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Drug resistance induces the upregulation of H_2S-producing enzymes in HCT116 colon cancer cells

Ashley A. Untereiner[1,*], **Athanasia Pavlidou**[2], **Nadiya Druzhyna**[1], **Andreas Papapetropoulos**[2,3], **Mark R. Hellmich**[4], and **Csaba Szabo**[1,*]

[1]Department of Anesthesiology, University of Texas Medical Branch, Galveston, TX 77555

[2]Laboratory of Pharmacology, Faculty of Pharmacy, National and Kapodistrian University of Athens, Greece

[3]Clinical, Experimental Surgery and Translational Research Center, Biomedical Research Foundation of the Academy of Athens, Greece

[4]Department of Surgery, University of Texas Medical Branch, Galveston, TX 77555

Abstract

Hydrogen sulfide (H_2S) production in colon cancer cells supports cellular bioenergetics and proliferation. The aim of the present study was to investigate the alterations in H_2S homeostasis during the development of resistance to 5-fluorouracil (5-FU), a commonly used chemotherapeutic agent. A 5-FU-resistant HCT116 human colon cancer cell line was established by serial passage in the presence of increasing 5-FU concentrations. The 5-FU-resistant cells also demonstrated a partial resistance to an unrelated chemotherapeutic agent, oxaliplatin. Compared to parental cells, the 5-FU-resistant cells rely more on oxidative phosphorylation than glycolysis for bioenergetic function. There was a significant increase in the expression of the drug-metabolizing cytochrome P450 enzymes CYP1A2 and CYP2A6 in 5-FU-resistant cells. The CYP450 inhibitor phenylpyrrole enhanced 5-FU-induced cytotoxicity in 5-FU-resistant cells. Two major H_2S-generating enzymes, cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) were upregulated in the 5-FU-resistant cells. 5-FU-resistant cells exhibited decreased sensitivity to the CBS inhibitor aminooxyacetate (AOAA) in terms of suppression of cell viability,

[*]Authors for correspondence: **Csaba Szabo**: The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555 (szabocsaba@aol.com) or **Ashley Untereiner**: The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555 (ashley.untereiner@utoronto.ca).
Current address: D University of Toronto, Faculty of Medicine, Medical Sciences Building, 1 King's College Circle, Toronto, ON, Canada, M5S 1A.

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Author disclosure statement

C.S. and M.R.H. are founders, officers and shareholders of CBS Therapeutics Inc., an UTMB spin-off company focusing on therapeutic approaches around H_2S biosynthesis inhibition in cancer cells. A.P. is a shareholder and officer of the same company.

For the other authors, no competing financial interests exist.

Author contributions

AAU – carried out molecular biology and pharmacological assays, contributed to the writing of the paper. TP – contributed to the production of the 5-FU-resistant HCT116 cells. ND – performed enzymatic activity analyses. AP – contributed to the design of the project and to data interpretation. MRH and CS – conceived and supervised the project and contributed to the writing of the paper.

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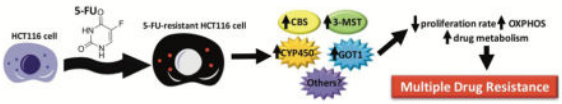
inhibition of cell proliferation and inhibition of oxidative phosphorylation. However, 5FU-resistant cells remained sensitive to the antiproliferative effect of benserazide (a recently identified, potentially repurposable CBS inhibitor). Taken together, the current data suggest that 5-FU resistance in HCT116 cells is associated with the upregulation of drug-metabolizing enzymes and an enhancement of endogenous H_2S production. The anticancer effect of prototypical H_2S biosynthesis inhibitor AOAA is impaired in 5-FU-resistant cells, but benserazide remains efficacious. Pharmacological approaches aimed at restoring the sensitivity of 5-FU-resistant cells to chemotherapeutic agents may be useful in the formulation of novel therapeutic strategies against colorectal cancer.

Graphical abstract



1. Introduction

Colorectal cancer is still the third most common cancer and the second most common cause of cancer-related death worldwide [1,2], with nearly 1.4 million cases a year and ~774,000 deaths worldwide [3]. The chemotherapeutic drug 5-fluorouracil (5-FU) remains widely used in the treatment of colorectal carcinoma.

5-FU is an analog of uracil with a fluorine atom substituted at the carbon-5 position of the pyrimidine ring in place of hydrogen. 5-FU, and other 5-fluorinated pyrimidines have been widely used in the treatment of colorectal cancer [4]. The efficacy of 5-FU is, at least in part, attributed to its ability to induce p53-dependent cell growth arrest and apoptosis. 5-FU is considered an S phase-active chemotherapeutic agent which inhibits cell proliferation and survival [5,6]. While some patients respond initially to chemotherapy, many advanced colorectal cancer patients eventually develop chemotherapy resistance disease, which is a major barrier to achieve effective therapy.

Cystathionine-β-synthase (CBS) is upregulated and hydrogen sulfide (H_2S) production is increased in various types of cancer including colon, ovarian, breast and lung cancer [7–12]. The functional consequence of increased cellular H_2S production is stimulation of cellular bioenergetics, tumor growth and proliferation (reviewed in [13]). The mechanisms involved in the stimulation of mitochondrial function by H_2S are multiple; they involve direct electron donation to Complex II of the mitochondrial electron transport chain [14–16] inhibition of mitochondrial cAMP phosphodiesterases, followed by cAMP-stimulated increases in mitochondrial electron transport [17], mitochondrial antioxidant effects [18,19], stimulation of mitochondrial DNA repair [12,20], direct stimulation of mitochondrial ATP synthase *via* posttranslational modification (*via* protein *S*-sulfhydration) [21] as well as the stimulation of lactate dehydrogenase activity (*via* protein *S*-sulfhydration) [22]. All of these effects occur at low-to-intermediate concentrations of H_2S, while at higher concentrations, inhibitory effects of H_2S on mitochondrial function become apparent – primarily mediated by the inhibition of mitochondrial Complex IV (cytochrome c oxidase) by H_2S [23,24]. In 2013, we discovered

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that CBS is overexpressed in colon cancer and that H_2S produced by it serves to support cellular bioenergetics and tumor angiogenesis. Pharmacological inhibition or silencing of CBS reduced tumor bioenergetic function, inhibited tumor angiogenesis and suppressed tumor growth [7]. In contrast, other H_2S-producing enzymes such as cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) were not differentially expressed in colon tumor tissue when compared to normal mucosa [7]. The mitochondrial enzyme 3-MST is a newly identified endogenous source of H_2S in various cells and tissues, including vascular endothelial cells [25]. We have recently demonstrated that 3-MST activity is inhibited by oxidative stress *in vitro* and speculated that this may exert adverse effects on cellular homeostasis [26]. Although we did not observe a significant upregulation of 3-MST in colon cancer [7], in other forms of cancer, an upregulation of 3-MST has been reported [12,27,28].

The aims of the present study were to examine whether H_2S homeostasis is altered in 5-FU resistant colon cancer and to characterize the molecular mechanisms that contributed to the development of the resistant phenotype, such as proteins involved in the extrusion and/or metabolism of the chemotherapeutic drug. We also tested how development of 5-FU resistance affected cellular bioenergetic status and sensitivity to CBS inhibition with either aminooxyacetic acid (a prototypical CBS inhibitor) or benserazide. Both of these compounds exert significant inhibitory effects *in vitro* and *in vivo.* The results of the present study demonstrate the upregulation of the H_2S-generating enzymes CBS and 3-MST during the development of 5-FU resistance in HCT116 cells, positively influencing cellular viability, bioenergetics and proliferation.

2. Materials and Methods

2.1 Cell culture

The parental human colorectal carcinoma cell line, HCT116 (ATCC, Manassas, VA, USA) was cultured in McCoy's 5A medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin as described [7]. Cells were grown in a 37°C, 5% CO_2 atmosphere.

To established an acquired resistant cell line, HCT116 cells were cultured in medium containing stepwise increased concentrations of 5-FU for 6 months to obtain a 5-FU-resistant HCT116 cell line. Briefly, HCT116 cells were cultured in fresh medium without drugs for 24 h. Subsequently, the medium was changed and 1 μM 5-FU (Cat. # F6627) in complete medium was added. HCT116 cells were exposed to 5-FU for 48–72 h, thereafter the 5-FU-treatment medium was removed and cells were allowed to recover (in normal medium) for about a week. When cells reached 70% confluence, the treatment process was repeated for several times until they were stable. Once stable, cells were subjected 3, 10 and finally 30 μM 5-FU treatment. Thereafter, the 5-FU-resistant cells were maintained in full medium supplemented with 30 μM 5-FU (to maintain 5-FU resistance).

