GLUTAMINE DEPRIVATION ENHANCES ANTITUMOR ACTIVITY OF 3-BROMOPYRUVATE THROUGH THE STABILIZATION OF MONOCARBOXYLATE TRANSPORTER-1

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ABSTRACT

Anticancer drug efficacy might be leveraged by strategies to target certain biochemical adaptations of tumors. Here we show how depriving cancer cells of glutamine can enhance the anticancer properties of 3-bromopyruvate, a halogenated analog of pyruvic acid. Glutamine deprival potentiated 3-bromopyruvate chemotherapy by increasing the stability of the monocarboxylate transporter-1 (MCT-1), an effect that sensitized cells to metabolic-oxidative stress and autophagic cell death. We further elucidated mechanisms through which resistance to chemo-potentiation by glutamine deprival could be circumvented. Overall, our findings offer a preclinical proof of concept for how to employ 3-bromopyruvate or other monocarboxylic-based drugs to sensitize tumors to chemotherapy.

INTRODUCTION

Cancer cells differ from untransformed ones by a plethora of bioenergetic and metabolic changes to sustain their high rate of growth and proliferation (1). Therefore, targeting metabolic adaptations has the potential to affect specifically cancer cells. In particular, inhibitors of glycolysis and drugs impairing mitochondrial oxidative phosphorylation (OXPHOS), have been exploited to target malignancies on the basis of their capability to elicit metabolic-oxidative stress, culminating with cell demise by apoptosis or autophagic cell death (2-5). To drive clinical translation of these drugs, growing interest is aimed at identifying metabolic conditions able to enhance their killing properties, improve selectivity towards neoplastic tissues and reduce side effects.

Glutamine complements glucose to meet the specific energetic and anabolic requirements of growing tumor cells (1, 6, 7). Indeed, its uptake and metabolism are strongly enhanced in cancer cells to fulfill ATP needs, to refuel Krebs cycle of metabolites constantly used for nucleotides and lipids biosynthesis as well as to modulates redox-homeostasis (6,7). To target glutamine-addicted tumors, growing interest has been recently arousing in the development of chemotherapeutics that

suppress glutamine-dependent anaplerosis (8,9) and lower blood glutamine levels (7, 10, 11). However, feasible antitumor strategies inhibiting glutamine uptake in cancer cells have not been explored yet.

3-Bromopyruvate (3-BrPA) is an halogenated and alkylating analog of pyruvic acid able to curb the growth of many tumors, frequently refractory to standard therapeutics, by compromising ATP synthesis through the inhibition of glycolysis and mitochondrial Complex II activity (12-16). In accordance with current challenges aimed at identifying metabolic conditions chemo-potentiating the effectiveness of antitumor drugs, our study argues for the ability of glutamine deprivation to sensitize carcinoma cells to the cytotoxic effects of 3-BrPA both *in vitro* and *in vivo*. We demonstrate that glutamine deprivation stimulates the intracellular uptake of 3-BrPA which results in the enhanced generation of metabolic-oxidative stress, mainly dependent on the inhibition of mitochondrial Complex II, responsible, in turn, for the final induction of autophagic cell death. Our results identify in monocarboxylate transporter-1 (MCT-1), the molecular determinant responsible for the enhanced uptake and cytotoxicity of 3-BrPA and, in glutamine synthetase expression, the molecular device exploited by cancer cells to circumvent 3-BrPA chemo-potentiation. Overall, our study represents proof of principle that anticancer effects can be potentiated by combinating monocarboxylic-based drugs with strategies to deprive tumors of glutamine.

MATERIALS AND METHODS

3-Bromopyruvate (3-BrPA), N-acetylcysteine (NAC), dimethylthiourea (DMTU) 3-Methyladenine (3-Ma), methionine sulfoximine (MSO), L-γ-glutamyl-p-nitroanilide (*GPNA*), 6-diazo-5-oxo-l-norleucine (DON), epigallocatechin gallate (EGCG), amino-oxyacetic acid (AOA), Ibuprofen, α-cyano-4-hydroxycinnamate (α-CHC), NH_4Cl, Staurosporine, 2-Deoxy-D-glucose (2-DG), cycloheximide (CHX), dichloroacetate (DCA), acridine orange, EDTA, EGTA, paraformaldehyde, propidium iodide, Triton X-100 were from Sigma; benzyloxycarbonyl-Val-Ala-DL-Asp fluoromethylketone (Z-VAD-fmk), was from Alexis; 2'7'-dichlorodihydrofluorescein

diacetate (DCFH-DA) from Invitrogen-Molecular Probes; goat anti-mouse and anti-rabbit IgG (heavy and light chains)–horseradish peroxidase conjugate were from Bio-Rad Laboratories. If not otherwise indicated, all other chemicals were obtained from Merck.

HeLa (human cervix carcinoma), DU145 (hormone insensitive and prostate antigen defective-human prostate carcinoma), PC-3 (bone metastasis-derived grade IV human prostatic adenocarcinoma), MCF-7 (pleural effusion-derived human mammary gland adenocarcinoma) and T-47D (pleural effusion-derived infiltrating ductal carcinoma of the mammary gland) cells were purchased from the European Collection of Cell Culture and grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS (fetal calf serum), 1% penicillin/streptomycin and 1% glutamine. All cell lines were periodically authenticated by morphologic inspection and tested negative for Mycoplasma contamination by PCR tests from 2010 to 2012. The cells were maintained at 37°C in a 5% CO_2 atmosphere in air and routinely trypsinized and plated at $4x10^4/cm^2$ on flasks. Cell viability was assessed by Trypan blue exclusion. Primary cervix endometrial cells (PCEC) were enzymatically isolated from the mouse uterus. Uterine tissues were obtained from immature (3.5 to 4 weeks of age) *C57BL/6* mice to avoid complications, related to estrous cycle. Isolated endometrial cells were resuspended in DMEM/F-12 culture medium containing 10% FBS, 1% NEAA, and 1% penicillin/streptomycin, plated at a density of about $1.4x10^6$ cells/ml onto collagen-coated cell culture plates and incubated at 37°C in a 5% CO_2 atmosphere in air for 3 days before being used.

If not otherwise indicated, glutamine deprivation was carried out by incubating cells for 12 hours in glutamine-free medium. Cell death was assessed after 24 hours of 3-BrPA incubation by the Trypan blue exclusion assay. A 10 mM solution of 3-BrPA (Sigma) was prepared just before the experiments by dissolving the powder in phosphate buffered saline (PBS), adjusted to pH 7.0 with NaOH . The antioxidants, the autophagy inhibitor, the pan-caspase inhibitor and the glutamine synthetase inhibitor were added upon glutamine deprivation and maintained throughout the experiment. The SLC1A5 inhibitor, the glutaminase inhibitor, the glutamate dehydrogenase

inhibitor and the transaminase inhibitor were pre-incubated for 12 hours in glutamine-containing medium and maintained throughout the experiment. The MCTs inhibitor and the SMCTs inhibitor were pre-incubated for 1 hour in glutamine-deprived medium and maintained throughout the experiment. Staurosporine and 2-DG were added in glutamine-containing or deprived medium. Cycloheximide was added in glutamine-containing or deprived medium after 6 hours of glutamine deprivation.

Cells were transfected by electroporation using a Gene Pulser Xcell system (Bio-Rad). siRNA were used to knock down MCT-1 and Atg5. Stable knockdown of SDHA expression was performed by transfecting cells with a pSuper vector carrying a shRNA sequence against SDHA (shSDHA). pCI-neo, pCDNA3 and pEGFP-C2 constructs were used to over-express MCT-1, DN-GS and LC3-EGFP, respectively.

