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A chirality-dependent action of vitamin C in suppressing Kirsten rat sarcoma mutant tumor growth by the oxidative combination: Rationale for cancer therapeutics

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Kirsten rat sarcoma (KRAS) mutant cancers, which constitute the vast majority of pancreatic tumors, are characterized by their resistance to established therapies and high mortality rates. Here, we developed a novel and extremely effective combinational therapeutic approach to target KRAS mutant tumors through the generation of a cytotoxic oxidative stress. At high concentrations, vitamin C (VC) is known to provoke oxidative stress and selectively kill KRAS mutant cancer cells, although its effects are limited when it is given as monotherapy. We found that the combination of VC and the oxidizing drug arsenic trioxide (ATO) is an effective therapeutic treatment modality. Remarkably, its efficiency is dependent on chirality of VC as its enantiomer D-optical isomer of VC (D-VC) is significantly more potent than the natural L-optical isomer of VC. Thus, our results demonstrate that the oxidizing combination of ATO and D-VC is a promising approach for the treatment of KRAS mutant human cancers.

Introduction

Kirsten rat sarcoma (KRAS) mutant cancers represent highly malignant oncologic disorders with poor clinical outcomes. Activating KRAS mutations occur predominately in pancreatic cancer and are found in almost all (95%) of pancreatic ductal adenocarcinomas (PDACs).[1] They are also common in biliary tract (33%), colorectal (32%), lung (19%), and ovarian (17%) cancers.[2] However, no effective therapies have been developed to treat KRAS mutant cancers, because the KRAS gene encodes a small GTPase with no distinctive pocket for targeting.[3,4]

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Abbreviations: ATO: arsenic trioxide; CI: combinational index; DHA: dehydroascorbate; D-VC: D-optical isomer of VC; KRAS: Kristen rat sarcoma; L-VC: L-optical isomer of VC; PDAC: pancreatic ductal adenocarcinoma; ROS: reactive oxygen species; VC: vitamin C
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What's new?

A new combination therapy could effectively fight Kirsten rat sarcoma (KRAS)-mutant cancers. Although KRAS mutations frequently crop up in human cancers, therapies targeting them have proved difficult to find. In our study, the authors tested a novel approach to kill the cancer cells by inducing oxidative stress with vitamin C and arsenic trioxide. The combo successfully killed cancer cells, with one surprising twist. When the researchers tested the therapy in a mouse xenograft model, they found that the tumor-shrinking action depended on the chirality of the vitamin C molecule. The D isomer showed much greater efficacy than the naturally occurring L isomer.

Hence, a specific and potent means of targeting this highly malignant oncogenic pathway is one of the most challenging and demanding tasks in oncology.

KRAS mutations lead to hyperactivation of the mitogen-activated protein kinase and phosphatidylinositol-3-OH kinase pathways, which accelerates cancer cell growth and proliferation. KRAS-dependent transformation results in a distinctive cellular metabolism with an unbalanced redox state due to high glucose consumption[5,6] leading to elevated generation of reactive oxygen species (ROS).[7] A vulnerable redox state has been actively explored as a therapeutic avenue to target KRAS mutant cancer cells by inducing oxidative stress.[8,9] Vitamin C ([VC] or also known as ascorbic acid) has attracted attention for its anticancer activity since 1974[10,11] and a recent study revealed its selective action to induce oxidative stress at high concentrations in KRAS mutant cancer cells.[9] A selective effect of VC on the cancer cells was linked to an elevated glucose uptake mediated by abundant expression of the glucose transporter GLUT1.[12,13] In the body, VC is oxidized to dehydroascorbate (DHA), which is actively absorbed by the cancer cells through GLUT1 because of its structural resemblance to glucose. Inside the cell, DHA is reduced back to VC at the expense of oxidizing glutathione (GSH). As a result, an extensive DHA reduction in KRAS mutant cells leads to oxidative stress.[9]