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2.2 Western blotting

Cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitor cocktail (Complete Mini EDTA-free, Roche Applied Science, Indianapolis, IN). Cell homogenates were resolved on 4–12% NuPage Bis-Tris acrylamide gels (Invitrogen), then transferred to nitrocellulose. Membranes were blocked in 10% non-fat dried milk and probed overnight with CBS (Proteintech Group, Inc., Rosemont, IL; Cat. # 14787-1-AP), CSE (Proteintech; Cat. # 12217-1-AP), 3-MST (Abcam; Cat. # ab85377), rhodanese (Proteintech; Cat. # 66018-1), MRP1 (Abcam; Cat. # ab24102), GOT1 (Abcam; Cat. # ab85857), CYP1A2 (Proteintech; Cat. # 19936-1-AP), CYP2A6 (Proteintech; Cat. # 21721-1-AP), or β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA; Cat. # 47778). After incubation with peroxidase conjugates the blots were detected on a CCD camera based detection system (GBox, Syngene USA, Frederick, MD). ImageJ was used for densitometric analysis.

2.3 Enzymatic activity of H_2S-generating enzymes

Cells were lysed in ice-cold NP40 lysing buffer (1% NP40; 150mM NaCl; 50mM Tris-Cl, pH 8.0) for 30 min on ice. Cell homogenates (~5–20 μl, 100–200 μg protein) were added to the reaction mixture (50 mM Tris HCL pH 8.0), which was supplemented with 2 mM homocysteine and 2 mM L-cysteine (CBS activity assay), 2 mM L-cysteine (CSE activity assay), or 2 mM 3-mercaptopyruvate (3-MST activity assay). Pyridoxal 5′-phosphate (PLP; 5 μM final concentration) was added to only the CBS and CSE reaction mixtures, followed by the addition of AzMc (7-azido-4-methylcoumarin; 10 μM final concentration) to all the reaction mixtures, bring the total volume of the reaction buffer to 200 μl. The enzyme activity mixture was incubated at 37 °C for 4 h. Immediately thereafter, fluorescence was measured at 450 nm *(via* Molecular Devices M2 microplate reader). 1% NP40 was used as a vehicle control.

2.3 Extracellular Flux Analysis

The XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies) was used to measure bioenergetic function as described [14,29]. Four key parameters of mitochondrial function (basal respiration, adenosine triphosphate (ATP) turnover, proton leak and maximal respiration) were assessed through the sequential use of 40 μM oligomycin (ATP synthase inhibitor), 13.5 μM FCCP (oxidative phosphorylation un-coupler) and 10 μM rotenone + 1 μM antimycin A (complex I and III inhibitors, respectively). The difference between the maximal and the basal respirations was considered the respiratory reserve capacity (the capacity of a cell to generate ATP *via* oxidative phosphorylation in response to increased demand for energy). A glycolytic stress test was used to estimate various parameters of cellular glycolysis (glycolysis, glycolytic capacity and glycolytic reserve), which was obtained with the sequential use of 25 mM glucose, 5 μM oligomycin (to block mitochondrial respiration and force the cells to rely on glycolysis for ATP production) and 100 mM 2-deoxyglucose (2-DG, a glucose analog and inhibitor of glycolytic ATP production). Glycolytic reserve was calculated as the difference between the glycolytic capacity and the glycolysis; this parameter is indicative of the cellular ability to increase the glycolytic rate upon increased energy demand. Acidification of carbon dioxide,

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the end product of the tricarboxylic acid (TCA) cycle, which can be converted to bicarbonate, is considered a major contributor to nonglycolytic acidification. Bioenergetic parameters were normalized to protein content *via* Lowry reagent (Bio-Rad) using BSA as a standard.

2.4 MTT and LDH assays

The MTT assay and LDH activity measurements were performed as previously described [29]. Briefly, after 48 h of treatment with 1-phenylpyrrole (Cat. # 131474), AOAA (Cat. # C13408) and/or 5-FU, cells were washed and incubated in medium containing 0.5 mg/ml, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Calbiochem, EMD BioSciences, San Diego, CA) for 1 h at 37°C in a 5% CO_2 atmosphere. The converted formazan dye was dissolved in DMSO. The plates were read on a Molecular Devices M2 microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability was calculated as the ratio of absorbance in treated cultures to absorbance in untreated control cultures.

LDH release was measured by mixing cell culture supernatant (30 μl) with 100 μl LDH assay reagent containing 110 mM lactic acid, 1.35 M nicotinamide adenine dinucleotide (NAD^+), 290 mM *N*-methylphenazonium methyl sulfate (PMS), 685 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2). The changes in absorbance were read kinetically at 492 nm for 15 min (kinetic LDH assay). LDH activity values are shown as Vmax (mOD/min).

2.5 Cell proliferation assays

For assessment of cell proliferation, the xCELLigence system (Roche Applied Science, Indianapolis, IN) was used as described [9,12]. Briefly, HCT116 cells were cultured until ~70% confluence. After 24 h, cells were detached by trypsin-EDTA and resuspended in fresh culture media at a concentration of 60,000 cells/ml. Cell suspension was added to each well (6,000 cells/well) of an E-plate 96, a specially designed 96-well microtiter plate containing interdigitated microelectrodes to noninvasively monitor the cell proliferation by measuring the relative change in the electrical impedance of the cell monolayer, a unitless parameter named cell index. After 24 h, cells were treated with 5-FU (1–30 μM) with or without AOAA (0.3–1 mM) and proliferation was monitored for 48 h.

2.6 Chemicals and statistical analysis

All chemicals and commercially available enzymes used in this study were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All data are presented as mean ± SEM and were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical analyses included Student *t* test or two-way ANOVA followed by Tukey's multiple comparisons were used to detect differences between groups. Statistical significance was considered at $P < 0.05$.

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3. Results

3.1 H_2S-generating enzymes are upregulated in 5-FU-resistant HCT116 cells

To elucidate the underlying mechanism for 5-FU resistance in CRC, we established an acquired resistant cell line, HCT116, through addition of increasing amount of 5-FU to the HCT116 parental cells for ~6 months. Thereafter, the 5-FU-resistant cells were maintained in medium supplemented with 30 μM 5-FU. MTT assay was used to assess cellular cytotoxicity. Parental HCT116 cells responded to 5-FU challenge (3–100 μM) *via* decreased MTT reduction, while 5-FU-resistant cells were not responsive to 5-FU treatment, whereby cell viability remained unchanged (Fig. 1A). 5-FU-resistant HCT116 cells proliferated at a markedly slower rate than the parental HCT116 cells. XCELLigence analysis revealed that 30 μM 5-FU treatment significantly inhibited cell proliferation of naive HCT116 cells, but was without antiproliferative effect in 5-FU-resistant HCT116 cells (Fig. 1B). (As a result of the different proliferation rates, XCELLigence data obtained from both cell lines were normalized to their respective, untreated control group.) Surprisingly, low concentrations of 5-FU (3 μM) significantly enhanced cell proliferation in parental HCT116 cells, and this effect became more pronounced in 5-FU-resistant cells (Fig. 1B). No changes were observed in cell necrosis *(via* LDH release assay) with 5-FU administration in either cell lines (Fig. 1C).

Furthermore, we observed partial resistance to the clinically-used anti-cancer drug, oxaliplatin, in the 5-FU-resistant cells with comparison to parental HCT116 cells (Fig. 2). From 1–100 μM, oxaliplatin significantly reduced MTT reduction in both resistant and parental cell lines (Fig. 2A); however, 5-FU-resistant HCT116 cell viability was significantly higher to that of HCT116 cell viability in the presence of 3–100 μM oxaliplatin (Fig. 2A). No significant changes were reported in cell necrosis *(via* LDH release assay) in either cell line in the presence of oxaliplatin (Fig. 2B). These findings indicate that the current protocol of 5-FU-resistance, in fact, creates multi-drug-resistant cells and predict that the development of 5-FU-resistance is associated with a significant degree of reprogramming of various cellular processes.

The physiological production of H_2S is due, in large part, to the activity of three enzymes: CBS, CSE, and 3-MST. Colon cancer cells contain high levels of CBS, whereby its product, H_2S, promotes the growth and proliferation of colorectal tumor cells [7]. Densitometric analysis of Western blots revealed a 49%, 63% and 107% increase in CBS, 3-MST and the H_2S-metabolizing enzyme rhodanese, respectively in 5-FU-resistant cells, compared to parental HCT116 cells (Fig. 3A and Fig. 3B). In agreement with the protein expression data, the enzymatic activities of CBS and 3-MST were enhanced in 5-FU-resistant cells compared to parental HCT116 cells (Fig. 3C). In contrast, CSE protein level and enzymatic activity were similar in both parental and 5-FU-resistant cells (Fig. 3).