$4x10^6$ HeLa cells were injected subcutaneously into adult female (6 weeks old) *Hsd*:*Athymic Nude-Foxn1nu* mice (Harlan Laboratories, Italy). After thirty days, animals were randomized into 4 different groups, containing five mice each one, which were intraperitoneally daily injected with freshly prepared 3-BrPA (1.25 mg/kg), GPNA (20 mg/kg) or the combination of both compounds. Tumor lengths (L) and widths (W) of sagittal sections were measured three times per week by manual caliper. After 14 days, mice were scarificed. Tumor weights were measured and tumor volumes were calculated by the *formula*: Tumor Volume = 0.5 x L x W^2. Tumor growth has been reported as % of tumor volume increase with respect to the beginning of treatment.

Intracellular ROS and AVOs levels were assessed cytofluorimetrically upon DCFH-DA and AO staining, respectively. Carbonylated proteins, ATP levels and intracellular glutamine concentration were detected using the Oxyblot Kit (Intergen), the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) and the glutamine assay kit (Abnova), respectively.

The activity of Complex II (succinate:coenzyme Q oxidoreductase activity), glutamine synthetase and extracellular lactate were assessed spectrophotometrically as described in detail in supplemental data.

Measurement of 3-BrPA levels in cell medium was achieved by NMR spectroscopy. In particular, HeLa cells were treated with 50 μM 3-BrPA-3-^{13}C (Sigma) for 1 hour. Cell media were harvested, centrifuged at 900 g for 5 min and supernatants incubated with 70% ethanol. After centrifugation at 20,000 g for 30 min, supernatant were dried and suspended in 20% D_2O, filtered and placed in a 5 mm NMR tube. ^{13}C spectra were obtained at 176.054 MHz by using a Bruker Avance 700 Ultrashield spectrometer.

Data significance was evaluated using a Student's t test corrected by Bonferroni's method. P values <0.05 were considered significant.

Clonogenic assay, qPCR subcellular fractionation and western blotting procedures as well as a complete and detailed description of all methods employed are available as Supplementary Data.

RESULTS

Glutamine deprivation potentiates antitumor activity of 3-BrPA

To evaluate the capability of glutamine to modulate sensitivity of carcinoma cells to 3-BrPA, we incubated glutamine-addicted cervix carcinoma HeLa cells in glutamine-free culture medium for different hours before challenging with sub-toxic doses of 3-BrPA and measured cell viability 24 hours after drug addition. **Fig.1A** shows that 12 hour-glutamine deprivation was able to sensitize HeLa cells to 3-BrPA cytotoxicity in a dose-dependent manner. Interestingly, glutamine withdrawal did not modulate the sensitivity of mouse primary cervix epithelial cells (PCEC), the untransformed counterpart of HeLa cells, to 3-BrPA challenge (**Fig. 1B**) suggesting that 3-BrPA chemo-potentiation is a cancer-specific phenomenon. To validate the ability of glutamine to increase 3-BrPA chemo-resistance in other cancer cells, we incubated human prostate carcinoma PC3 and DU145 cells with 3-BrPA after glutamine deprivation. Similarly to HeLa cells, glutamine withdrawal increased 3-BrPA cytotoxicity in both these cell lines (**Suppl. Fig. 1A**). Next, we investigated whether the enhancement of 3-BrPA effects could be phenocopied by the

pharmacological inhibition of glutamine uptake. Therefore, HeLa cells were incubated for 12 hours with L-γ-glutamyl-p-nitroanilide (GPNA), the specific inhibitor of SLC1A5, the main transporter mediating glutamine uptake in carcinoma cells (17) and, then, exposed to 3-BrPA. Results obtained demonstrated that GPNA pre-treatment was effective in enhancing HeLa cells sensitivity to 3-BrPA (**Fig. 1C**) and in lowering intracellular glutamine content (**Suppl. Fig. 1B**).

To assess the long-term effects of 3-BrPA chemo-potentiation on cell growth and survival, clonogenic assay was performed on glutamine-starved or GPNA-preincubated HeLa cells. Glutamine deprivation, as well as SLC1A5 inhibition, reduced the clonogenic and reproductive potential of HeLa cells after 3-BrPA challenge (**Suppl. Fig. 1C**). Next we evaluated the capability of GPNA to potentiate anticancer activity of 3-BrPA *in vivo*. To this aim, nude mice harboring HeLa tumor xenografts were treated with 3-BrPA (1.25 mg/kg), GPNA (20mg/kg) or the combination of both compounds for 14 days. **Fig. 1D** shows that, although the drugs had no effect when used alone, they significantly affected tumor growth when used in combination. Then, we explored whether apoptosis was engaged for the execution of 3-BrPA-induced cell death elicited upon glutamine restrictive conditions. As displayed in **Suppl. Fig. 1D**, no significative changes in the apoptotic extent was measured in 3-BrPA-treated cells with respect to untreated counterparts, regardless of glutamine supplementation. Moreover, the pan-caspase inhibitor Z-VAD-*fmk*, failed to inhibit 3-BrPA cytotoxicity (**Suppl. Fig. 1E**), thereby allowing us to exclude apoptosis in cell death execution.

Autophagy is involved in 3-BrPA-induced cell death upon glutamine withdrawal

Since apoptosis was not associated with 3-BrPA-induced cell death, we evaluated whether autophagy was induced in our conditions. Therefore, we measured the levels of acidic vesicular organelles (AVOs), produced upon 3-BrPA challenge. Results obtained in HeLa cells (**Fig. 2A**) as well as PC3 and DU145 cells (**Suppl. Fig. 2A**) indicated that glutamine deprivation generated

AVOs, whose levels further increased upon 3-BrPA treatment. Similar evidence were obtained inhibiting glutamine uptake by GPNA incubation (**Fig. 2A**). To define the autophagic nature of 3-BrPA-induced AVOs, we measured, by Western blotting, the levels of the autophagosome-associated form of LC3 (LC3-II) and the autophagy-degraded protein p62. In line with data from literature (17), glutamine-deprived as well as GPNA incubated cells underwent autophagy (**Fig. 2B**). Moreover, glutamine withdrawal and GPNA pre-treatment primed HeLa cells to undergo a higher autophagic activity upon 3-BrPA challenge, as indicated by the enhanced accumulation of LC3-II and degradation of p62 with respect to their 3-BrPA-untreated counterparts (**Fig 2B**). To corroborate this data, the intracellular distribution of LC3 in LC3-EGFP transfected HeLa cells was examined. Glutamine deprivation induced a change in the distribution of LC3 fluorescence from a diffuse to a punctate cytosolic pattern (**Fig. 2C**), typical of cells undergoing autophagy. Moreover, an enhanced percentage of LC3-GFP dotted cells was observed upon 3-BrPA challenge in glutamine-starved cells with respect to their unchallenged counterparts (**Fig. 2C**). Co-incubation with NH_4Cl, which blocks autophagic degradation, enhanced 3-BrPA-induced accumulation of LC3-GFP punctuated cells, confirming that 3-BrPA citotoxicity is associated with a sustained autophagic flux in absence of glutamine. Enhanced autophagy induction was observed in PC3 and DU145 cells, as well (**Suppl. Fig. 2B**). Furthermore, Western blot analysis of excised tumor tissues indicated that the slower tumor growth observed in GPNA/3-BrPA-cotreated mice was associated with a higher autophagic activity compared with both vehicle and single drugs-treated mice (**Fig. 2D**). As autophagy can mediate cell death (type-II or autophagic cell death), we evaluated its contribution in 3-BrPA cytotoxicity. To this aim, we treated cells with 3-methyladenine (3-Ma), an inhibitor of initiation steps of autophagy, and evaluated cell viability upon 3-BrPA treatment. In line with the pro-survival role of autophagy upon nutrient limitation, 3-Ma induced a slight increase of cell death in glutamine-deprived cells (**Fig. 2E**). However, it significantly decreased 3-BrPA chemo-potentiation, as the percentage of dead cells was almost halved (**Fig. 2E**) and inhibited autophagosomes formation, as demonstrated by the significant decrease of LC3-II levels (**Suppl.**

Fig. 2C). This finding was substantiated by experiments carried out in PC3 and DU145 cells, as well (**Suppl. Fig. 2D**). To confirm the role of autophagy in cell demise, we silenced the expression of Atg5, an essential protein for autophagosomes formation. siRNA-mediated knock down of Atg5 decreased cell death extent upon 3-BrPA addition (**Fig. 2F** and **Suppl. Fig. 2E**) and reduced autophagy (**Suppl. Fig. 2F**), thereby resembling the effects of 3-Ma.

