Supplementary Materials for:

Tumor cells have decreased ability to metabolize H_2O_2: Implications for pharmacological ascorbate in cancer therapy

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by

Claire M. Doskey[1], Visarut Buranasudja[1], Brett A. Wagner[2], Justin G. Wilkes[3], Juan Du[3], Joseph J. Cullen[2,3,4], Garry R. Buettner[1,2,*]

[1] Interdisciplinary Graduate Program in Human Toxicology

[2] Free Radical & Radiation Biology Program in the Department of Radiation Oncology

[3] Department of Surgery

[4] Veterans Affairs Medical Center

The University of Iowa, Iowa City, IA 52242

*Corresponding Author:

Garry R. Buettner, PhD
Free Radical & Radiation Biology
Department of Radiation Oncology
Med Labs B180
The University of Iowa
Iowa City, IA 52242
garry-buettner@uiowa.edu
Tel: +1 319-335-8015

Supplementary Figures

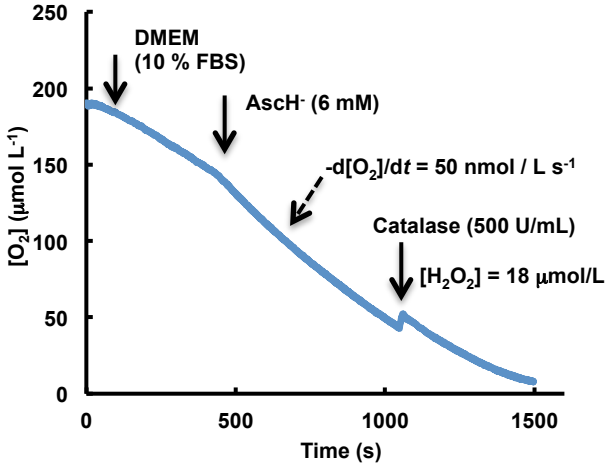

Fig. S1. Ascorbate is oxidized in DMEM generating a flux of H_2O_2. The increase in the rate of oxygen consumption upon the introduction ascorbate (6.0 mM) in DMEM with 10 % FBS is about 50 nmol L^{-1} s^{-1}. Addition of catalase leads to a return of oxygen, which indicates that 18 µmol L^{-1} of H_2O_2 accumulated in the medium. This is consistent with previous observations on the oxidation of ascorbate in cell culture medium [1, 2]. If cells were present at the typical cell densities used in this work, the level of H_2O_2 that would accumulate would be below the limit of detection of a Clark electrode; the removal of this H_2O_2 by cells is a central theme of this work. If this extracellular H_2O_2 did not react further, then the amount of H_2O_2 that would accumulate in the medium would be 50% of the oxygen consumed. Here the concentration of oxygen in the medium decreases by about 150 µM at the time of addition of catalase; if all of this O_2 were to accumulate as H_2O_2, then the addition of catalase would have yielded an increase in the concentration of O_2 of ≈75 µM. We observed an increase of about 18 µM. This indicates that some of the H_2O_2 formed is slowly being removed, likely being reduced to H_2O. It is not fully appreciated by many that the serum used in cell culture medium contains heme peroxidase activity [3]. These sera peroxidases will be activated by H_2O_2 leading to the removal of H_2O_2 and the oxidation of substances in the medium by these activated peroxidases [3]. A Clark electrode was employed for this experiment, not only to determine the rate of oxygen consumption, but also to demonstrate the formation of H_2O_2. Because high levels of ascorbate are present in the medium, typical fluorescent probes coupled with horseradish peroxidase to determine the levels of H_2O_2 in medium cannot be employed because of interfering chemistry.

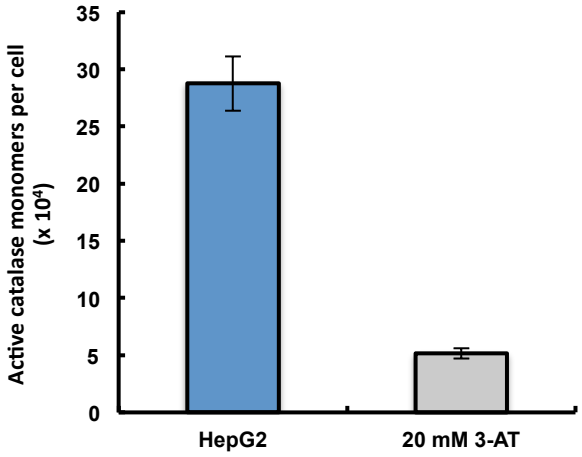

Fig. S2. 3-Amino-1,2,4-triazole inhibits catalase in HepG2 cells. Catalase activity decreased to ≈20% of control upon treatment with 20 mM 3-AT (*n* = 3, error bars are standard error of the mean).