Materials and Methods

Cell culture and cell lines

The human HCT116 (Research Resource Identifier [RRID]: CVCL_0291), RKO (RRID:CVCL_0504), HT-29 (RRID:CVCL_0320), human pancreatic cancer (HPAC) (RRID: CVCL_3517) and MRC-5 (RRID:CVCL_0440) cell lines were obtained from the American Type Cell Culture Collection (ATCC, Rockville, MD). Mouse AK192 and human HKh-2 (RRID:CVCL_9797) cells derived from HCT116 cell line by deleting the KRAS mutant allele were described previously and provided from the original sources by Drs. Haoqiang Ying and Senji Sirasawa.[6,14] All studied human cell lines have been authenticated using the short tandem repeat profiling within the last 3 years and all culture experiments were performed with mycoplasma-free cells. Cells were cultured at 37°C in a humidified incubator and maintained in the Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (catalog #DFL13 from Caisson Labs) containing 17.5 mM of glucose and supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and penicillin (100 units/ml)-streptomycin (100 μg/ml). For the drug treatments, the cells were split to 50% confluence previous day in the same medium containing 17.5 mM glucose supplemented with 10% FCS, 2 mM glutamine and penicillin (100 units/ml)–streptomycin (100 μg/ml); the cells were then incubated with the indicated concentrations of the drugs. For HCT116 cells to obtain 50% confluence, 500,000 (a half million) cells were plated into six-well cell culture dishes in 2 ml cell culture medium a day prior the drug treatment.

Drug preparations

The following drugs were obtained from Sigma: VC (ascorbic acid, catalog #A7506), D-vitamin C (D-[−] isoascorbic acid, catalog #856061) and arsenic trioxide ([ATO], catalog #A1010). We prepared 340 mM stock of VC by dissolving 30 g of VC in 400 ml of the autoclaved phosphate-buffered saline (PBS), stirring and then slowly adding 39 g of sodium bicarbonate (NaHCO3, catalog #S5761) while continuously stirring for 5 min as described previously.[15] The addition of sodium bicarbonate changed the acidic pH of VC from pH 2.3 to a physiological pH 7.35 that was critical for injections into mice. After checking pH, we adjusted the VC solution to 500 ml by PBS. The solution was further filtered, aliquoted and stored in a −20°C freezer. Similar steps were taken to prepare 340 mM stock of D-optical isomer of VC (D-VC). The stock of ATO was prepared at a 330 mM concentration by dissolving 13 g of ATO in 1 N sodium hydroxide (NaOH) and adjusting the total volume to 200 ml. After ATO had been dissolved, it was filtered, aliquoted and stored in a −20°C freezer.

Apoptosis assay

Cell apoptosis was evaluated by flow cytometry. The cells were harvested 48 or 72 hr after treatment with VC, ATO or with both compounds. The cells were resuspended at a density of 1×10^6 cells/ml in 1× binding buffer after three PBS washes. The cells were then stained with fluoresce inisothiocyanate (FITC)-Annexin-V and propidium iodide (PI) for 30 min at 4°C in the dark using the FITC-Annexin-V: PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). The cells were analyzed using BD FACSCanto II flow cytometer according to the manufacturer's instructions to detect early and late apoptosis. All experiments were performed in triplicate.

Measurement of intracellular ROS

HCT116 cells were treated with VC, D-VC, ATO or combination of VC or D-VC with ATO for 72 hr and then collected by

trypsinization and incubated with MitoSOX (2 μM) for 30 min at 4°C in the dark before being analyzed by flow cytometry.