Mitochondrial Complex II inhibition accounts for 3-BrPA-induced metabolic-oxidative stress upon glutamine deprivation

In accordance with data from literature, we hypothesized that 3-BrPA chemo-potentiation could result from the generation of bioenergetics crisis elicited by the simultaneous dampening of glutaminolytic route, caused by glutamine withdrawal, and the inhibition of glycolysis, elicited by 3-BrPA (scheme of **Suppl. Fig. 3A**). To address this issue, we measured intracellular ATP content (**Fig. 3A**) and extracellular lactate levels (**Suppl. Fig. 3B**) after 3-BrPA treatment. In line with the ability of glutamine to sustain ATP synthesis and lactate generation, glutamine-deprived HeLa cells exhibited a more pronounced reduction in both analytes levels in resting conditions and whenever treated with 3-BrPA, with respect to their glutamine-fed counterparts. To unravel the role of glycolytic dampening in 3-BrPA cytotoxicity, we supplemented glutamine-free medium with methyl pyruvate (MP), the cell-permeable form of the glycolytic endpoint product pyruvate. MP refueling did not prevent decrease of ATP and viability upon 3-BrPA challenge (**Suppl. Fig. 3C**). Moreover, the failure of glutamine starvation to chemo-potentiate 2-Deoxy-D-glucose (2-DG) (**Suppl. Fig. 3E**), a glycolytic inhibitor more efficient in reducing lactate levels then 3-BrPA (**Suppl. Fig. 3D**), suggests that 3-BrPA chemo-potentiation was not dependent on glycolysis block in our experimental conditions. Next, we challenged HeLa cells with 3-BrPA upon pharmacological inhibition of specific glutaminolytic enzymes (scheme of **Suppl. Fig. 3A)**. Inhibition of glutaminolysis did not chemo-potentiate 3-BrPA (**Suppl. Fig. 3F**). Furthermore, supplementation of

glutamine-free medium with glutamate, the first metabolite generated from oxidative deamination of glutamine, did not prevent 3-BrPA cytotoxicity (**Suppl. Fig. 3F**), suggesting that reduction of glutaminolytic flux was not responsible for 3-BrPA chemo-potentiation.

It has been previously reported that reactive oxygen species (ROS) underlie 3-BrPA cytotoxicity (18). To evaluate whether 3-BrPA affected cellular redox state, we measured the intracellular levels of ROS (**Fig. 3B**) and protein carbonyls (**Fig. 3C**), by-products of ROS-mediated damages, after 3 hours of 3-BrPA treatment. Results provided evidence that both 3-BrPA-generated analytes accumulated preferentially in glutamine-deprived HeLa cells than in glutamine-supplemented counterparts. Moreover, incubation of HeLa cells with the antioxidants and radical scavangers N-acetylcysteine (NAC) or dimethylthiourea (DMTU) prevented 3-BrPA-mediated cell death (**Fig. 3D**), induction of autophagy (**Fig. 3E**), protein carbonylation (**Suppl. Fig. 4A**) and ATP loss (**Suppl. Fig 4B**), providing the functional link between the generation of pro-oxidant conditions, the onset of energetic stress and the execution of cell death by autophagy.

3-BrPA has been reported to inhibit mitochondrial Complex II (13, 19, 20). In line with these data, we found that 3-BrPA elicited a greater inhibition of Complex II activity in glutamine-starved cells with respect to glutamine-fed counterparts (**Fig. 3F**). Electrons escaping from the redox core of Complex II, negatively affected by mutations or chemically inhibited, partially reduce O_2 and produce ROS (3, 20, 21); furthermore, ROS generated by OXPHOS inhibitors are prone to affect cellular energetics and elicit autophagic cell death (5, 22, 23). Therefore, we hypothesized that accumulation of ROS elicited by 3-BrPA-mediated Complex II inhibition could underlie the induction of type-II cell death upon glutamine withdrawal. To verify this hypothesis, we blunted Complex II activity by stably silencing the expression of SDHA (**Suppl. Fig. 5A and 5B**), the subunit responsible for succinate oxidation and electrons entering into the redox core of complex II (24, 25). Silenced SDHA cells (shSDHA) showed lower amount of protein carbonyls (**Fig. 3G**) and maintained higher ATP levels (**Suppl. Fig. 5C**) upon 3-BrPA treatment. Moreover, they were almost insensitive to 3-BrPA-indued cytotoxicity as demonstrated by the lower cell death extent

(**Fig. 3H**) and autophagic activity (**Suppl. Fig. 5D**) with respect to their controls. Moreover, in line with their lower sensitivity to the drug (**Suppl. Fig. 5E)**, we found that PCEC were characterized by a lower Complex II activity with respect to tumor counterparts (**Suppl. Fig. 5E)**, thereby substantiating the role of Complex II as pivotal target underlying 3-BrPA cytotoxicity.

Chemo-potentiation of 3-BrPA depend on the reduced degradation of MCT-1

To unravel the mechanisms responsible for the potentiation of 3-BrPA-induced metabolic-oxidative stress, we postulated that removal of glutamine from culture medium might stimulate the intracellular drug uptake. To evaluate drug internalization, we measured the decrease of 3-BrPA levels in cell media after 1 hour of treatment, by means of NMR spectroscopy. Glutamine-deprived cells displayed a significant decrease of extracellular 3-BrPA content with respect to the glutamine-supplemented counterparts (**Fig. 4A**), indicating that the presence of this amino acid in cell medium strongly influenced the efficiency of drug internalization. 3-BrPA belongs to the class of monocarboxylic acid drugs whose uptake are mediated by H^+-linked (MCTs) and Na^+-coupled (SMCTs) monocarboxylate transporters. To discriminate which subset could be involved in 3-BrPA internalization and cytotoxicity, we measured the viability of 3-BrPA-treated HeLa cells co-incubated alternatively with α-cyano-4-hydroxycinnamate (α-CHC), a specific pharmacological inhibitor of MCTs, and ibuprofen, a blocker of SMCTs. α-CHC, but not ibuprofen, rescued HeLa cells from 3-BrPA-induced cell death upon glutamine starvation (**Suppl. Fig. 6A**), suggesting that MCTs-dependent 3-BrPA uptake could be operative in our experimental conditions. α-CHC preferentially inhibits MCT-1 member of MCTs family (26). Therefore, we hypothesized that this transporter could be involved in the enhanced uptake of 3-BrPA upon glutamine starvation. To address this issue, we analyzed by Western blotting the intracellular content of MCT-1 in dependence on glutamine availability. Twelve hour-glutamine deprived (**Fig. 4B**) and GPNA-treated HeLa cells (**Suppl. Fig 6B**) displayed higher levels of MCT-1, with respect to their