3.2 CYP450 and GOT1 proteins, but not MRP1, are upregulated in 5-FU-resistant cells

Members of the ATP-binding cassette (ABC) transporter family proteins are one of the most studied mechanisms of cancer drug resistance, including multidrug resistance-associated protein 1 (MRP1). ABC proteins are involved in reducing drug accumulation by enhancing

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efflux [30]. We found no change in the protein level of MRP1 in 5-FU-resistant cells compared to parental HCT116 cells (Fig. 4). Glutamate oxaloacetate transaminase 1 (GOT1), also known as aspartate transaminase or AST, is an essential enzyme in the malate/aspartate shuttle [31,32]. Densitometric analysis showed a ~40% increase in GOT1 protein level in 5-FU-resistant cells compared to parental HCT116 cells (Fig. 4).

Various cytochromes P450 (CYP) - including isoforms 1A2 and 2A6 - are involved in drug metabolism in cancer cells [33–35]. We observed a significant increase in the protein levels of CYP1A2 and CYP2A6 in 5-FU-resistant HCT116 cells compared to parental HCT116 cells (Fig. 4). Densitometric analysis of Western blots revealed a 73% and 56% increase in CYP1A2 and CYP2A6, respectively in 5-FU-resistant cells (Fig. 4A and Fig. 4B).

We next tested whether or not treatment with the CYP450 inhibitor, phenylpyrrole, would sensitize 5-FU-resistant cells to 5-FU treatment. Both resistant and parent cell lines were treated with 100 μM phenylpyrrole (or its vehicle control), followed by increasing concentrations of 5-FU (i.e. 1–100 μM). There was a steady decline in parental HCT116 cell viability with increasing 5-FU concentration (Fig. 5A). Parental HCT116 cell viability did not further decline with the co-administration of 100 μM phenylpyrrole and 5-FU (Fig. 5A). In contrast, a significant enhancement of 5-FU cytotoxicity (at 100 μM) was noted in 5-FU-resistant cells in the presence of phenylpyrrole (Fig. 5B). These data suggest that increased metabolism (inactivation) of 5-FU (but not increased extrusion of 5-FU from the resistant cells) may underlie, at least in part, the drug resistance observed in the 5-FU-resistant cells in the current study.

3.3 The efficacy of AOAA is impaired in 5-FU-resistant cells

We previously showed that the prototypical CBS inhibitor aminooxyacetic acid (AOAA) suppresses the proliferation of colon cancer cells *in vitro* and reduces tumor growth *in vivo* [7,9]. AOAA is a potent pharmacological inhibitor of both CBS and CSE [36]. To assess whether the excessive generation of H_2S contributed to 5-FU resistance in the 5-FU-resistant HCT116 cells, cells were treated with a combination of AOAA and 5-FU. 5-FU-resistant cells developed a resistance against AOAA treatment; in 5-FU-resistant cells AOAA only decreased viability at millimolar concentrations (i.e. 1–3 mM), and even then, it only produced a partial effect (Fig. 6A, top panel). When co-treatment of AOAA and 5-FU was tested, the 5-FU-resistant cells remained insensitive to the combination of drugs and cell viability maintained (Fig. 6B, top panel). Fig. 6B (bottom panel) shows that a significant increase in cell necrosis (*via* LDH release assay) was only noted at the highest concentration of AOAA and 5-FU combination; in these conditions both resistant and parental cell lines responded with a comparable degree of LDH release (Fig. 6B, bottom panel).

In agreement with our previous findings [7,9], AOAA treatment significantly inhibited HCT116 cell proliferation (Fig. 7). 5-FU-resistant cells showed resistance to AOAA (Fig. 7). At 300 μM AOAA produced a stimulatory effect on cell proliferation in the 5-FU-resistant cells, while at 1 mM AOAA, only a minor (statistically not significant) inhibitory effect was observed on the proliferation of 5-FU-resistant HCT116 cells (Fig. 7).

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When analyzing bioenergetic responses, we noted that 5-FU-resistant cells had a comparable basal respiration rate as parental control cells, but an increased respiratory spare capacity (in response to the uncoupling agent FCCP) (Fig. 8). AOAA markedly suppressed respiratory spare capacity in control cells, but only had a slight inhibitory effect in 5-FU-resistant cells (Fig. 8D). 5-FU-resistant cells exhibited a lower basal glycolytic rate compared to the parental HCT116 cells and AOAA inhibited glycolysis to a comparable degree in both wild-type and resistant cells (Fig. 9). Taken together, the bioenergetic data indicate that the 5-FU resistant cells, compared to the parental controls, tend to rely more on oxidative phosphorylation than on glycolysis. Also, the 5-FU-resistant cells become more resistant to the oxidative-phosphorylation-inhibitory effects of AOAA, than against its glycolysis-inhibitory effects. However, the functional data (cell viability and cell proliferation) suggest that whatever small suppressive effect AOAA exerts on bioenergetic parameters in the 5-FU-resistant cells, it is insufficient to produce any significant suppression of cell viability or cell proliferation.

3.4 The efficacy of benserazide is largely maintained in 5-FU-resistant HCT116 cells

To further assess the effect of CBS inhibition in 5-FU-resistant HCT116 cells, cells were treated with benserazide. We have recently reported that benserazide, a compound that is clinically used as a DOPA decarboxylase inhibitor, inhibits CBS activity *in vitro* and colon tumor growth *in vivo* [37]. Parental HCT116 cells treated with benserazide at concentrations of 30 μM and above showed a significant decrease in cell viability and an increase in LDH (Fig. 10). By comparison, although 5-FU-resistant HCT116 cells showed resistance to benserazide at the low concentration of 30 μM, the drug maintained efficacy at higher concentrations; reducing cell viability and increasing cell death (LDH release) (Fig. 10).

4. Discussion

The major findings of the present study are the following: 1) protein levels and enzymatic activities of the H_2S-generating enzymes, CBS and 3-MST are significantly upregulated in 5-FU-resistant HCT116 cells. Rhodanese, an enzyme involved in the oxidation and thus removal of endogenous H_2S, was also upregulated in 5-FU-resistant cells; 2) 5-FU-resistant cells express elevated CYP1A2 and CYP2A6 proteins and pharmacological data suggest that increased drug metabolism may contribute to the development of drug resistance; 3) 5-FU-resistant HCT116 cells are resistant (in terms of cell proliferation and MTT/LDH parameters) to the anticancer effect of the prototypical CBS inhibitor AOAA, even though partial effects of AOAA on bioenergetic parameters remain detectable; 4) 5-FU-resistant cells remain largely responsive to the antiproliferative effect of the “non-professional” (repurposable) CBS inhibitor benserazide.

Many different molecular mechanisms have been implicated in the resistance to cytotoxic chemotherapy or targeted therapy, particularly for the acquired resistance [30,33–35,38,39]. Although upregulation of drug transporters has been previously implicated in drug resistance [30], we did not detect an upregulation of ABC proteins. We did, however, detect an upregulation of CYP450 enzymes, which have also been previously implicated in oncological studies [33–35]. CYP450 enzymes are key players in the phase I-dependent

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metabolism of drugs and other xenobiotics and these enzymes mediate the metabolic activation of numerous precarcinogens and participate in the inactivation and activation of anticancer drugs. The presence of functionally active CYP450 enzymes in tumor cells can inhibit the efficacy of chemotherapy-mediated tumor cell death through the deactivation of various anti-cancer drugs [33]. In the current study, CYP1A2 and 2A6 protein levels were significantly upregulated in 5-FU-resistant cells. Furthermore, we observed a partial restoration of 5-FU cytotoxicity in 5-FU-resistant cells upon co-treatment of phenylpyrrole (a CYP450 inhibitor). Therefore, these results suggest that CYP450-mediated metabolism may contribute to HCT116 cell resistance to 5-FU, and subsequently the CBS inhibitor AOAA.

In addition to the upregulation of CYP450 enzymes, we have also observed a significant upregulation of GOT1 protein. GOT1 is an essential component of the malate/aspartate shuttle [31]. In cancer cells, this mechanism has been shown to link glycolysis to the transfer of electron donors into the mitochondria [40]. Moreover, the function of GOT1 is linked to glutaminolysis, another partially cancer-selective metabolic pathway and anti-cancer target [31]. It is possible that the elevated level of GOT1 seen in 5-FU-resistant cells may have affected some of the cells' metabolic parameters and may have affected the balance of oxidative phosphorylation and glycolysis. However, the role of GOT1 and its possible relationship with the H_2S pathway has not been fully explored in the current study and this remains to be a subject of future investigations.