Animal study and immunohistochemical analysis

The animal study complied with the protocol that has been approved by the University of Texas MD Anderson Cancer Center (#0000193). A total 1.5 million HCT116 cancer cells were transplanted into nude mice subcutaneously, and the drugs were injected after 10 days, when tumors became larger than 0.8 cm in diameter. The excised xenograft tumors and livers were examined by the immunohistochemical analysis. Formalin-fixed, paraffin embedded tissues were stained with hematoxylin and eosin. For immunohistochemical analysis, tumor tissue slides were incubated in a microwave oven in citrate buffer (pH 6.0) for 15 min for epitope retrieval. The slides were then incubated with cleaved Caspase 3 (Cell Signaling Technology, Danver, MA, product number 9661, 1:100 dilution) at 4°C for 18 hr. After the secondary antibody incubation, the slides were dehydrated and stabilized with mounting medium and the images were acquired with a Leica DM1000 microscope. Cleaved Caspase 3-positive cells were counted at ×400 magnification in five or six randomly selected areas in tumor samples.

Dual drug combination assay

HCT116 cells were plated in 96-well plates and treated with various concentrations of VC, either alone or in combination with ATO, for 48 hr. Cell viability was determined using a colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell culture medium was replaced prior to the application of MTT solution because the presence of VC in the medium interfered with the dye staining. Synergistic effects were determined using the Chou-Talalay method to calculate the combinational index (CI).[16,17]

Statistical analysis

Statistical significance was determined using an unpaired Student's *t*-test or analysis of variance by GraphPad Prism 6 (Graph Pad Software, San Diego, CA). Data were considered to be significant when p values are <0.05. Sample sizes and animal numbers were chosen on the basis of the results of pilot studies performed in the laboratory.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Results

The ATO and VC combination is cytotoxic to the KRAS mutant cancer cells

We assumed that if VC alone is sufficient to induce oxidative stress in KRAS mutant cancer cells, the combination of VC and another oxidizing reagent would effectively enhance