glutamine-fed and GPNA-untreated counterparts. Glutamine deprivation-induced MCT-1 up-regulation was also observed in PC3 and DU145 cells (**Suppl. Fig. 6C**) and was coupled to the enhanced localization of the transporter in the plasma membrane (**Fig. 4C)**. As documented in literature (27), MCT-1 up-regulation was not due to a stimulation of MCT-1 transcription. Indeed, no significative differences in MCT-1 mRNA levels were measured in HeLa cells regardless of glutamine supplementation (**Suppl. Fig. 6E**). This evidence prompted us to postulate a role of glutamine as modulator of MCT-1 stability. To assess this issue, we starved cells of glutamine for 6 hours; then, cell media were replaced with glutamine-free or glutamine-containing ones in the presence of cycloheximide for additional 3 hours. Glutamine refueled cells showed a deeper reduction in MCT-1 protein content with respect to glutamine-deprived cells, indicating that this amino acid affects MCT-1 protein degradation (**Fig. 4D**). To evaluate whether the up-regulated levels of MCT-1 could facilitate the drug uptake, we stably over-expressed MCT-1 (pCI-MCT-1) (**Suppl. Fig. 7A**). pCI-MCT-1 displayed a significant decrease of extracellular 3-BrPA levels with respect to their mock counterparts, indicating that MCT-1 up-regulation strongly stimulated drug internalization (**Fig. 4E**). Interestingly, MCT-1 overexpression was sufficient to increase 3-BrPA toxicity, as demonstrated by cell death (**Fig. 4F**), ATP drop (**Suppl. Fig. 7B**) and protein carbonylation (**Suppl Fig. 7C**) with respect to mock counterparts. In agreement with the enhanced sensitivity of pCI-MCT-1 cells to the drug, siRNA-mediated MCT-1 silencing (**Suppl. Fig. 7D**) almost completely abolished 3-BrPA chemo-potentiation (**Fig. 4G**). These results were supported by the reduction of protein carbonyls content (**Suppl. Fig. 7E**), the maintenance of ATP levels (**Suppl Fig. 7F**) and inhibition of autophagic flux (**Suppl. Fig. 7G**). The link between MCT-1 up-regulation and enhancement of 3-BrPA cytotoxicity, prompted us to investigate whether the cytotoxic effects of other monocarboxylic drugs could be potentiated upon glutamine withdrawal, by capitalizing on MCT-1 up-regulation. To this aim, we evaluated the cytotoxicity of dichloroacetate (DCA), anticancer drug able to inhibit pyruvate dehydrogenase kinase (28, 29) in dependence on glutamine availability and MCT-1 expression. Similarly to 3-BrPA, DCA was able

to be chemo-potentiated upon glutamine deprivation (**Suppl. Fig. 7H**) or inhibition of its uptake (**Suppl. Fig. 7I**) as well as by MCT-1 overexpression (**Suppl. Fig. 7J**), providing a proof of principle that cytotoxic effects of monocarboxylic chemotherapeutics can be enhanced by strategies limiting glutamine availability.

Glutamine synthetase is involved in circumvention of 3-BrPA chemo-potentiation

Glutamine synthetase (GS), the enzyme catalyzing glutamine biosynthesis, is expressed differentially among cancer cells, representing one of genetic determinants of cell type-specific glutamine independence (30). On the basis of the tight liaison among glutamine availability, MCT-1 expression and cell sensitivity to 3-BrPA, we hypothesized that glutamine deprivation should predicts 3-BrPA chemo-potentiation only in glutamine-addicted tumors, expressing low levels of GS. To validate this assumption, we compared GS activity among different tumor cells. MCF-7 and T-47D, two breast cancer cell lines, are characterized by a higher GS activity with respect to HeLa, DU145 and PC3 cells (**Fig. 5A**). Consistent with this feature, glutamine content of glutamine deprived MCF-7 and T-47D was significantly higher than the other three carcinoma cells (**Suppl. Fig. 8A**). Moreover, MCF-7 and T-47D become sensitive to 3-BrPA only upon GS inhibition, achieved either by incubating cells with methionine sulfoximine (MSO), the specific pharmacological inhibitor of GS (**Fig. 5B**) or over-expressing a dominant-negative form of this enzyme (DN-GS) (**Suppl. Fig. 8B and 8C**). In accordance with these results, MCT-1 up-regulation was observed only in glutamine-free medium upon inhibition of GS activity (**Fig. 5C and Suppl. Fig. 8D**). Overall, these results identify the molecular strategy exploited by cancer cells to circumvent 3-BrPA chemo-potentiation.

DISCUSSION

Targeting metabolic adaptations of cancer cells has the potential to chemo-potentiate antitumor drugs (2, 9, 31). In line with this concept, we demonstrate that depriving cancer cells of glutamine or drugging the glutamine transporter SLC1A5 by GPNA represent a feasible strategy to potentiate antitumor effects of 3-BrPA both *in vitro* and *in vivo*, setting the stage for its clinical use against glutamine-addicted malignancies (**Suppl. Fig. 9**). Although 3-BrPA has antitumor effect on multiple cancer cell types, no trials attesting its clinical use are so far documentable, probably due to the putative side effects resulting from its alkylating properties. Therefore, in line with recent investigations focused on identifying strategies to chemo-potentiate 3-BrPA effectiveness (15, 32, 33), here we show that reduction of intracellular glutamine pool enhances the antitumor efficacy of 3-BrPA *in vivo*, avoiding thus, its putative deleterious noxious effects on normal cells.

Bringing further insights into the mechanism of action, we demonstrate that, upon glutamine withdrawal, 3-BrPA cytotoxicity depends on the generation of metabolic-oxidative stress mainly resulting from the inhibition of Complex II. Although we did not investigate the 3-BrPA target sites into Complex II, this report is the first elucidating the effective contribution of Complex II inhibition in the induction of 3-BrPA-mediated cell death. Moreover, strengthening the growing consensus depicting Complex II as a therapeutic target in cancer (3), our data highlights the preferential usage of ROS-generating Complex II inhibitors against epithelial cancers, on the basis of the higher Complex II activity and, consequently, the greater capability to generate oxidative stress, with respect to untransformed epithelial cells.

Some reports have described autophagy stimulation in response to 3-BrPA challenge (34), but no evidence of its involvement as mechanism of death elicited by 3-BrPA had been provided so far. We demonstrate that, upon glutamine withdrawal, autophagy contributes to the execution of 3-BrPA-induced cell death. Interestingly, supporting the well-recognized capability of many chemotherapeutics to induce autophagic cancer cell death *in vivo* (35, 36), our results strengthen the feasible application of therapeutic regimens exploiting autophagy as an effective cancer cell killing strategy, particularly to target apoptosis-resistant cells.

We provide evidence that 3-BrPA chemo-potentiation depends on its more efficient uptake. Indeed, glutamine starvation primed cells to a higher intracellular drug internalization, thus resulting in a sustained metabolic-oxidative stress that culminates in cell death. 3-BrPA belongs to the class of monocarboxylic acid drugs whose uptake is mediated by both MCTs and SMCTs (37). Although a strong correlation between MCTs expression and 3-BrPA sensitivity has been already reported (38, 39), kinetic measurements documenting its affinity for 3-BrPA are available only for SMCT-1, which rather is epigenetically silenced during tumorigenesis (18, 40). Furthermore, 3-BrPA is effective in reducing the viability of many tumors defective of SMCT-1 expression (18), including cancer cells employed in this study (data not shown). Therefore, the effective uptake of 3-BrPA in cancer cells through this subset of transporters remains questionable, making MCTs, which are overexpressed in many tumors (41), the most reasonable candidates for its internalization (18, 39). In agreement with these observations, our study indicate MCT-1 as a candidate for 3-BrPA uptake upon glutamine deprivation. Indeed, *i*) MCT-1 half-life is increased upon glutamine starvation, *ii*) 3-BrPA internalization and cytotoxicity is stimulated by MCT-1 overexpression and *iii*) MCT-1 silencing prevents 3-BrPA-induced metabolic-oxidative stress and cell death. The tight liaison between glutamine availability, MCT-1 expression and 3-BrPA cytotoxicity is further highlighted by the observation that cells able to maintain a high intracellular glutamine content, in absence of its exogenous supplementation, do not up-regulate MCT-1 levels, thereby circumventing 3-BrPA chemo-potentiation. We substantiate that the independence on exogenous glutamine of these cell types is due to a high GS activity which, ensuring a high intracellular glutamine content, restrains them from up-regulating MCT-1 and chemo-potentiating 3-BrPA. Besides providing a mechanistically explanation of resistance to 3-BrPA chemo-potentiation, our findings elucidate also the metabolic rationale for the assessment of the GS levels in tumors as a valuable tool in predicting response to the combined therapy described in this study. Indeed, MSO the only specific GS inhibitor is a toxic compound. Therefore, 3-bromopyruvate chemo-potentiation may not be

applicable to tumors which are capable of synthesizing glutamine, for as long as a safe procedure is designed to inhibit GS activity.