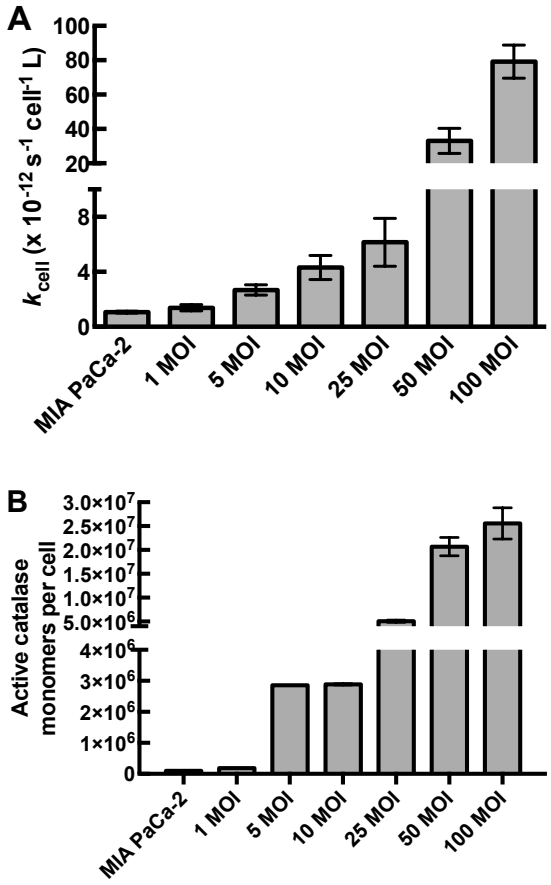

Fig. S3. Transduction of MIA PaCa-2 cells with adenovirus catalase (0-100 MOI) increases capacity of cells to remove H_2O_2 (k_{cell}).

(A) Transduction of MIA PaCa-2 cells with adenovirus catalase (1-100 MOI) resulted in 1.5- to 80-fold increase in the rate constant by which these cells remove H_2O_2 (n = 4, error bars are standard error of the mean).

(B) Catalase activity after transduction with 1-100 MOI adenovirus catalase increased 1.5- to 2,500-fold from basal catalase activity of MIA PaCa-2 cells.

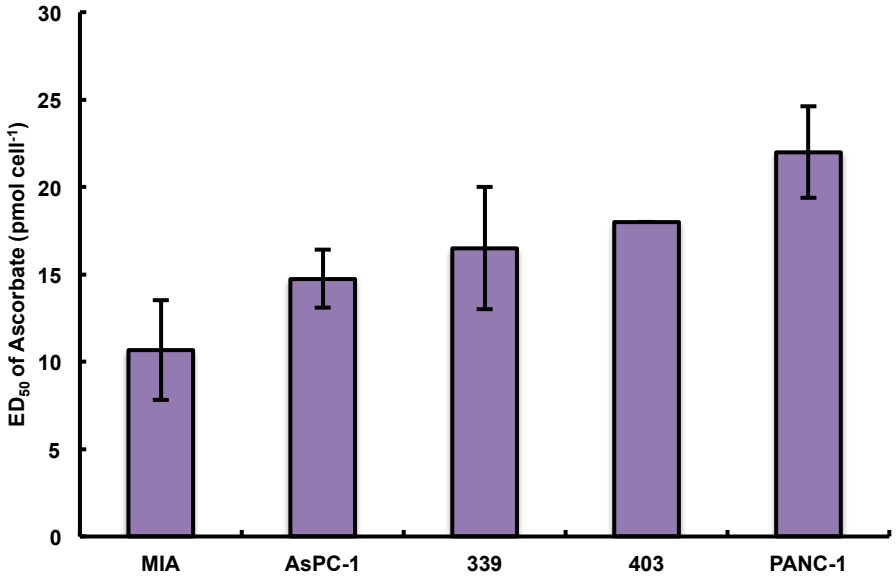

Fig. S4. Sensitivity to ascorbate varies across pancreatic cancer cell lines. The ED_{50} of ascorbate was determined in MIA PaCa-2, AsPC-1, 403, 339, and PANC-1 cell lines using a clonogenic survival assay. The dose of ascorbate needed to decrease clonogenic survival by 50 % varied across pancreatic cancer cell lines. Some uncertainties are very small.

