targeting Bcl-2 family members with the BH3-mimetic AT-101 leads to a maximal 10% reduction in $\Delta\psi_m$ at 12 hours and that this

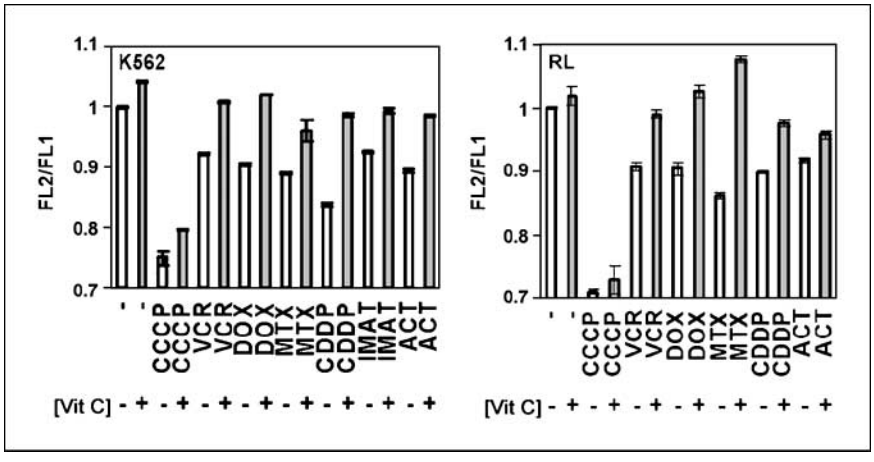

Figure 6. Vitamin C antagonizes mitochondrial membrane depolarization mediated by antineoplastic agents. Cells were stained with 10 μg/mL JC-1, washed, and equilibrated for 1 h at 37°C. K562 and RL cells either untreated (−) or loaded (+) with 18 and 8.5 mmol/L vitamin C (*Vit C*), respectively, were exposed to antineoplastic agents for 6 h, and mitochondrial membrane potential was measured as the ratio of 530 to 585 nm fluorescence. Maximal depolarization was determined by exposure to 100 nmol/L carbonyl cyanide 3-chlorophenylhydrazone (*CCCP*). The results are representative of at least three separate experiments. The differences in mitochondrial membrane potential between chemotherapy-treated cells with and without pretreatment with vitamin C were statistically significant ($P < 0.003$ in RL cells, $P < 0.0005$ in K562 cells; Student's *t* test). Three separate experiments were conducted in triplicate. *Columns,* mean values of a representative experiment; *bars,* SD.

apparently mild depolarization induced further depolarization and apoptosis at later time points both *in vitro* and *in vivo* (32).

Discussion

The finding that vitamin C antagonized the cytotoxic effects of such a wide range of antineoplastic agents was unexpected. We had originally hypothesized that vitamin C would antagonize the cytotoxic effects of antineoplastic agents that use ROS to mediate some activity. Our data indicate, however, that pretreatment with dehydroascorbic acid attenuates the antineoplastic activity of chemotherapeutic agents, including highly selective agents, that do not lead to ROS generation. Whereas it is possible that small effects on ROS may exert disproportionate effects on cytotoxicity, it seems more likely that other mechanisms are involved. A recent study suggested that both vitamin C and *N*-acetylcysteine may diminish tumorigenesis through effects on hypoxia inducible factor-1–related pathways rather than by preventing direct oxidative damage to DNA (33). Whereas many antineoplastic agents target DNA, our findings suggest that the spectrum of activity of vitamin C is substantially broader than *N*-acetylcysteine, which antagonized only cisplatin cytotoxicity. It has been shown that vitamin C antagonizes fas ligand-mediated mitochondrial membrane depolarization (30). Previous studies have also shown that cisplatin, doxorubicin, etoposide, and several other agents result in mitochondrial membrane depolarization (34, 35). We have expanded the number of antineoplastic agents that lead to rapid mitochondrial membrane depolarization and have found that this depolarization can be inhibited by vitamin C. A substantial body of work has shown that mitochondrial membrane depolarization plays an important role in regulating cell death (36, 37). Thus, mitochondrial membrane depolarization may be a common mechanism that contributes to the cytotoxicity of many chemotherapeutic agents, including highly selective agents, such as imatinib.