Importantly, in our studies, we detected a significant upregulation of CBS and 3-MST in 5-FU-resistant HCT116 cells. CBS is selectively upregulated in various human colon cancer lines (HCT116, HT-29, LoVo) as well as in patient-derived colon tumor specimens [7,8]. Although 3-MST was not previously found to be upregulated in colon tumor tissue, we observed a significantly upregulation in the 5-FU-resistant cells. Pharmacological inhibition of CBS *(via* AOAA treatment) was markedly less effective in suppressing cell viability in 5-FU-resistant HCT116 cells than in parental cells. The mechanism responsible for the loss of AOAA's efficacy may be due to the fact that: (a) other H_2S-producing enzymes, such as the 3-MST system are also contributing to H_2S production in these cells and therefore inhibition of CBS results in a lesser suppression of cellular H_2S levels; (b) AOAA may be metabolized and inactivated by the upregulated cytochrome P450 enzymes and/or; (c) in 5-FU-resistant cells other pathways are mobilized to maintain cell viability and cell proliferation and these pathways are not affected by AOAA. The fact that AOAA remained partially effective in suppressing various bioenergetic parameters in 5-FU-resistant cells, and yet it failed to affect functional parameters (viability, proliferation) indirectly supports the last hypothesis.

Consistent with our findings, an earlier study that exposed CT26 mouse colon carcinoma cancer cells to cytotoxic concentrations of hydrogen peroxide to yield a potentially lethal damage-recovered cells population, showed that the recovered cells increased expression of CBS, CSE and 3-MST [41]. Furthermore, the cells with upregulated H_2S-producing enzyme expression underwent a significant degree of dedifferentiation, which was, at least in part, due to elevation of intracellular NAD^+ levels in the damage-recovered cells [41]. However, in the CT26 study, the effect of H_2S biosynthesis inhibitors on the proliferation or viability of parental or damage-recovered cells was not evaluated.

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Endogenous H_2S is cleared at least in part, through the mitochondrial sulfide oxidation pathway. The major oxidation products of H_2S are thiosulfate and sulfate whose ratio and production rate vary in a tissue-specific manner. The sulfide oxidation pathway begins with sulfide quinone oxidoreductase (SQR) and includes a sulfur dioxygenase, sulfite oxidase and rhodanese. Rhodanese has a 60% homology to 3-MST [42]. Rhodanese catalyzes the formation of thiosulfate primarily *via* transferring sulfhydryl groups to cyanide, forming thiocyanate and sulfate [42]. However, rhodanese also has the ability to produce H_2S from thiosulfate [43,44]. Furthermore, Kimura *et al.* showed that rhodanese utilizes H_2S to generate polysulfides (i.e. H_2S_3) [45]. There are some reports indicating that rhodanese expression is altered in cancer cells compared to non-transformed cells [46–48]; however, changes in rhodanese expression during drug resistance, prior the current study, have not been investigated. The current results indicate a substantial upregulation of rhodanese in 5-FU-resistant cells; however, the functional importance of this change remains to be explored. It appears that the increased H_2S degradation that would be expected from increased rhodanese is not able to counterbalance the increased H_2S biosynthesis, as evidenced by the direct measurement of H_2S production in tissue homogenates (Fig. 3C). One would expect that if upregulation of rhodanese (or other H_2S-degrading pathways) were a major factor contributing to the phenotype of the 5-FU-resistant cells, then pharmacological inhibition of H_2S production (e.g. with AOAA) would maintain most of its efficacy in 5-FU-resistant cell line; however, this is not the case, according to our data. Future experiments remain to be conducted to investigate the functional role of rhodanese upregulation in drug resistance.

One of the observations of the current study is that 5-FU-resistant HCT116 cells proliferated at a slower basal rate than parental HCT116 cells. It is possible that the excessively elevated endogenous H_2S level in 5-FU-resistant cells produced an anti-mitogenic effect in resistant cells. Indeed, others have demonstrated an antiproliferative effect of H_2S (at millimolar concentrations) in various cell lines *via* the induction of the cyclin-dependent kinase inhibitor p21Cip1, a protein associated with cell cycle arrest [49,50]. Wu and colleagues [49] observed that 1 mM NaHS (an H_2S concentration similar to those found in the human colon) induced a substantial accumulation of HT-29 cells (a colon epithelial cell line) at the G_0/G_1-phase and an upregulation of the cyclin-dependent kinase inhibitor p21Cip1 in a time-dependent manner. The AMPK/mTOR cascade plays a central role in mediating the antiproliferative effect of H^2S. Wu et al. [49] also demonstrated that H_2S increased the phosphorylation of AMPK at Thr-172, whereby the pharmacological inhibition of AMPK by compound C prevented the anti-mitogenic effect of H_2S in both HT-29 and HCT116 cells. Additionally, another group showed that H_2S inhibited cell proliferation and induced cell cycle arrest *via* the induction of p21Cip1 in Ca9-22 cells (a human gingival epithelial cell line) [50]. Further work is needed to delineate the pathways and mechanisms responsible for the slower proliferation rate observed in the 5-FU-resistant HCT116 cells.

We can only speculate on the nature of the genes and pathways that are altered in the current 5-FU-resistant cell population and further work remains to be conducted to evaluate the possible connections between these alterations and the upregulation of various H_2S pathways. For example, it has been previously suggested that 5-FU leads to the activation of ataxia-telangiectasia mutated serine/threonine protein kinase (ATM), AMP kinase (AMPK) and PGC-1α, which could promote mitochondrial biogenesis and thus stimulate OXPHOS

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[51–53]. In fact, H_2S was shown to enhance the expression and activity of PGC-1α leading to increased mitochondrial biogenesis in primary hepatocytes [54]. The potential connection between the H_2S pathway and the PGC-1α pathway in the current experimental paradigm remains to be further investigated.

We previously showed that pharmacological inhibition of CBS with AOAA significantly reduced the growth rate of patient-derived colon cancer xenografts and reduced tumor blood flow to the tumor xenografts in nude mice [7]. We also showed that AOAA significantly decreased tumor growth and size in nude mice bearing subcutaneous HCT116 cancer cell xenografts [9]. Based on the current results, we anticipate that the efficacy of AOAA would diminish in mice bearing relapsing/multi-drug resistant colon cancer cell xenografts. However, the current data showing that the anticancer efficacy of another CBS inhibitor, benserazide may be encouraging in this respect. Benserazide has been identified by a screening campaign that was seeking to identify clinically used, and potentially repurposable drugs for CBS inhibition [37]. The mode of benserazide's inhibition of CBS is likely related to its metabolic conversion to an active metabolite, which interferes with the PLP prosthetic group in the active center of CBS [37]. Since benserazide likely affects several enzymes (its "original" mode of action is inhibition of DOPA decarboxylase, another PLP-dependent enzyme) it is possible that some of its antitumor effects rely on the combination of these additional targets. Benserazide is not known to be metabolized by those cytochrome enzymes that we found upregulated in the 5-FU-resistant HCT116 cells. Our working hypothesis, therefore, is that the 5-FU-resistant colon cancer cells metabolize benserazide to a lesser degree than other compounds (e.g. 5-FU or AOAA), and this metabolic stability may account for its maintained antiproliferative efficacy in 5-FU-resistant cells. Further studies are needed to test whether the efficacy of benserazide *in vivo* is maintained in animals bearing drug-resistant colon cancer cell lines.

The prognosis of advanced colorectal cancer remains poor [55]. Chemotherapy with 5-FU has been the first line treatment option for advanced colorectal cancer, but many patients develop cancer relapse after initial treatment. Therefore, investigating cellular molecular mechanisms leading to anti-cancer drug resistance is of paramount importance. By further understanding the molecular mechanisms of drug resistance - including the mechanism and functional consequences of enhanced H_2S generation in 5-FU-resistant colon cancer - may be useful to formulate rational strategies to counteract multidrug resistance.

Acknowledgments

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Abbreviations

2-DG	2-deoxyglucose
3-MST	3-mercaptopyruvate sulfurtransferase
5-FU	5-Fluorouracil
AA	antimycin A

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ABC	ATP-binding cassette
AMPK	AMP kinase
AOAA	aminooxyacetic acid
ATM	ataxia-telangiectasia mutated serine/threonine protein kinase
ATP	adenosine triphosphate
AzMc	7-azido-4-methylcoumarin
CBS	cystathionine-β-synthase
CSE	cystathionine-γ-lyase
CTL	control
CYP450	cytochromes P450
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GOT1	glutamate oxaloacetate transaminase 1
H_2S	hydrogen sulfide
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride
MRP1	multidrug resistance-associated protein 1
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
OCR	oxygen consumption rate
oligo	oligomycin
PGC-1α	peroxisome proliferator-activated receptor-γ- coactivator-1α
PMS	*N*-methylphenazonium methyl sulfate
PPR	proton production rate
Rot	rotenone
SQR	sulfide quinone oxidoreductase
TCA	tricarboxylic acid