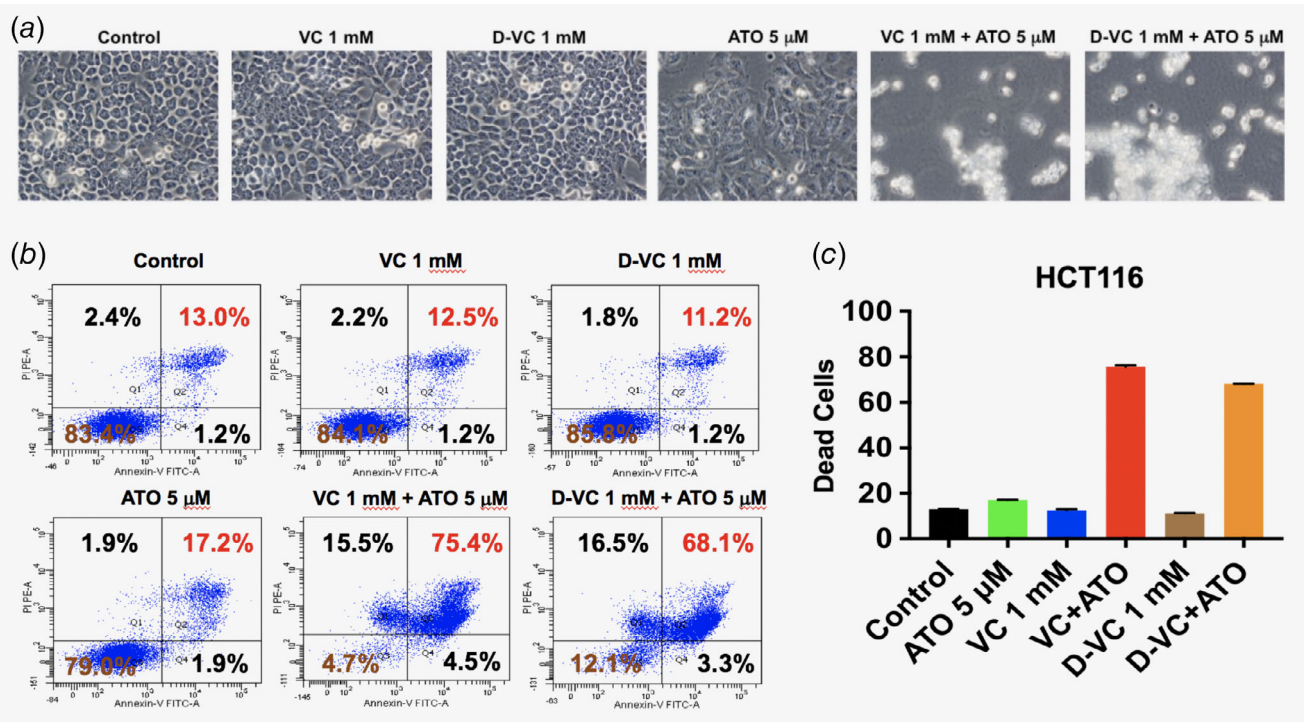

Figure 1. Induction of apoptosis by the arsenic trioxide (ATO) and vitamin C (VC) combination in the Kirsten rat sarcoma mutant cancer cells. (*a*) The images of HCT116 human cancer cells after the treatment with phosphate-buffered saline (control), 1 mM VC, 1 mM D-optical isomer of VC (D-VC), 5 μM ATO, 1 mM VC + 5 μM ATO or 1 mM D-VC and 5 μM ATO for 48 hr. (*b*) The apoptotic assay histograms of the cells described in (*a*) using Annexin-V and propidium iodide staining and flow cytometric analysis. (*c*) The graphical presentation of the apoptotic analysis shown in (*b*).

oxidative stress and lead to cytotoxic effects with high selectivity toward the cancer cells. We selected a potent oxidizing drug ATO as the second compound, which is effective in treating acute promyelocytic leukemia.[18] To test this hypothesis, human HCT116 (KRAS mutant) cancer cells were treated with 1 mM VC, 5 μM ATO or with both compounds. We observed a substantial cell death only in cells treated with the drug combination, as visualized by a massive detachment of cells (Fig. 1*a*), suggesting that the ATO/VC combination had cytotoxic impact.

An analysis of the detached cells using the Annexin-V/PI flow cytometric apoptotic assay indicated that the cells were apoptotic.[19] The cells treated with VC alone showed basal cytotoxicity of 12.5% as compared to 13% in the nontreated control cells (Figs. 1*b* and 1*c*). Previously, it was shown that the cytotoxic effect of VC in HCT116 cells was glucose dependent and that VC was highly effective when cells were maintained in a low glucose (2 mM) medium.[9] In the cell culture medium containing a physiological glucose concentration (5.5 mM), VC alone did not induce a cytotoxic effect. Besides, ATO alone induced only a moderate increase in cytotoxicity (17.2%), but the combination of ATO and VC induced a potent apoptotic response with 75.4% of cells undergoing apoptosis after 48 hr of treatment (Figs. 1*b* and 1*c*). The combined action of ATO and VC resulted in a synergistic apoptotic impact as indicated by a substantial (45.7%) increase in cell death above their additive cytotoxic effect (29.7%). The synergistic action of ATO and VC was further supported by the results of our CI study, which revealed CI values less than 1 (CI < 1), as calculated by the Chou-Talalay method,[16,17] shown in Supporting Information Figure S1. Of note, the effect of VC was not restricted to its natural L-optical isomer of VC (L-VC) since its enantiomer (D-VC) showed similar behavior inducing potent cytotoxicity (68.1%) in cells only in combination with ATO (Figs. 1*b* and 1*c*). These findings indicate that both optical isomers of VC (L-VC or D-VC) act synergistically with the oxidizing drug ATO to induce the effective elimination of HCT116 cancer cells that are known to carry the oncogenic KRAS^{G13D} allele.[14]

Sensitivity to the oxidative combination is dependent on the oncogenic KRAS expression