Whereas the effects of vitamin C on intracellular targets in the nucleus or the cytoplasm, for example, cannot be excluded, we hypothesize that the mitochondria are an important site of action for vitamin C. Vitamin C enters the mitochondria, and recent work shows that transport into the mitochondria occurs through the glucose transporters (18, 38, 39). Although treatment with antineoplastic agents does not generally lead to increased intracellular ROS, it is possible that vitamin C in the mitochondria plays a role in quenching local ROS. Alternatively, vitamin C in the mitochondria may help to stabilize respiratory electron transport, thereby preserving the most efficient means of energy generation and maintaining overall cellular fitness. The role of vitamin C in protecting mitochondria from cytotoxic agents is supported by the finding that vitamin C antagonizes the cytotoxic effects of actinonin. Actinonin specifically inhibits human mitochondrial peptide deformylase, an enzyme whose central function seems to be the deformylation of the 13 proteins encoded by mitochondria, all of which play vital roles in electron transport and subsequent energy generation (31). Although the precise mechanism and kinetics through which vitamin C mediates its protective effects remain unknown, the finding that vitamin C protects cells from actinonin toxicity suggests that the ability of vitamin C to stabilize the mitochondria plays a central role in exerting its cytoprotective profile.

Taken together, our data show that pharmacologic concentrations of intracellular vitamin C antagonize the therapeutic cytotoxic effects of antineoplastic chemotherapeutic agents. This finding could have important clinical relevance given the wide use of vitamin C as a nutritional supplement. These results suggest that supplementary vitamin C may have adverse consequences in patients who are receiving cancer chemotherapy. Such a supposition, however, would need confirmation in other therapeutic models or in human trials. Whereas we have used dehydroascorbic acid in our experimental model to achieve high intracellular concentrations of vitamin C, there is evidence from murine models of human prostate cancer xenografts that ascorbic acid may be converted to dehydroascorbic acid in the peritumor milieu, leading to higher intracellular concentrations of vitamin C (40). Although we studied cells derived from hematopoietic malignancies, intracellular vitamin C accumulation has been observed in cell lines derived from solid tumors, as well as solid tumor xenografts(40), suggesting that our observations are unlikely to be unique to hematologic cancers. This is supported by clinical evidence showing that intratumor concentrations of vitamin C are higher in cancer cells than in adjacent normal tissue in patients who did not take supplementary vitamin C (41). It was notable that the concentration of vitamin C measured in the tumors of the mice in this study was similar to the concentration of vitamin C that can be achieved in human leukocytes with oral vitamin C supplementation (42, 43), suggesting that our study conditions were relevant to clinical conditions. Therefore, it is possible that vitamin C supplementation may alter the effectiveness of commonly used chemotherapy agents and adversely influence treatment outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 4/21/2008; revised 7/3/2008; accepted 7/25/2008.

Grant support: New York State Department of Health (M.L. Heaney and D.W. Golde), NIH grants CA30388 (D.W. Golde) and CA55349 (D.A. Scheinberg), Leukemia and Lymphoma Society Scholar in Research Award (O.A. O'Connor), Doris Duke Distinguished Clinical Scientist Award (D.A. Scheinberg), and Lewis Family Foundation (M.L. Heaney).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work is dedicated to the memory of our friend and colleague, David W. Golde, M.D.

We thank Dr. William Bornmann for supplying the imatinib mesylate and Dr. Anna Kenney for critical review of the manuscript.

References

1. Cameron E, Pauling L, Leibovitz B. Ascorbic acid and cancer: a review. Cancer Res 1979;39:663–81.
2. Golde DW. Vitamin C in cancer. Integr Cancer Ther 2003;2:158–9.
3. Cameron E, Pauling L. Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A 1978;75:4538–42.
4. Lutzky J, Astor MB, Taub RN, et al. Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells. Cancer Res 1989;49:4120–5.
5. Cossarizza A, Franceschi C, Monti D, et al. Protective effect of *N*-acetylcysteine in tumor necrosis factor-α-induced apoptosis in U937 cells: the role of mitochondria. Exp Cell Res 1995;220:232–40.
6. Dai J, Weinberg RS, Waxman S, Jing Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 1999;93:268–77.