The capability of glutamine to regulate MCT-1 stability finds support in studies documenting the capability of this amino acid to modulate protein turnover by stimulating protein ubiquitination (42). Intracellular glutamine availability, indeed, is known to negatively affect the stability of *i*) p65 subunit of NF-kB transcription factor, thereby protecting against inflammatory conditions (43) and *ii*) GS, thereby maintaining in check the intracellular concentration of glutamine upon resting conditions and allowing its biosynthesis upon nitrogen starvation (44). Generally, membrane proteins stability depends on their trafficking from plasma membrane to endosomal/lysosomal compartments. As ubiquitination facilitates endocytosis of such proteins, thereby reducing their cell-surface localization and intracellular abundance (45, 46), it is reasonable to hypothesize that glutamine might regulate MCT-1 turnover by inducing its ubiquitination and delivery to the endosomal/lysosomal-degradation pathway. Work is presently underway in our laboratory to deeply address this issue.

MCT-1 overexpression is part of an adaptive reprogramming aimed to extrude lactate from highly glycolytic cancer cells in the hypoxic regions of solid tumors (47). This transporter, indeed, has been identified as a therapeutic target in cancer, because its inhibition decreases intracellular pH, causing cell death (26, 41). However, emerging findings describe MCT-1 as an inward transporter. According to the "stromal-epithelial" lactate shuttle in tumors, MCT-1 allows the uptake of lactate, ketones and other metabolites released by stromal fibroblasts in the extracellular milieu to refuel epithelial cancer cells within the same tumors (46). Interestingly, although MCT-1 expression is affected by hypoxia (48), no data documenting its modulation in response to nutrient availability had been provided so far. Although we did not investigate the role of MCT-1 up-regulation in glutamine-deprived cells, at least one hypothesis can be formulated. Interruption of glutamine availability in glutamine-addicted cells induces compensatory mechanisms catalyzed by the pyruvate carboxylase, allowing cells to use glucose-derived pyruvate, rather than glutamine, for

Krebs cycle anaplerosis (49). Therefore, we can hypothesize that MCT-1 up-regulation could be part of a biochemical reprogramming enabling glutamine-deprived cancer cells to take up extracellular lactate or pyruvate to fulfill anaplerotic requirements by sustaining pyruvate carboxylase activity. Hence, on the basis of its elevated expression in tumors and modulation in response to environmental conditions, MCT-1 could be considered as a "Trojan horse", that allows chemotherapeutics structurally related to monocarboxylates to enter into cancer cells and selectively kill them. Although our study identify 3-BrPA as a feasible candidate in exploiting metabolic adaptations of glutamine-deprived tumors, it can be suggested that other monocarboxylic anticancer drugs, such as dichloroacetate could be chemo-potentiated *in vivo* by capitalizing on MCT-1 stabilization. Therefore, this study represents a proof of concept that anticancer effects can be enhanced by combining monocarboxylic-based chemotherapeutics with strategies depriving glutamine pool of tumor cells. Although this strategy could be potentially adopted for treating every glutamine addicted tumors, glutamine deprivation may be of limited value for treatment of brain malignancies. Indeed, since glutamine is a molecule critical for neurotransmission and neuron-astrocyte metabolic cycles, its deprivation could severely impair function of brain region involved. Moreover, it is known that glioma cells react to glutamine deprivation with modification of amino acid transport involving the different ASC system variants (50), the consequences of which for 3-BrPA therapy could be difficult to predict.

In conclusion, our results identify the metabolic conditions to increase the selectivity and sensitivity of 3-BrPA targets in neoplastic tissues and the mechanisms of tumor resistance to its chemo-potentiation, thereby driving the process of clinical translation of this drug for glutamine-addicted malignancies.

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FIGURE LEGENDS

Fig. 1. Glutamine withdrawal induces chemo-potentiation of 3-BrPA antitumor effects.

3-BrPA cytotoxicity in glutamine-deprived HeLa (**A**) PCEC (**B**) and GPNA-treated HeLa cells (**C**). Data represent the means±S.D. of five independent experiments. For (A) and (C), *P<0.05; ***P <0.001 compared with glutamine-fed control cells; For (A), † P<0.05; $^{\dagger\dagger\dagger}P$<0.001 compared

with 3-BrPA-untrated cells**;** $^{###}P<0.001$. For (C), $^{\dagger\dagger\dagger}P<0.001$. In (B), the % of dead cells induced by 150 μM 3-BrPA was significant (***$P<0.001$) compared to 3-BrPA-untreated cells, irrespective of glutamine supplementation. (**D**) Analysis of antitumor effect of 3-BrPA/GPNA combined treatment on HeLa xenografts. T*op*, growth of tumors in the four experimental groups: *points*, mean tumor growth (% of tumor volume increase with respect to the beginning of treatment); *bars*, SD. *Center*, tumor weights in the four experimental groups: *points*, mean tumor weight (g); *bars*, SD. *$P<0.05$ with respect to control and both monotherapy groups. *Bottom*, photograph representative of five excised tumors in each experimental group.

Fig. 2. Autophagy contributes to the execution of 3-BrPA-induced cell death upon glutamine withdrawal.

(**A**) Analysis of AVOs levels. Data are expressed as % of cells with AVOs and represent the mean±SD of five independent experiments. *$P<0.05$; ***$P<0.001$ compared with glutamine-fed control cells; $^{\dagger\dagger}P<0.01$. Western blot of LC3 and p62 in HeLa (**B**) and excised xenografts (**D**). Actin was used as a loading control. The immunoblots shown are representative of three that gave similar results. Molecular masses are given in kDa to the left. (**C**) Fluorescence analysis of autophagic flux. *Top*, images from one representative experiment of five with similar results; *bottom*, % of dotted (autophagic) LC3-EGFP positive cells. Data are means±S.D. of five independent experiments. *$P<0.05$; ***$P<0.001$ versus glutamine-fed control cells. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\#}P<0.05$; $^{\#\#}P<0.01$. Assessment of 3-BrPA chemo-potentiation upon pharmacological inhibition of autophagy induction (**E**) or knock-down of ATG5 (**F**). Data are means±S.D. of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with glutamine-deprived control (E) or siScr cells (F); $^{\dagger\dagger}P<0.01$

Fig. 3. Complex II inhibition is responsible for the generation of 3-BrPA-induced metabolic-oxidative stress upon glutamine deprivation.

(**A**) ATP measurement. Data represent the mean±SD of eight independent experiments. **P*<0.05; ***P*<0.01, ****P*<0.001 compared with glutamine-fed control cells; $^{\dagger\dagger}P$<0.01. (**B**) Analysis of ROS production. Data are expressed as % of DCF^{+} cells and represent the mean±SD of four independent experiments. **P*<0.05; ****P*<0.001 compared with glutamine-fed control cells; $^{\dagger\dagger}P$<0.01. (**C**) Protein carbonyls determination. Immunoblot from one representative experiment of three with similar results. Actin was used as a loading control. Molecular masses are given in kDa to the left. Effect of antioxidants on 3-BrPA chemo-potentiation (**D**) and induction of autophagy (**E**) upon glutamine withdrawal. Data in (D) represent the means±S.D. of five independent experiments. **P*<0.05; ****P*<0.001 compared with control cells; $^{\dagger\dagger\dagger}P$<0.001. Immunoblots in (E) from one representative experiment of three with similar results are shown. Actin was used as a loading control. Molecular masses are given in kDa to the left. (**F**) Measurement of Complex II activity. Data represent the means ± S.D. of four independent experiments. **P*<0.05; ****P*<0.001 compared with glutamine-fed control cells; $^{\dagger\dagger}P$<0.01. (**G**) Effect of SDHA knockdown on modulation of protein carbonylation. Actin was used as a loading control. Immunoblot from one representative experiment of three with similar results. Molecular masses are given in kDa to the left. (**H**) 3-BrPA chemo-potentiation upon knock-down of SDHA. Data are expressed as percentage of glutamine-deprived control cells and represent the mean±SD of five independent experiments. **P*<0.05; ****P*<0.001 compared with shScr control cells; $^{\dagger\dagger}P$<0.01.