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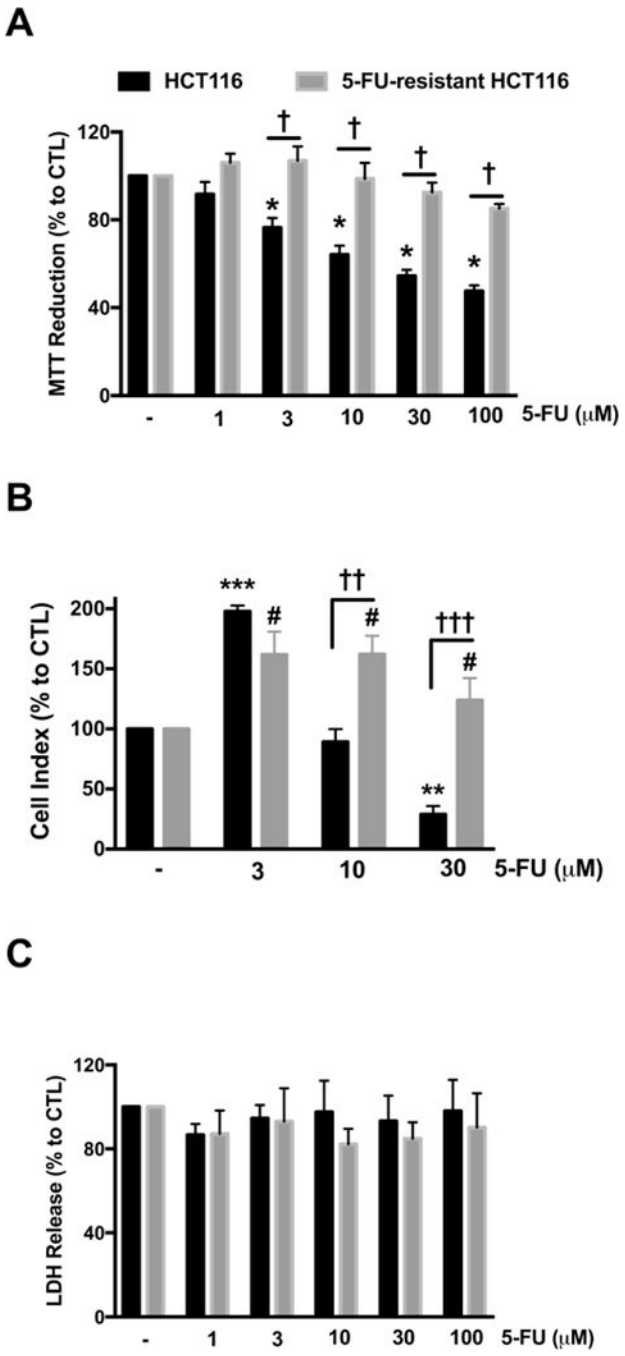

Figure 1. Characterization of 5-FU-resistant HCT116 cells

HCT116 and 5-FU-resistant HCT116 cells were treated with 5-FU (3–100 μM) for 48 h. **(A)** MTT reduction in both HCT116 and 5-FU-resistant HCT116 cells treated with 5-FU. **(B)** HCT116 and 5-FU-resistant HCT116 cells were seeded in xCELLigence plates and proliferation was monitored for 24 h. Thereafter, cells were treated with or without 5-FU (3–30 μM) and proliferation was monitored for another 48 h. Figure shows statistical analysis of cell proliferation endpoints normalized to cell index and expressed as a percentage of the corresponding control group. **(C)** Necrotic cell death determined by LDH release in HCT116

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and 5-FU-resistant HCT116 cells treated with 5-FU. Data represent mean ± SEM. $n = 4$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ (based on two-way ANOVA corrected with Tukey's post-hoc test).

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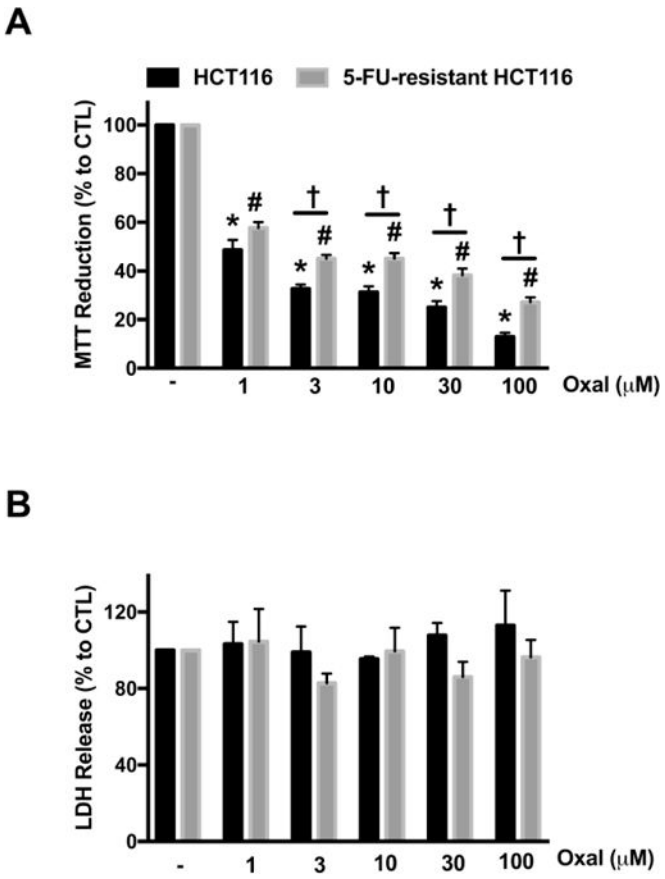

Figure 2. 5-FU-resistant HCT116 cells exhibit partial resistance to oxaliplatin

HCT116 and 5-FU-resistant HCT116 cells were treated with oxaliplatin (1–100 μM) for 48 h. **(A)** MTT reduction in both HCT116 and 5-FU-resistant HCT116 cells treated with oxaliplatin. **(B)** Necrotic cell death determined by LDH release in HCT116 and 5-FU-resistant HCT116 cells treated with oxaliplatin. Data represent mean ± SEM. $n = 5–8$ per group. $^{*}P < 0.05$ *vs* HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test). Oxal: oxaliplatin.

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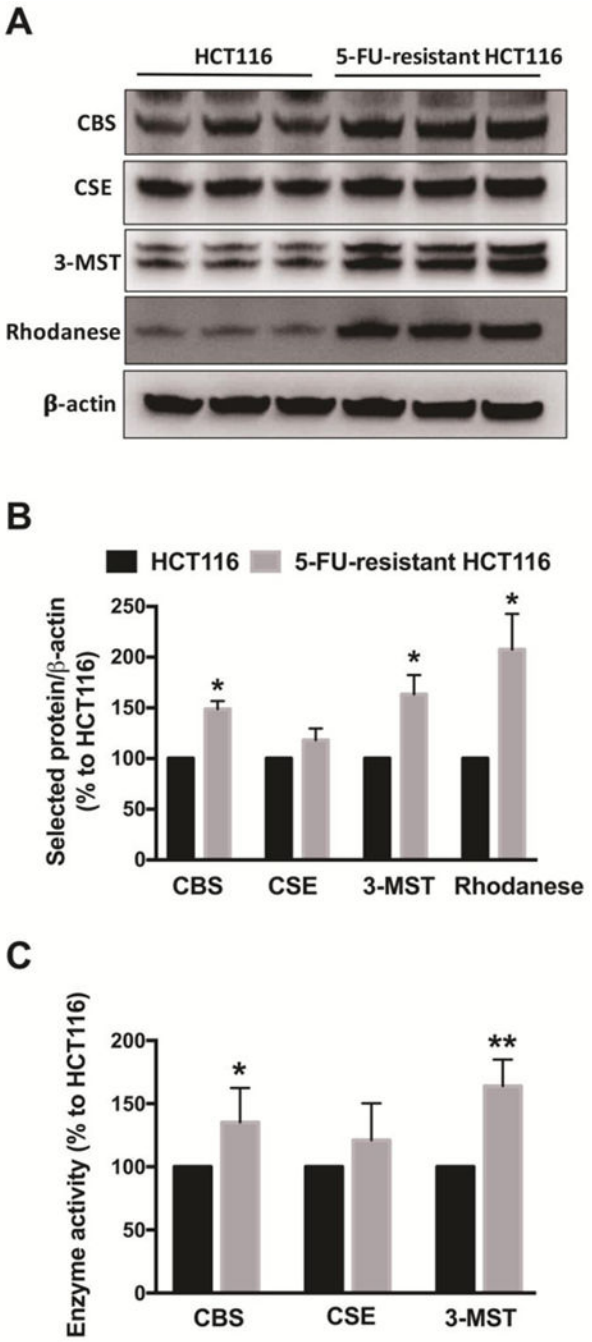

Figure 3. Proteins and the enzymatic activities of major H_2S-generating enzymes are upregulated in 5-FU-resistant HCT116 cells

(A) Representative western blot images of CBS, CSE, 3-MST and rhodanese are shown. **(B)** The density of the H_2S-producing enzymes was normalized to that of β-actin and expressed as a percentage of the corresponding HCT116 group. $n = 3–6$ per group. $*P < 0.05$ vs. HCT116 CTL. **(C)** The enzymatic activity of CBS, CSE and 3- MST were analyzed as described in Material and Methods and expressed as a percentage to the corresponding HCT116 group. $n = 3–4$ per group. Data represent mean ± SEM. $*P < 0.05$, $**P < 0.01$ vs. HCT116 cells (based on unpaired Student's *t* test).