7. Grad JM, Cepero E, Boise LH. Mitochondria as targets for established and novel anti-cancer agents. Drug Resist Updat 2001;4:85–91.
8. Karasavvas N, Carcamo JM, Stratis G, Golde DW. Vitamin C protects HL60 and U266 cells from arsenic toxicity. Blood 2005;105:4004–12.
9. Tsukaguchi H, Tokui T, Mackenzie B, et al. A family of mammalian Na+-dependent L-ascorbic acid transporters. Nature 1999;399:70–5.
10. Vera JC, Rivas CI, Fischbarg J, Golde DW. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature 1993;364:79–82.
11. Vera JC, Rivas CI, Zhang RH, Farber CM, Golde DW. Human HL-60 myeloid leukemia cells transport dehydroascorbic acid via the glucose transporters and accumulate reduced ascorbic acid. Blood 1994;84:1628–34.
12. Guaiquil VH, Farber CM, Golde DW, Vera JC. Efficient transport and accumulation of vitamin C in HL-60 cells depleted of glutathione. J Biol Chem 1997;272:9915–21.
13. Guaiquil VH, Vera JC, Golde DW. Mechanism of vitamin C inhibition of cell death induced by oxidative stress in glutathione-depleted HL-60 cells. J Biol Chem 2001;276:40955–61.
14. Galleano M, Aimo L, Puntarulo S. Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. Toxicol Lett 2002;133:193–201.
15. Peterkofsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 1991;54:1135–40S.
16. Levine M, Dhariwal KR, Washko P, et al. Ascorbic acid and reaction kinetics *in situ*: a new approach to vitamin requirements. J Nutr Sci Vitaminol Tokyo 1992; Spec No:169–72.
17. Rebouche CJ. Ascorbic acid and carnitine biosynthesis. Am J Clin Nutr 1991;54:1147–52S.
18. Li X, Cobb CE, Hill KE, Burk RF, May JM. Mitochondrial uptake and recycling of ascorbic acid. Arch Biochem Biophys 2001;387:143–53.
19. Li X, Cobb CE, May JM. Mitochondrial recycling of ascorbic acid from dehydroascorbic acid: dependence on the electron transport chain. Arch Biochem Biophys 2002;403:103–10.
20. Lutsenko EA, Carcamo JM, Golde DW. Vitamin C prevents DNA mutation induced by oxidative stress. J Biol Chem 2002;277:16895–9.
21. May JM, Mendiratta S, Qu ZC, Loggins E. Vitamin C recycling and function in human monocytic U-937 cells. Free Radic Biol Med 1999;26:1513–23.
22. Vera JC, Rivas CI, Velasquez FV, Zhang RH, Concha II, Golde DW. Resolution of the facilitated transport of dehydroascorbic acid from its intracellular accumulation as ascorbic acid. J Biol Chem 1995;270:23706–12.
23. Agus DB, Gambhir SS, Pardridge WM, et al. Vitamin C crosses the blood-brain barrier in the oxidized form through the glucose transporters. J Clin Invest 1997;100:2842–8.
24. Huang J, Agus DB, Winfree CJ, et al. Dehydroascorbic acid, a blood-brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke. Proc Natl Acad Sci U S A 2001;98:11720–4.
25. Bertino JR, O'Connor OA. Oncologic Disorders. In: Carruthers SG, Hoffman BB, Melmon KL, Nierenberg DW, editors. Clinical Pharmacology. 4th ed. New York: McGraw-Hill; 2000. p. 799–871.
26. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996;2:561–6.
27. Meister A. Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 1994;269:9397–400.
28. Yuhas JM, Spellman JM, Culo F. The role of WR-2721 in radiotherapy and/or chemotherapy. Cancer Clin Trials 1980;3:211–6.
29. Perez-Cruz I, Carcamo JM, Golde DW. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. Blood 2003;102:336–43.
30. Witenberg B, Kletter Y, Kalir HH, et al. Ascorbic acid inhibits apoptosis induced by X irradiation in HL60 myeloid leukemia cells. Radiat Res 1999;152:468–78.
31. Lee MD, She Y, Soskis MJ, et al. Human mitochondrial peptide deformylase, a new anticancer target of actinonin-based antibiotics. J Clin Invest 2004;114:1107–16.
32. Paoluzzi L, Gonen M, Gardner JR, et al. Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in *in vitro* and *in vivo* models of B-cell lymphoma. Blood 2008;111:5350–8.
33. Gao P, Zhang H, Dinavahi R, et al. HIF-Dependent Antitumorigenic Effect of Antioxidants *In vivo*. Cancer Cell 2007;12:230–8.
34. Decaudin D, Geley S, Hirsch T, et al. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res 1997;57:62–7.
35. Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000;92:1042–53.
36. Marchetti P, Castedo M, Susin SA, et al. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996;184:1155–60.
37. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626–9.
38. Sagun KC, Carcamo JM, Golde DW. Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury. FASEB J 2005;19:1657–67.
39. Fujimoto Y, Matsui M, Fujita T. The accumulation of ascorbic acid and iron in rat liver mitochondria after lipid peroxidation. Jpn J Pharmacol 1982;32:397–9.
40. Agus DB, Vera JC, Golde DW. Stromal cell oxidation: a mechanism by which tumors obtain vitamin C. Cancer Res 1999;59:4555–8.
41. Langemann H, Torhorst J, Kabiersch A, Krenger W, Honegger CG. Quantitative determination of water- and lipid-soluble antioxidants in neoplastic and non-neoplastic human breast tissue. Int J Cancer 1989;43:1169–73.
42. Bergsten P, Amitai G, Kehrl J, Dhariwal KR, Klein HG, Levine M. Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes. Depletion and reaccumulation. J Biol Chem 1990;265:2584–7.
43. Levine M, Conry-Cantilena C, Wang Y, et al. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc Natl Acad Sci U S A 1996;93:3704–9.