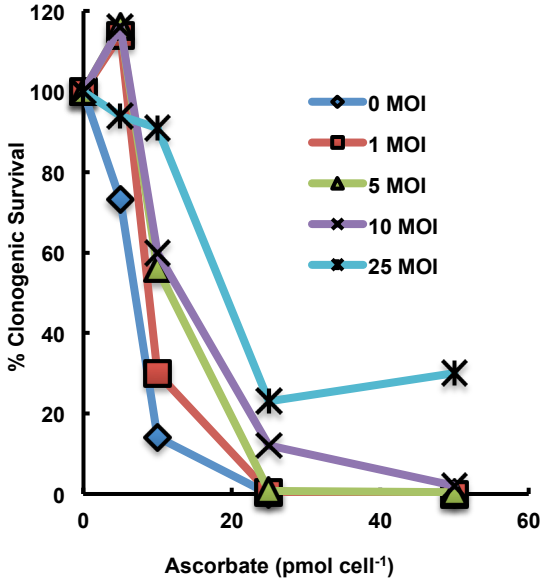

Fig. S5. Transduction of MIA PaCa-2 cells with adenovirus catalase (0-25 MOI) increases resistance to ascorbate. MIA PaCa-2 cells were transduced with adenovirus catalase at 0-25 MOI and then exposed to ascorbate (0-50 pmol cell^{-1}). The dose-response curves shifted to the right with increasing transduction-MOI of adenovirus catalase.

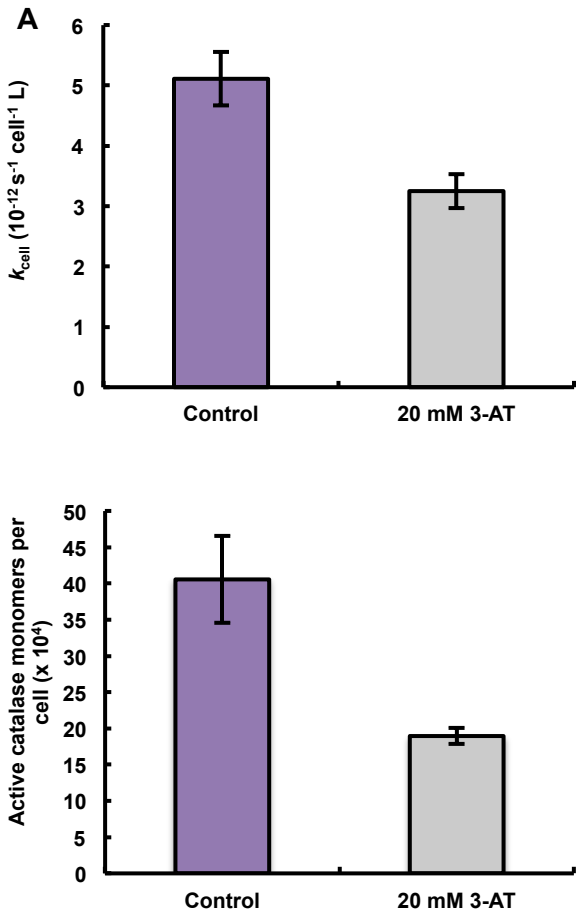

Fig. S6. 3-Amino-1,2,4-triazole inhibits catalase and decreases the rate constant for removal of H_2O_2 in PANC-1 cells.

(A) The rate constant at which PANC-1 cells remove H_2O_2 after treatment with 3-AT decreased 1.5-fold compared to untreated PANC-1 cells (*n* = 4, error bars are standard error of the mean).

(B) The effective number of active catalase molecules per cell following treatment with 3-AT decreased 2-fold compared to untreated PANC-1 cells (*n* = 3, error bars are standard error of the mean).