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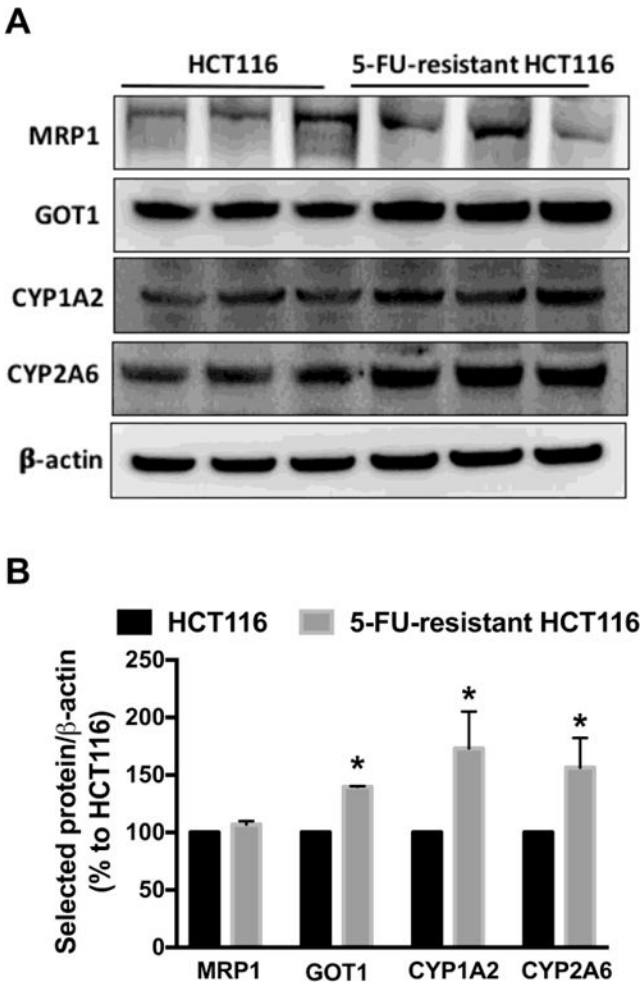

Figure 4. GOT1 and CYP450 proteins are upregulated in 5-FU-resistant HCT116 cells

(A) Representative western blot images of MRP1, GOT1, CYP1A2 and CYP2A6 are shown. **(B)** The density of the selected proteins was normalized to that of β-actin and expressed as a percentage of the corresponding HCT116 group. $n = 3–4$ per group. Data represent mean ± SEM. *$P < 0.05$ vs. HCT116 CTL (based on Student's *t* test).

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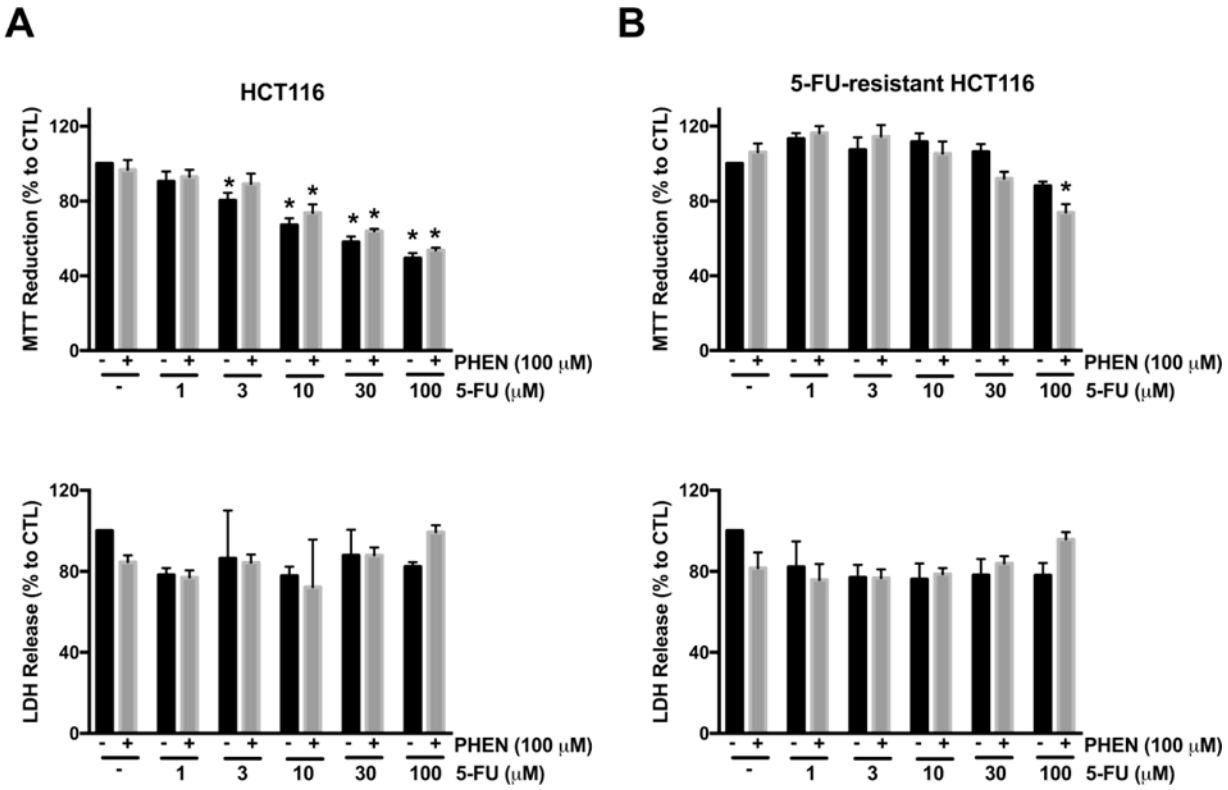

Figure 5. Inhibition of CYP450 enzymes sensitizes 5-FU-resistant HCT116 cells to 5-FU treatment

HCT116 **(A)** and 5-FU-resistant HCT116 cells **(B)** were treated with 5-FU (1–100 μM) and/or 100 μM phenylpyrrole, a potent CYP450 inhibitor, for 48 h. Top panels in both **(A)** and **(B)** shows cell viability *via* MTT reduction under various treatment conditions. $n = 3–5$, for each group. Bottom panels in both **(A)** and **(B)** show necrotic cell death determined by LDH release under various treatment conditions. $n = 4$ per group. Data represent mean ± SEM. $^{*}P < 0.05$ *vs.* HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resisant CTL (based on two-way ANOVA corrected with Tukey's post-hoc test). PHEN: phenylpyrrole.

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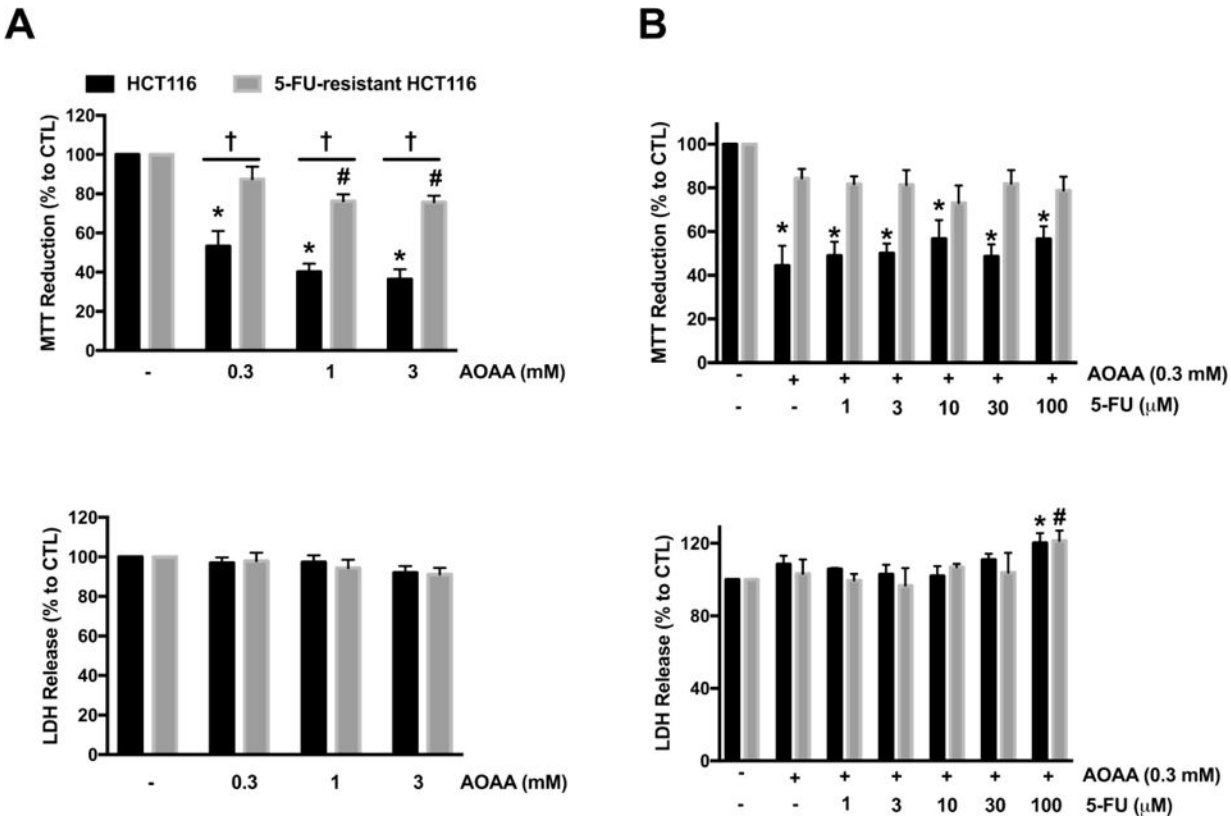