To determine whether the observed pronounced sensitivity of HCT116 (KRAS^{G13D} mutant) cancer cells to the combination of ATO/VC is indeed linked to the oncogenic KRAS allele, we used an isogenic cell line HKh2, which is derived from HCT116 cells by deleting KRAS mutant alelle.[14] The combination of ATO and VC in the HKh2 cells induced a much weaker apoptotic response, without a potentiating effect (as was 4.7% below of the additive effect of drugs). The cytotoxic response in HKh2 cells (Supporting Information Fig. S2) to the drug combination was sixfold lower than that observed in the parental (HCT116) KRAS mutant cells (Supporting Information Fig. S3) even at a higher drug dosage. Conversely, AK192 cells (a mouse PDAC cell line) expressing oncogenic KRAS^{G12D} were highly sensitive to the ATO/VC combination, as observed by the induction of a potent cytotoxic impact in the range of 91% (Supporting Information Fig. S4).[6] A high sensitivity to the ATO/VC combination was also observed in HPAC cells, the human PDAC cell line carrying the KRAS^{G12D} mutant allele,[20] with 56.7% of cells undergoing cell death (Supporting Information Fig. S5). The drug combination targeted KRAS mutant cancer cells with high specificity, as we did not observe a potent cytotoxic impact of the

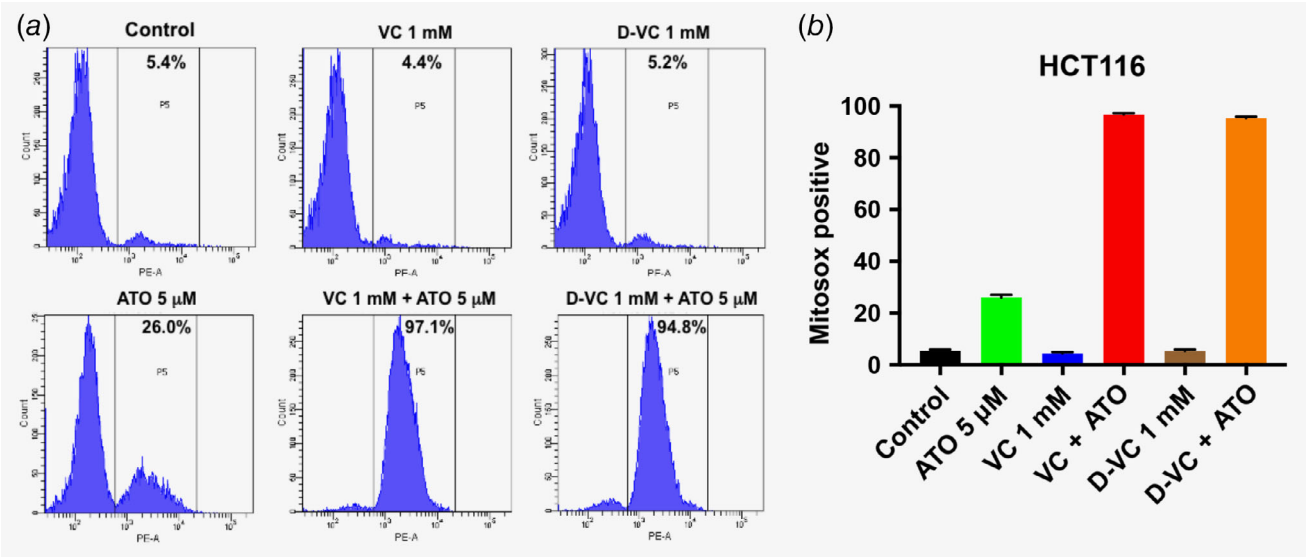

Figure 2. Cytotoxic oxidative stress is induced by the arsenic trioxide (ATO) and vitamin C (VC) combination in the Kirsten rat sarcoma mutant cancer cells. (*a*) Reactive oxygen species-detecting histograms of the cells with the similar treatment described in Figure 1. HCT116 cancer cells were treated with phosphate-buffered saline (control), 1 mM VC, 1 mM D-optical isomer of VC (D-VC), 5 μM arsenic trioxide (ATO), 1 mM VC and 5 μM ATO or 1 mM D-VC and 5 μM ATO for 48 hr and after treatments were incubated with 2 μM MitoSOX for 30 min and analyzed by flow cytometry. (*b*) The graphical presentation of the ROS detection analysis shown in (*a*).