Supplementary Discussion

The potential benefits of high-dose vitamin C (*i.e.*, ascorbic acid, referred to as pharmacological ascorbate, P-AscH$^-$), given by IV delivery as an adjuvant in the treatment of cancer, has gained considerable interest upon the uncovering of a new mechanism for its anticancer effects [4, 5, 6]. Intravenous ascorbate, but not oral ascorbate, produces high plasma concentrations, which are in the range that are cytotoxic to tumor cells. Clinical data show that when ascorbate is given orally, fasting plasma concentrations are tightly controlled at < 100 μM [7]. In contrast, when ascorbate is administered intravenously, plasma concentrations as high as 20 - 30 mM are safely achieved with few side effects [8]. Thus, it is clear that intravenous administration of ascorbate can yield very high plasma levels, while oral delivery does not.

These high levels of ascorbate act as a pro-drug for the delivery of hydrogen peroxide to tumors [4, 5, 6]. This H_2O_2 appears to be significant in the anticancer mechanism. The present work uses absolute quantitative approaches to establish the central role for H_2O_2 derived from the oxidation of ascorbate.

There are three distinct arenas for the current research on the use of P-AscH$^-$ to treat cancer, **Fig. S7**. The research represented by **Node 1** is focused on how to achieve the highest possible flux of H_2O_2 in tumors. The research represented by **Node 2** is addressing how cells protect themselves from this high flux of H_2O_2, *i.e.* what enzyme systems and redox active molecules contribute to maintaining a low ambient level of intracellular H_2O_2. **Node 3** represents the effects of P-AscH$^-$ on small molecules, proteins, as well as pathways and networks and the subsequent response to the intracellular H_2O_2 resulting from **1** and **2.**

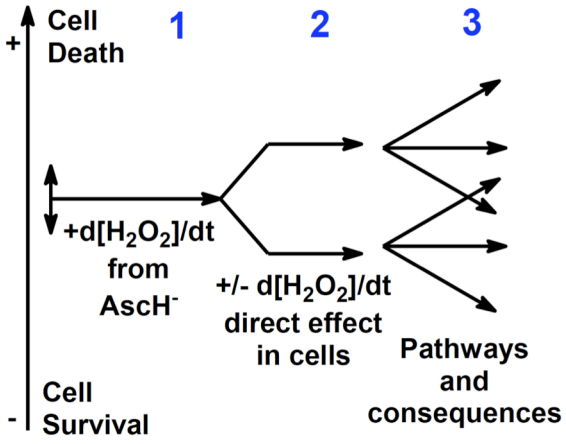

Fig. S7. Overview of our model for the mechanism of P-AscH$^-$.

Node 1: P-AscH$^-$ readily oxidizes to produce a high flux of H_2O_2 in both the extracellular and intracellular space. The ordinate reflects in part this flux of H_2O_2 and the resulting biological response. This H_2O_2 is the essential mediator of the cytotoxicity of AscH$^-$ toward cancer cells. The greater the flux of H_2O_2, the greater the cellular effects; this flux is a function of [AscH$^-$], pH, and catalytic metals.

Node 2: This represents how cells handle the H_2O_2. An important concept is that the effect on cells is a function of cell density, *i.e.* the critical parameter is flux per cell, *i.e.* moles of H_2O_2 cell^{-1} s^{-1}; the response will vary with this flux and the ability of the intracellular peroxide-removal enzyme system to remove this H_2O_2. Cells with high capacity for removal of H_2O_2 are less affected; on the other hand inhibiting the removal of H_2O_2 will result in greater effects on the cell.

Node 3: These modulators represent the effects of small molecules as well as pathways and networks on the response to the intracellular H_2O_2 resulting from **1** and **2**, *e.g.* the influence of intracellular labile iron, ATP levels, GAPDH, GSH, SVCT2 activity, NOX activation, HIF, autophagy, apoptotic signaling, DNA damage and repair, *etc.*

Data from cell culture experiments indicate that P-AscH$^-$ induces selective oxidative stress and cytotoxicity in cancer cells *vs.* normal cells by a mechanism involving the production of H_2O_2 [4, 5, 6, 9, 10]. In these studies the addition of various forms of both extracellular and intracellular catalase, which remove H_2O_2, reverse the toxicity induced by pharmacological P-AscH$^-$ in a variety of cancer cell lines, while normal cells are resistant. Thus, in cell culture the

chemistry of small molecules in the medium that react with H_2O_2 and thereby remove it must also be considered.