Figure 6. 5-FU-resistant HCT116 cells are resistant against AOAA treatment

(A) HCT116 and 5-FU-resistant HCT116 cells were treated with AOAA (0.3–1 mM) for 48 h. Top panel: MTT reduction in both HCT116 and 5-FU-resistant HCT116 cells treated with 5-FU. Bottom panel: Necrotic cell death determined by LDH release in HCT116 and 5-FU-resistant HCT116 cells treated with AOAA. $n = 5–8$ per group. **(B)** HCT116 and 5-FU-resistant HCT116 cells were treated with 5-FU (1–100 μM) with or without 0.3 mM AOAA for 48 h. Top panel: MTT reduction in both HCT116 and 5-FU-resistant HCT116 cells; bottom panel: necrotic cell death determined by LDH release in HCT116 and 5-FU-resistant HCT116 cells. $n = 3–5$ per group. Data represent mean ± SEM. $^{*}P < 0.05$ *vs* HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test).

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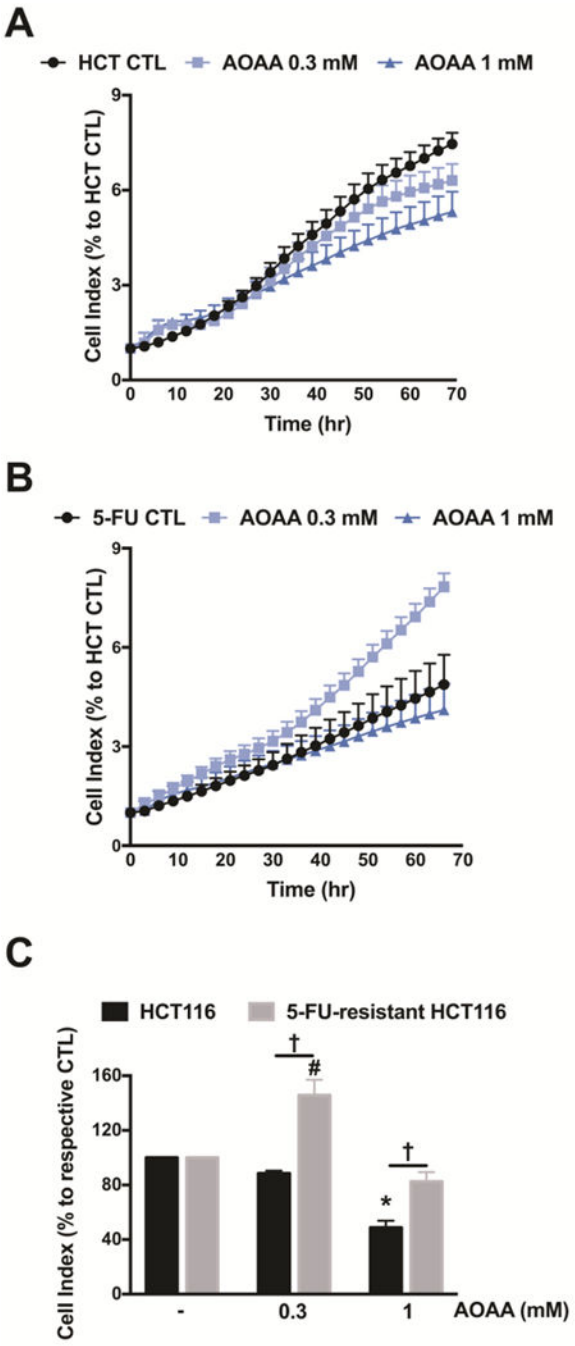

Figure 7. The antiproliferative effects of AOAA is absent in 5-FU-resistant HCT116 cells

Both cell lines were seeded in xCELLigence plates and proliferation was monitored for 24 h. Thereafter, cells were treated with or without AOAA (0.3–1 mM), and proliferation was monitored for another 48 h. Cell proliferation is shown over time for HCT116 cells **(A)** and 5-FU-resistant HCT116 cells **(B)** under various treatments. **(C)** Statistical analysis of cell proliferation endpoints normalized to cell index and expressed as a percentage of the corresponding control group. Data represent mean ± SEM. $n = 3–4$ per group. $^{*}P < 0.05$ *vs.*

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HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test).

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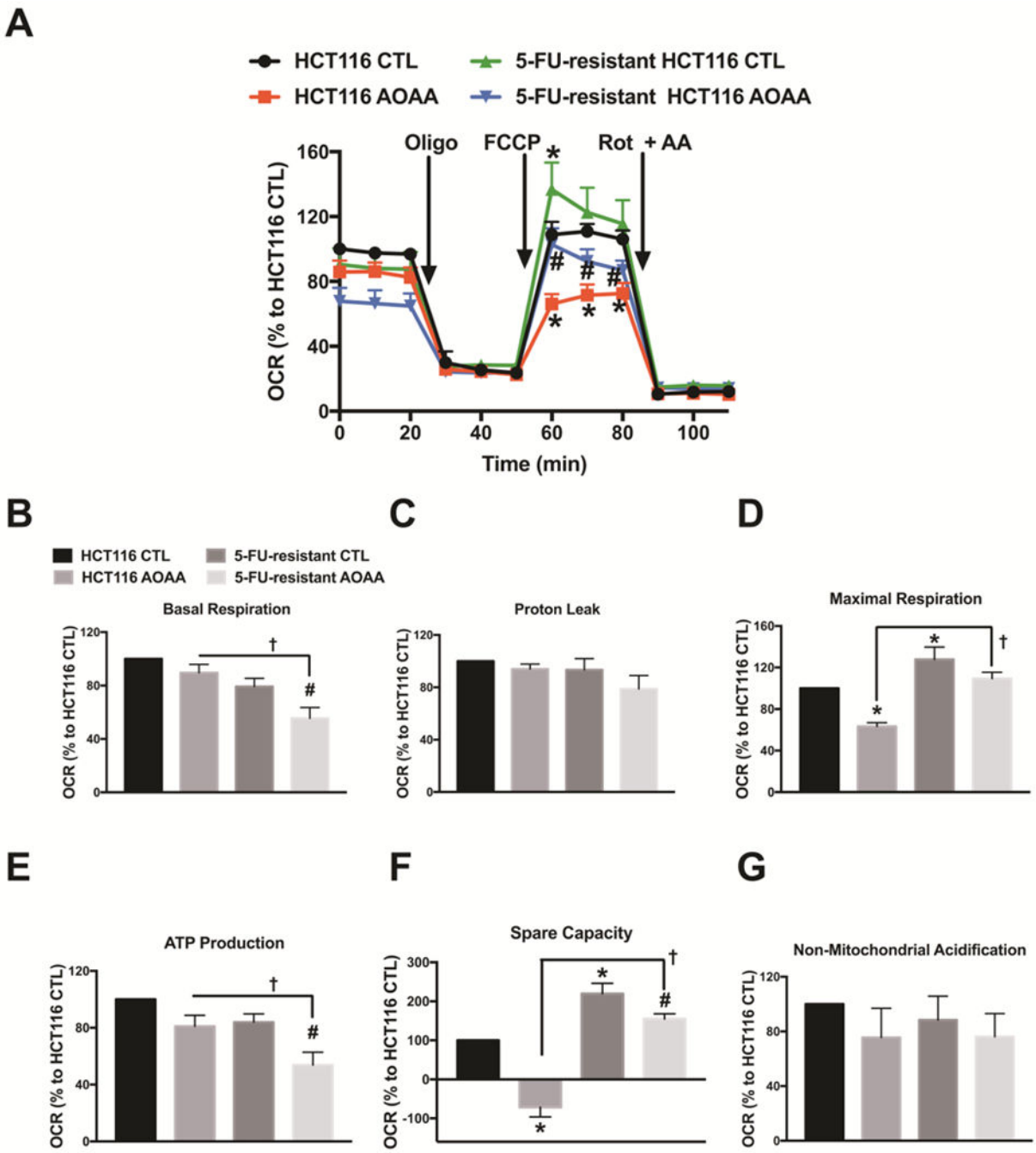

Figure 8. Comparison of aerobic respiration in control and 5-FU-resistant HCT116 cells; effect of AOAA

(A) Oxygen consumption rate (OCR) in HCT116 and 5-FU-resistant HCT116 cells (FCCP: carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, a mitochondrial oxidative phosphorylation uncoupler; Rot: rotenone; and AA: antimycin A; inhibitors of complex I and III, respectively). **(B–E)** Comparison of cellular bioenergetics parameters based on OCR. Data represent mean ± SEM. $n = 4–6$ per group. $^{*}P < 0.05$ *vs.* HCT116 CTL; $^{\#}P <$

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0.05 *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test).