drug combination in KRAS wild-type HT29 (17.4%) or RKO (22.2%) colorectal cancer cells (Supporting Information Figs. S6 and S7) or human MRC5 primary fibroblasts (14.4%), as shown in Supporting Information Figure S8. These results indicate that ATO and VC act synergistically to induce a potent cytotoxic effect selectively in KRAS mutant cancer cells.

If ATO and VC work together by inducing a cytotoxic oxidative stress, an excessive generation of ROS will accompany the action of drugs in KRAS mutant cancer cells. To determine whether the generation of ROS is provoked by the drug combination, we transiently incubated HCT116 cancer cells with the superoxide-dependent fluorogenic dye MitoSOX Red after the drug treatments. The flow cytometry analysis revealed a basal ROS accumulation in approximately 5% of the control and VC or D-VC treated cells. Up to 26% more ROS was detected in the ATO treated cells, whereas an increase of up to 94% was observed in cells treated with the ATO/VC or ATO/D-VC combination (Figs. 2*a* and 2*b*). Most of the cells treated by both drug combinations had high ROS levels and underwent active cell death (Fig. 1). It is likely that the accumulation of ROS in most cells treated with the drug combinations reflects not only the apoptotic (Annexin-V and PI-positive) cells but also the necrotic (PI-positive) or early apoptotic (Annexin-V positive) cells that are known to be also associated with the generation of ROS.[21–23] To link a cytotoxic impact of the oxidative drug combination to ROS generation, the cells were incubated with the drugs and reducing compound N-acetyl cysteine (NAC).[24] We found that NAC was effective in blocking not only generation of ROS but also the cytotoxic impact caused by the drug combination (Supporting Information Fig. S9). Thus, a cytotoxic impact