Fig. 4. Gluatamine-deprivation induced MCT-1 stabilization dictates 3-BrPA chemo-potentiation.

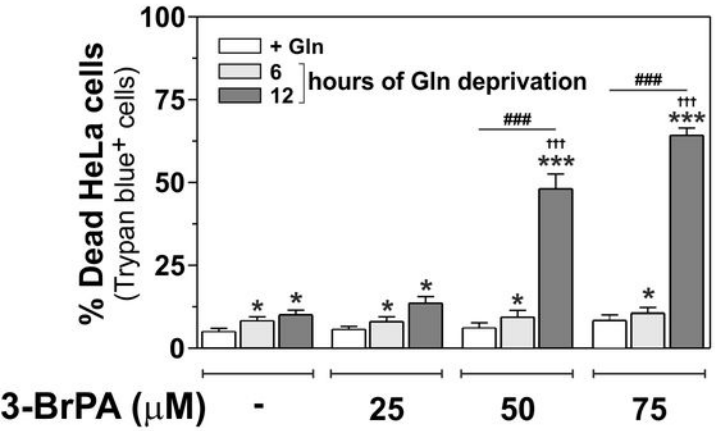
(**A**) NMR spectra of extracellular 3-BrPA abundance. *Top*, 3-BrPA in cell-unconditioned glutamine-supplemented (a) or deprived (b) medium. *Bottom*, 3-BrPA in cell-conditioned glutamine-supplemented (c) or deprived (d) medium. Spectra from one experiment of three with similar results are shown. Intracellular content (**B**), plasma membrane localization (**C**) and turnover of MCT-1 (**D**). Immunoblots from one representative experiment of three with similar results and

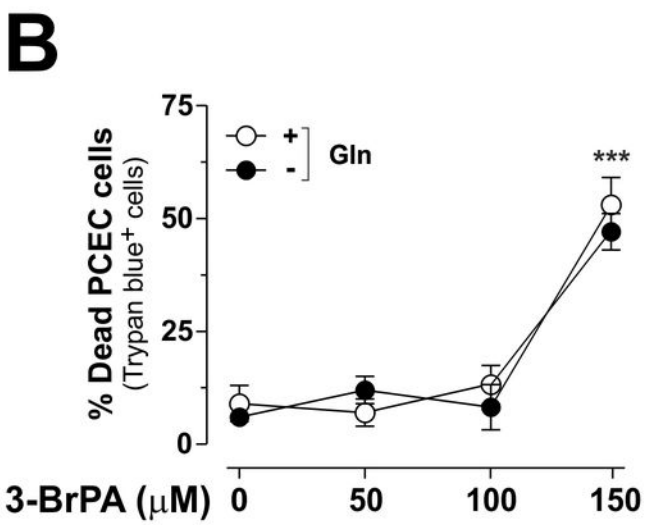
their densitometric analyses are shown. Actin was used as a loading control. Molecular masses are given in kDa to the left. For (B), $^{**}P<0.01$. For (D), $^{*}P<0.05$, $^{**}P<0.01$, compared to glutamine-deprived control cells; $^{\dagger\dagger}P<0.01$. (**E**) NMR spectroscopy determination of extracellular 3-BrPA in MCT-1 overexpressing cells (pCI-MCT-1) with respect to mock counterparts. Data represent the mean±SD of three independent experiments. $^{**}P<0.01$. 3-BrPA cytotoxicity in MCT-1-overexpressing (**F**) or MCT-1 silenced (siMCT-1) cells (**G**). Data represent the mean±SD of three independent experiments. For (F), $^{***}P<0.001$. For (G), $^{*}P<0.05$, $^{***}P<0.001$ compared to siScr control cells; $^{\dagger\dagger}P<0.01$.

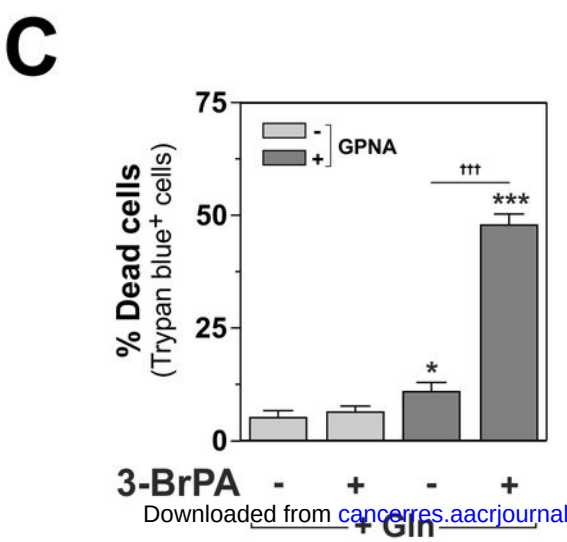
Fig. 5. Glutamine synthetase is exploited by cancer cells to circumvent 3-BrPA chemo-potentiation.

(**A**) GS activity. Absorbance measured at λ=540 nm is proportional to the enzyme activity and represents the mean±SD of four independent experiments. $^{*}P<0.05$. (**B**) 3-BrPA chemo-potentiation upon GS inhibition. Data represent the mean±SD of five independent experiments; $^{*}P<0.05$; $^{***}P<0.001$ compared with glutamine-fed control cells; $^{\dagger\dagger}P<0.01$. (**C**) Effect of GS inhibition on the intracellular levels of MCT-1 in MCF-7 (*top*) and T-47D (*bottom*) cells. The immunoblots from one representative experiment of three with similar results and their densitometric analyses are shown. Actin was used as a loading control. Molecular masses are given in kDa to the left. $^{*}P<0.05$.