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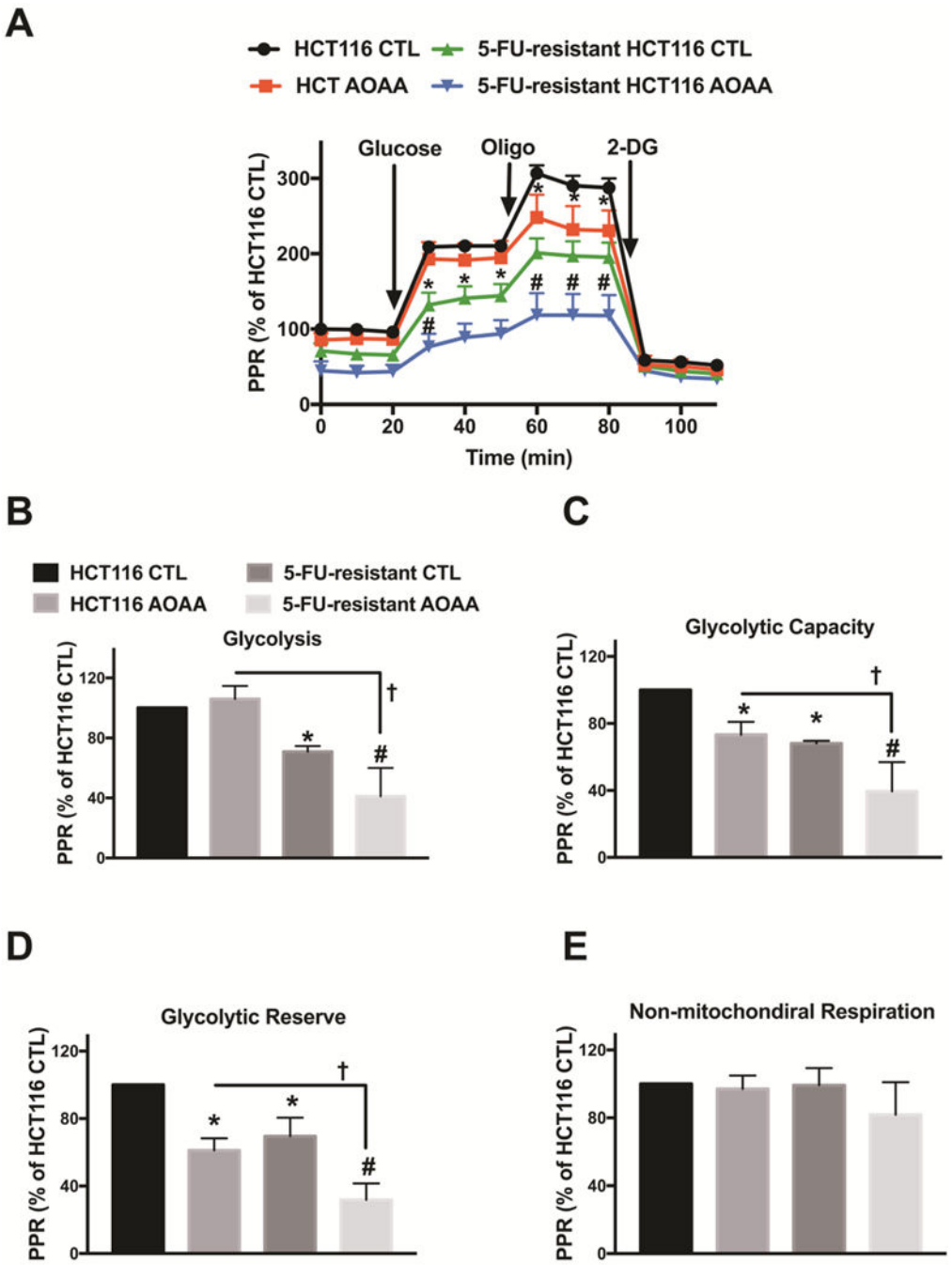

Figure 9. Comparison of glycolysis in control and 5-FU-resistant HCT116 cells; effect of AOAA

HCT116 and 5-FU-resistant HCT116 cells were treated with or without 0.3 mM AOAA for 1 h. Extracellular Flux Analysis was then performed. **(A)** Changes in the prolife of proton production rate (PPR) in HCT116 or 5-FU-resistant HCT116 cells (oligo: oligomycin, an inhibitor of ATP synthase; 2-DG: 2-deoxy-D-glucose; an inhibitor of glycolysis). **(B–E)** Comparison of cellular bioenergetics parameters based on PPR. Data represent mean ± SEM. $n = 4–6$ per group. $^{*}P < 0.05$ vs. HCT116 CTL; $^{\#}P < 0.05$ vs. 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test).

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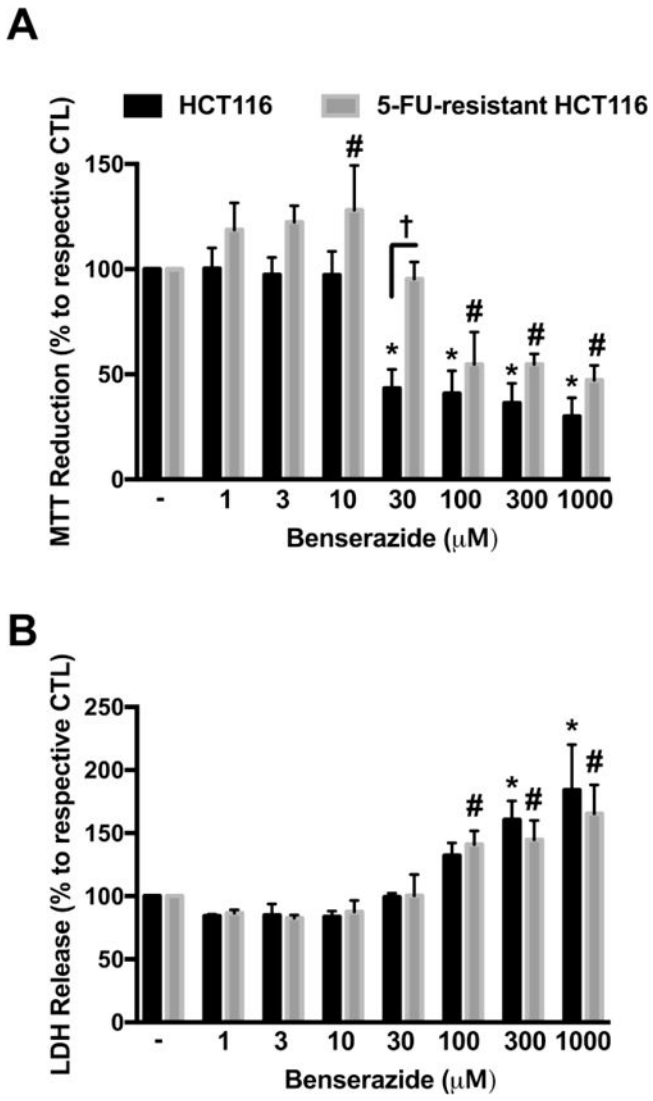

Figure 10. 5-FU-resistant HCT116 cells respond to benserazide treatment

HCT116 and 5-FU-resistant HCT116 cells were treated with benserazide (1–1000 μM) for 48 h. **(A)** MTT reduction in both HCT116 and 5-FU-resistant HCT116 cells treated with benserazide. **(B)** Necrotic cell death determined by LDH release in HCT116 and 5-FU-resistant HCT116 cells treated with benserazide. $n = 4$ per group. $^{*}P < 0.05$ *vs* HCT116 CTL; $^{\#}P < 0.05$ *vs.* 5-FU-resistant HCT116 CTL; $^{\dagger}P < 0.05$ (based on two-way ANOVA corrected with Tukey's post-hoc test).