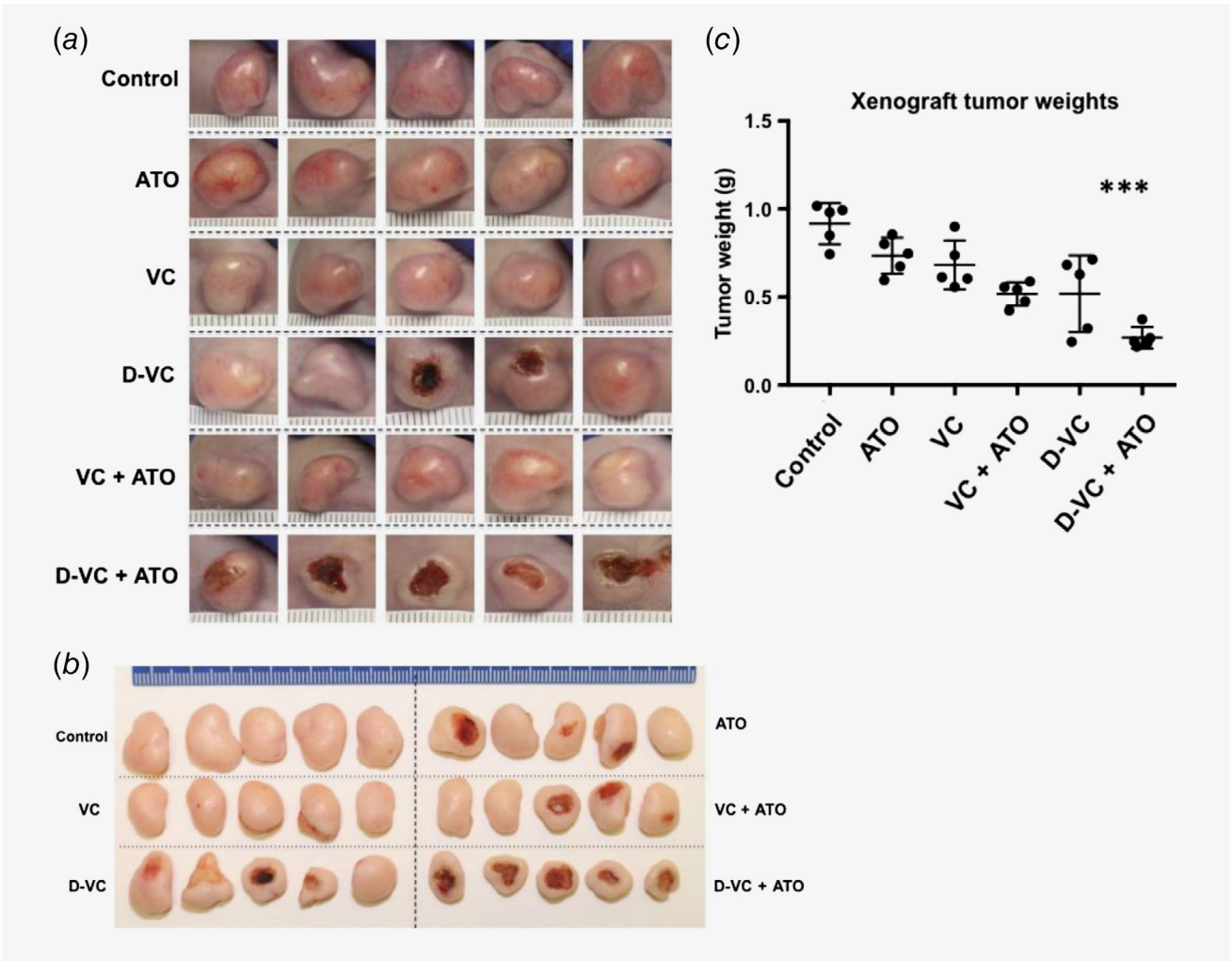

Figure 3. A stereodependent action of vitamin C (VC) in the suppression of Kirsten rat sarcoma mutant tumor growth by the arsenic trioxide (ATO) and VC combination. (*a*) The images of xenograft tumors after nine drug injections. HCT116 human cancer cells were transplanted into nude mice. After 10 days when the tumors had reached at least 0.8 cm in diameter, the mice were injected daily with phosphate-buffered saline (control), ATO (7 mg/kg), VC (1.5 g/kg), D-optical isomer of VC (D-VC; 1.5 g/kg), VC and ATO or D-VC and ATO. (*b*) Images of the excised xenograft tumors shown in (*a*) after the final (15th) drug injection. (*c*) Graphical presentation of the weight of the xenograft tumors shown in (*b*). One-way analysis of variance confirmed a significant difference in tumor weight across the six groups (p = 1.02e−06***).

induced by the drug combination in KRAS mutant cancer cells is mediated by generation of ROS.

A chirality dependent action of VC in suppressing KRAS mutant tumor growth

Consistent with this hypothesis, the action of ATO is significantly potentiated when it is combined with VC, which leads to cytotoxic oxidative stress selectively in KRAS mutant cancer cells. Considering that the ATO/VC combination is effective in cell culture, we tested it in a xenograft mouse cancer model. HCT116 cancer cells were transplanted into nude mice subcutaneously and the drugs were injected after 10 days, when tumors became larger than 0.8 cm in diameter. The stocks of VC and D-VC for mouse injections were prepared by adding sodium bicarbonate according to the method used in a previous study.[15]

To increase the efficacy of the drug combination, we introduced a 2 hr time interval between the ATO and VC injections. In addition, to eliminate the blood glucose fluctuations in mice during drug administration, food was withdrawn 2 hr prior to the ATO injections and replaced 2 hr after the VC injections. In this manner, the dose of VC was decreased from a reported daily dosage of[9] 4 g/kg to 1.5 g/kg, which was not toxic in combination with ATO.