Fig. 1







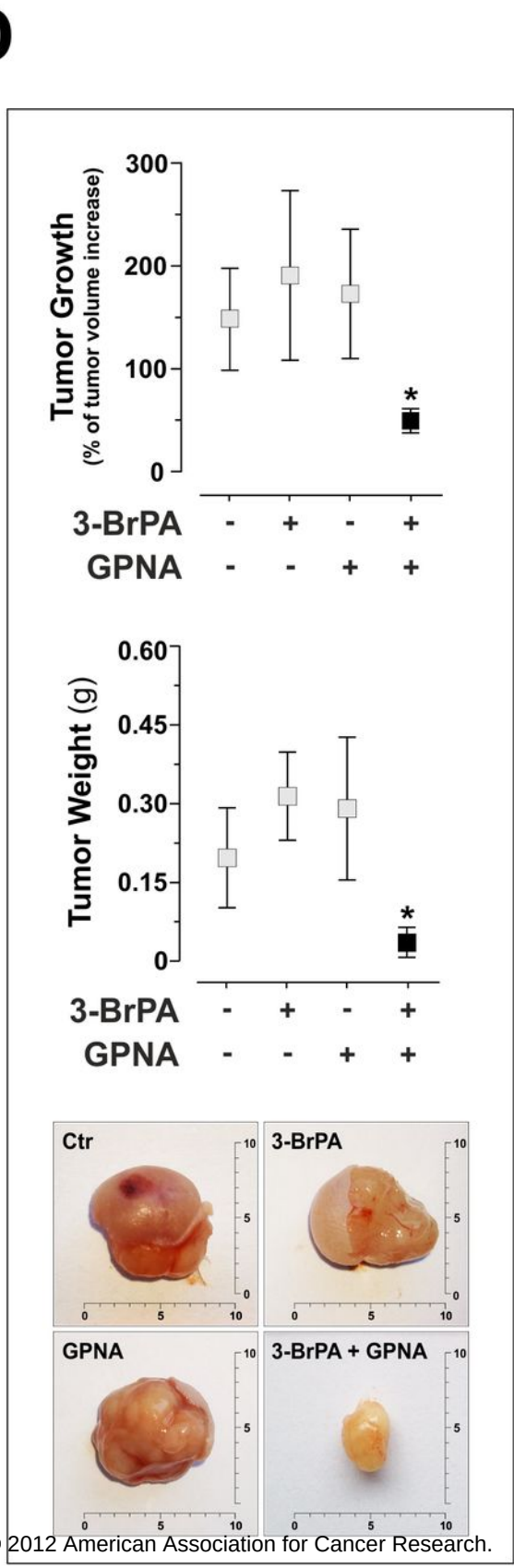


Fig. 2

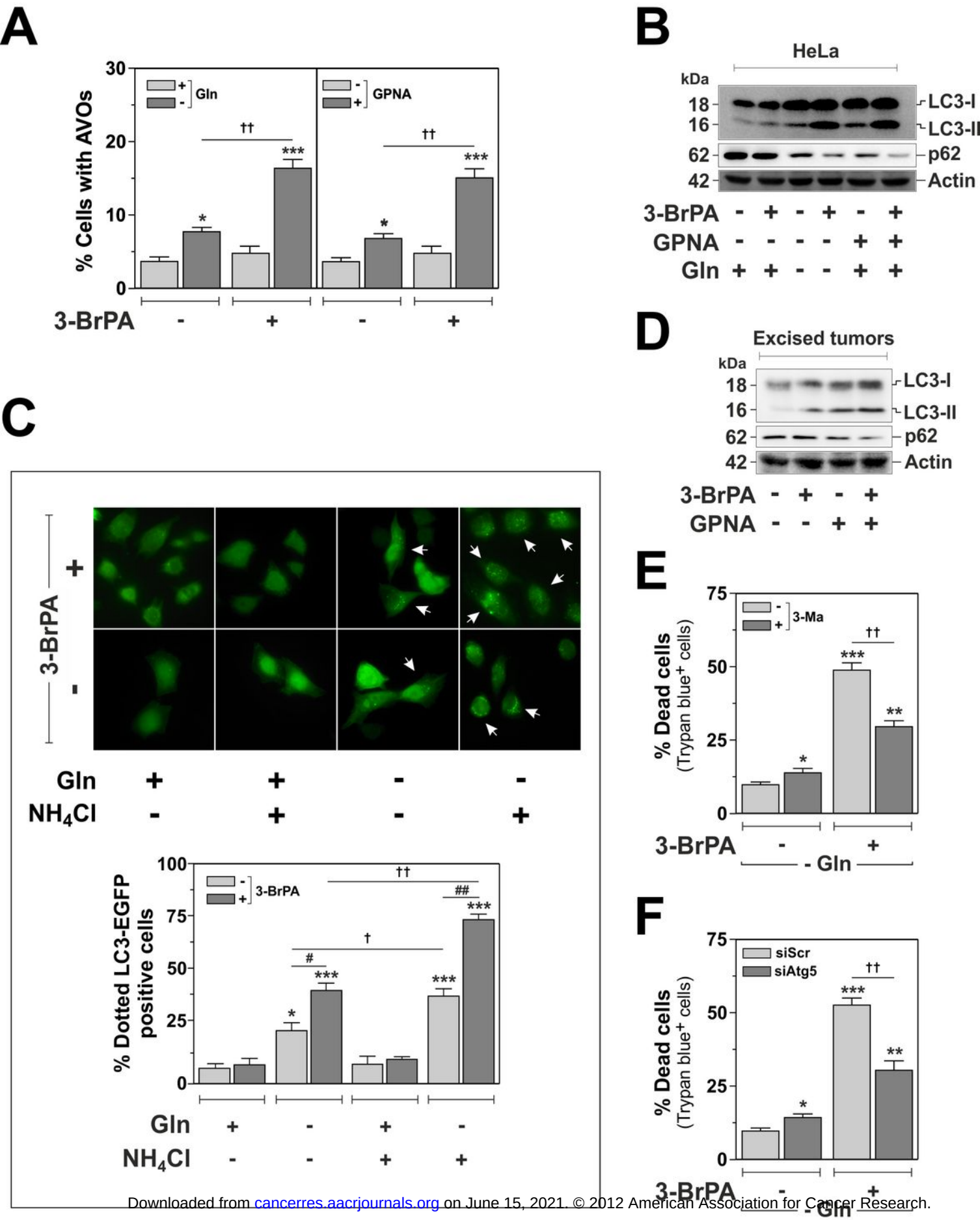


Fig.3

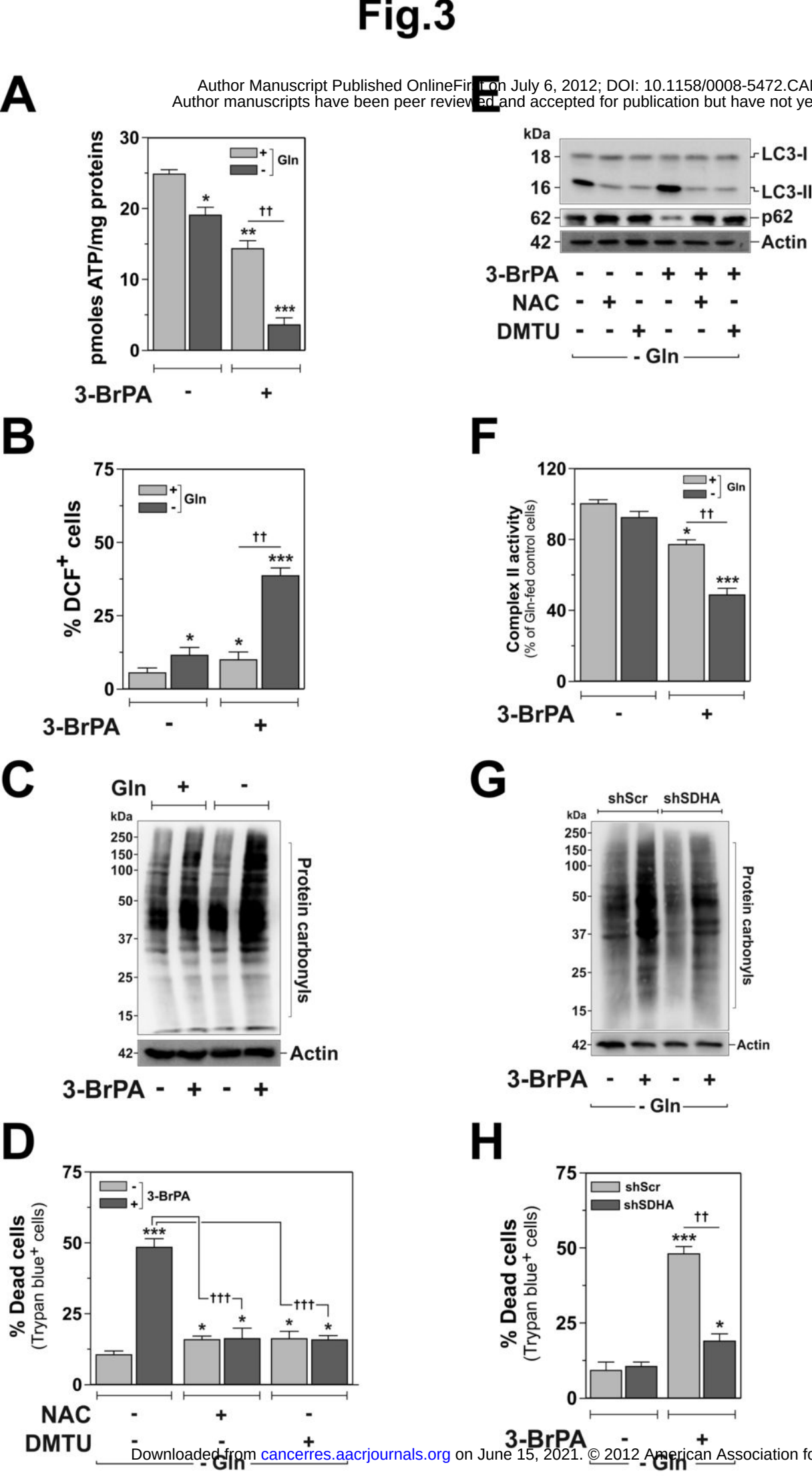


Fig. 4

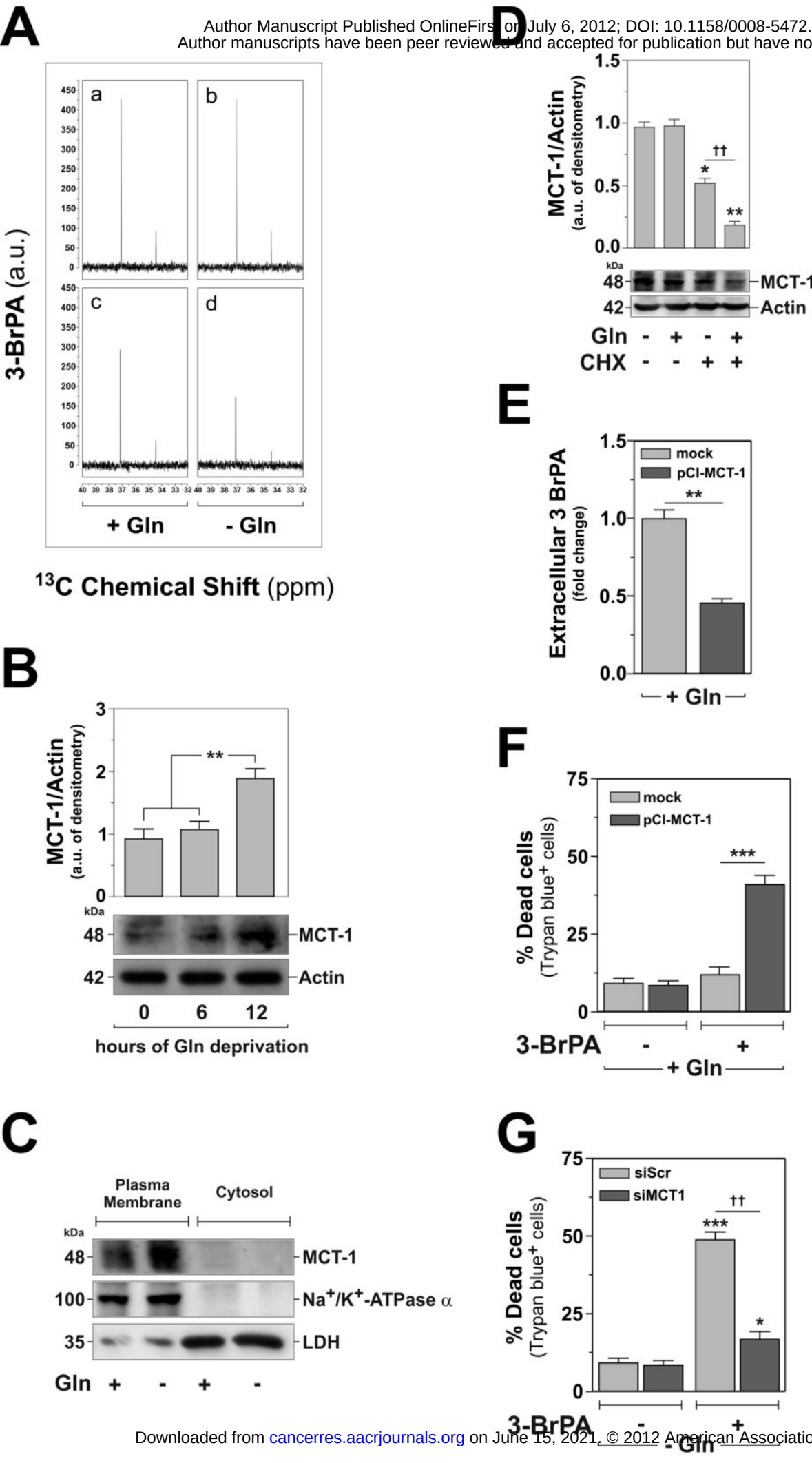
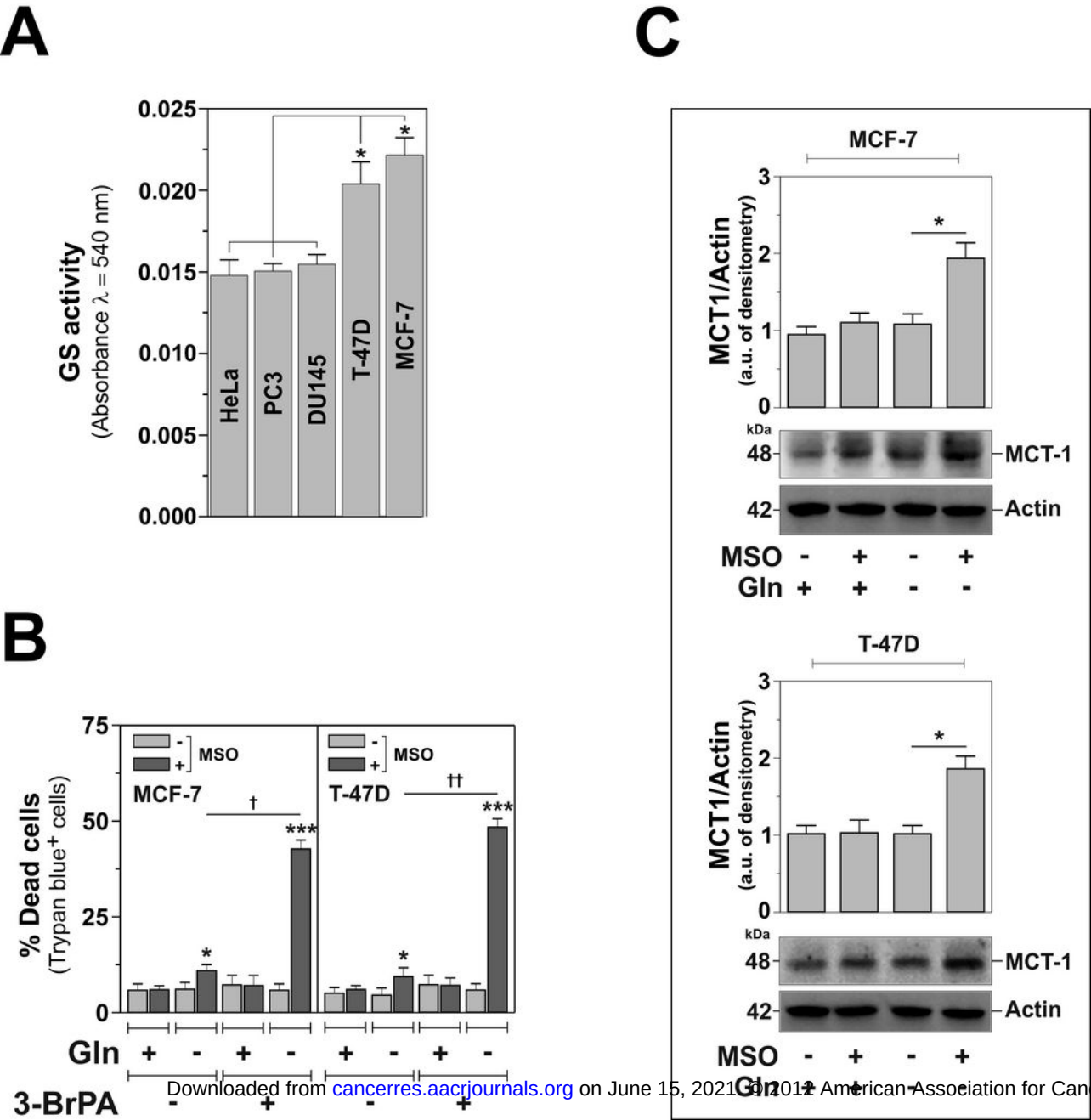


Fig. 5



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GLUTAMINE DEPRIVATION ENHANCES ANTITUMOR ACTIVITY OF 3-BROMOPYRUVATE THROUGH THE STABILIZATION OF MONOCARBOXYLATE TRANSPORTER-1

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