For example thiol compounds, such as cysteine, N-acetylcysteine (NAC) and glutathione (GSH), as well as pyruvate, and even certain iron compounds, will react directly with H_2O_2, thereby removing it. This chemistry is in addition to changes in the redox biochemistry that these substances may bring about in cells.

Iron as catalyst and protectant

Catalytic metals, such as iron and copper can serve as catalysts for the oxidation of ascorbate [11]. These same metals can also react with H_2O_2. Thus, in cell culture experiments, higher levels of extracellular iron can actually protect cells from the detrimental effects of extracellular H_2O_2 [12, 13]. However, it must be kept in mind that not all iron or copper species will catalyze the oxidation of ascorbate; the coordination environment and resulting thermodynamics and kinetics are central to the catalytic efficiency [14].

Kinetic considerations

The second-order rate constant for the reaction of cysteine with H_2O_2 is k_{Cys} = 1.1 M^{-1} s^{-1} (pH 7.4, 37 °C) [15]; from the apparent pK_a of the thiol proton, estimates for the second-order rate constant for GSH would be, $k_{GSH} \approx 0.3$ M^{-1} s^{-1} (pH 7.4, 37 °C) and for NAC, $k_{NAC} \approx 0.05$ M^{-1} s^{-1}. This value for k_{NAC} is somewhat smaller than 0.85 M^{-1} s^{-1} (pH 7.4) estimated by Aruoma *et al.* [16]. Analysis of published data indicates that the rate constant for the reaction of pyruvate with H_2O_2 is $k_{pryuvate}$ = 2 M^{-1} s^{-1} (pH 7.4, 37 °C) [17, 18]; the rate constant for the parallel decarboxylation reaction with oxaloacetate would be similar. Thus, the direct chemistry of these and similar substances that will remove H_2O_2 cannot be overlooked.

For example in a recent study on potential mechanisms of P-AscH$^-$ some of these compounds were included in the cell culture medium at concentrations of 5 mM [19]. At this concentration, the pseudo first-order rate constant for the removal of H_2O_2 by GSH/GS$^-$ would be:

$$k'_{GSH/GS^-} = (5 \times 10^{-3}\ M) \times (0.3\ M^{-1}\ s^{-1})$$
$$= 1.5 \times 10^{-3}\ s^{-1}$$

The cell density was reported as ≈3500 cells/200 µL = 17 x 10^6 cell L^{-1}. If the cells have a robust capacity to remove H_2O_2, *e.g.* k_{cell} = 5 x 10^{-12} s^{-1} $cell^{-1}$ L, then the pseudo first-order rate constant for the removal of H_2O_2 by cells would be:

$$k'_{cell} = (17 \times 10^6\ cell\ L^{-1})\ (5 \times 10^{-12}\ s^{-1}\ cell^{-1}\ L)$$
$$= 0.09 \times 10^{-3}\ s^{-1}$$

Thus, ≈95 % of the H_2O_2 formed upon the oxidation of ascorbate in the cell culture medium would be consumed by GSH/GS$^-$, thereby protecting the cells. If in a similarly designed experiment the cell culture medium contained pyruvate at 5 mM, then

$$k'_{pyruvate} = (5 \times 10^{-3}\ M) \times (2\ M^{-1}\ s^{-1})$$
$$= 10 \times 10^{-3}\ s^{-1}$$

or 99 % of the H_2O_2 produced would be removed by pyruvate. Thus, a significant role for H_2O_2 in the mechanism of toxicity of P-AscH$^-$ in the experiments of Yun *et al.* [19] needs to be considered [20].

Clearly quantitative approaches provide a much richer context in which to analyze experimental results. Unfortunately, most laboratory assays provide relative changes or relative

levels of species or enzyme activities in some arbitrarily defined unit. This information is not directly useful in modeling, even the very simple kinetic model above, without a path to arrive at absolute levels. Absolute quantitation can provide context and crosschecks for data from different types of experiments and provide guidance as to what mechanistic possibilities are most probable and the most important.

Absolute quantitative data, such as provided in this work, can be compared directly to quantitative data generated by other laboratories around the world. Quantitative approaches lead to much improved reproducibility, greater efficiencies in the world's research enterprise, resulting in a more rapid rate of discovery that can be translated to improve human health [21, 22].

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