Within the first week of injections, we observed massive tumor shrinkage in mice that had received the injections of the ATO/D-VC combination. After the ninth injection, all five mice in the combination group showed massive tumor shrinkage to the degree that macroscopic identification was only possible because of the development of scar tissue. Only two scarred tumors were observed in the group of mice injected with D-VC alone (Fig. 3*a*). However, mice injected with ATO or ATO and VC exhibited formation of initial scars on some tumors only after the 12th injection (Supporting Information Fig. S10). After the 15th (final) drug injection, a tumor analysis indicated that the ATO/D-VC combination was the most effective treatment in suppressing tumor growth, with an average tumor weight at least 70% (3.4-fold) lower than that in the control group (Figs. 3*b* and 3*c*). Suppression of tumor growth was also detected in mice treated with the ATO/VC combination where: tumor weights were 44% (1.77-fold) lower than were those in the control group. In contrast, ATO alone resulted in an only 20% decrease in tumor weight and VC and D-VC were even less effective with high variations. Consistent with tumor size effects, the tumors from mice treated with the ATO/D-VC combination had the highest rate of apoptosis (as determined by cleaved Caspase 3 detection) compared to those from mice treated with a single agent or the ATO/VC combination (Supporting Information Figs. S11 and S12). Importantly, we did not observe a substantial drug induced toxicity because the histological analysis of mouse livers did not detect any adverse abnormalities or signs of necrosis (Supporting Information Fig. S13) that was coherent with no lethality or weight loss in mice (Supporting Information Fig. S14). Thus, we found that, contrary to the results of the cell culture studies, a nonnatural enantiomer of VC (D-VC), in combination with ATO, was far more potent in suppressing KRAS mutant tumor growth than was the natural VC form (L-VC).

Discussion

We report here that ATO and VC in combination act synergistically by selectively inducing a cytotoxic oxidative stress in KRAS mutant cancer cells. The mechanism of a self-destructing ROS generation remains unknown, but it is becoming evident that KRAS mutant cancer cells accumulate ROS under oxidative stress. An elevated glucose consumption rate by these highly malignant cancer cells is likely to impose a metabolic state that is sensitive to oxidative stress. The indicated working concentration of VC at 1 mM concentration does not limit a potential clinical application of the ATO and VC combination because the intravenous VC administration bypassed limitation of its oral administration by increasing the drug dosage 100 times and by reaching maximum safe administration of VC to 10 mM.[25] Importantly, we found a remarkable difference in efficacy between the L-VC and D-VC enantiomers when they were combined with ATO in the xenograft mouse model, while both combinations showed similar potency according to the cell culture study. This finding suggests that both VC isomers use a similar mechanism in KRAS mutant cancer cells *via* provoking an oxidative stress but the distinctive pharmacokinetics of D-VC made it more superior to its natural L-VC form in the animal model. It has been reported that the oxidizing of VC to DHA is a chirality-dependent reaction that occurs at an approximately eight times faster rate for L-VC.[26] It is possible that a slow oxidation of D-VC results in a more sustained accumulation of DHA in the blood circulation and subsequently promotes a higher absorbance of DHA by the cancer cells. Although a chirality-specific pharmacokinetics of VC have yet to be defined, we demonstrated that ATO and D-VC represented a very potent combination for targeting KRAS mutant cancer cells in the mouse xenograft model.

In summary, we identified a promising oxidizing drug combination in the treatment of KRAS mutant human cancers or other cancers with high glucose consumption. The potential clinical benefits of the VC enantiomers in combination with ATO have yet to be explored.

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