Glutamine Deprivation Enhances Antitumor Activity of 3-Bromopyruvate through the Stabilization of Monocarboxylate Transporter-1

Simone Cardaci¹, Salvatore Rizza¹, Giuseppe Filomeni¹,², Roberta Bernardini¹, Fabio Bertocchi², Maurizio Mattei¹, Maurizio Paci², Giuseppe Rotilio¹,³, and Maria Rosa Ciriolo¹,³

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 deprivation potentiated 3-bromopyruvate chemotherapy by increasing the stability of the monocarboxylate transporter-1, an effect that sensitized cells to metabolic oxidative stress and autophagic cell death. We further elucidated mechanisms through which resistance to chemopotentiation by glutamine deprivation 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.

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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 toward 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 modulate 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 a 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 chemopotentiating 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 show that glutamine deprivation stimulates the intracellular uptake of 3-BrPA that 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 chemopotentiation. Overall, our study represents proof-of-principle that anticancer effects can be potentiated by combining monocarboxylic-based drugs with strategies to deprive tumors of glutamine.

Materials and Methods

3-BrPA, N-acetylcysteine (NAC), dimethylthiourea (DMTU), 3-Methyladenine (3-Ma), methionine sulfoximine (MSO), L-glutamyl-p-nitroanilide (GPNA), 6-diazo-5-oxo-l-norleucine, epigallocatechin gallate, amino-oxyacetic acid, Ibuprofen, α-cyano-4-hydroxycinnamate (α-CCH), NH₄Cl, Staurosporine, 2-Deoxy-D-glucose (2-DG), cycloheximide (CHX), dichloroacetate (DCA), acidine orange, EDTA, EGTA, paraformaldehyde,
propidium iodide, and Triton X-100 were from Sigma; benzylxoycarbonyl-Val-Ala-Asp (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 human prostate carcinoma), 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 Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% 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% CO2 atmosphere in air and routinely trypsinized and plated at 4 × 10^4/cm^2 onto collagen-coated cell culture plates and incubated at 37°C in a 5% CO2 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 mmol/L solution of 3-BrPA (Sigma) was prepared just before the experiments by dissolving the powder in PBS, adjusted to pH 7.0 with NaOH. The antioxidants, the autophagy inhibitor, the complex II inhibitor, and the transaminase inhibitor were added upon glutamine deprivation and maintained throughout the experiment. The activity of complex II (succinate:coenzyme Q oxidoreductase activity), glutamine synthetase, and extracellular lactate dehydrogenase were measured and tumor volumes were calculated by the formula: Tumor volume = 0.5 × L × W^2. Tumor growth has been reported as % of tumor volume increase with respect to the beginning of treatment.

Intracellular reactive oxygen species (ROS) and acidic vesicular organelles (AVO) levels were assessed by confocal microscopy. In particular, HeLa cells were treated with subtoxic doses of 3-BrPA and measured cell viability 24 hours after drug addition. Figure 1A shows that 12-hour treatment with 3-BrPA caused a significant reduction in cell viability. A total of 4 × 10^6 HeLa cells were injected subcutaneously into adult female (6 weeks old) Hsd: Athymic Nude-Foxn1nu mice (Harlan Laboratories). After 30 days, animals were randomized into 4 different groups, containing 5 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 3 times per week by manual caliper. After 14 days, mice were sacrificed. Tumor weights were measured and tumor volumes were calculated by the formula: Tumor volume = 0.5 × L × W^2. Tumor growth has been reported as % of tumor volume increase with respect to the beginning of treatment.

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 subtoxic doses of 3-BrPA and measured cell viability 24 hours after drug addition. Figure 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 PCECs, the untransformed counterpart of HeLa cells,
to 3-BrPA challenge (Fig. 1B), suggesting that 3-BrPA chemopotentiation is a cancer-specific phenomenon. To validate the ability of glutamine to increase 3-BrPA chemoresistance 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 (Supplementary Fig. S1A). Next, we investigated whether the enhancement of 3-BrPA effects could be phenocopied by the pharmacologic inhibition of glutamine uptake. Therefore, HeLa cells were incubated for 12 hours with GPNA, the specific inhibitor of SLC1A5, the main transporter mediating glutamine uptake in carcinoma cells (17) and, then, exposed to 3-BrPA. Results obtained showed that GPNA pretreatment was effective in enhancing HeLa cells sensitivity to 3-BrPA (Fig. 1C) and in lowering intracellular glutamine content (Supplementary Fig. S1B).

To assess the long-term effects of 3-BrPA chemopotentiation on cell growth and survival, clonogenic assay was carried out 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 (Supplementary Fig. S1C). Next we evaluated the capability of GPNA to potentiate antitumor activity of 3-BrPA in vivo. To this aim, nude mice harboring HeLa xenografts were treated with 3-BrPA (1.25 mg/kg), GPNA (20 mg/kg), or the combination of both compounds for 14 days. Figure 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 Supplementary Fig. S1D, no significant changes in the apoptotic extent were 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 (Supplementary Fig. S1E), thereby allowing us to exclude apoptosis in cell death execution.

**Figure 1.** Glutamine withdrawal induces chemopotentiation 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 ± SD of 5 independent experiments. For A and C, *, P < 0.05; ***, P < 0.001 compared with glutamine-fed control cells. For A, †††, P < 0.001 compared with 3-BrPA-untreated cells; ###, P < 0.001. For C, †††, P < 0.001. In B, the % of dead cells induced by 150 μmol/L 3-BrPA was significant (***, P < 0.001) compared with 3-BrPA–untreated cells, irrespective of glutamine supplementation. D, analysis of antitumor effect of 3-BrPA/GPNA combined treatment on HeLa xenografts. Top, growth of tumors in the 4 experimental groups: points, mean tumor growth (% of tumor volume increase with respect to the beginning of treatment); bars, SD. Middle, tumor weights in the 4 experimental groups: points, mean tumor weight (g); bars, SD. †, P < 0.05 with respect to control and both monotherapy groups. Bottom, photograph representative of 5 excised tumors in each experimental group.
Autophagy is involved in 3-BrPA–induced cell death upon glutamine withdrawal

Because 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 AVOs, produced upon 3-BrPA challenge. Results obtained in HeLa cells (Fig. 2A) as well as PC3 and DU145 cells (Supplementary Fig. S2A) indicated that autophagosome degradation 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 pretreatment 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). Coincubation with NH4Cl, which blocks autophagic degradation, enhanced 3-BrPA–induced accumulation of LC3-GFP–punctate cells, confirming that 3-BrPA cytotoxicity is associated with a sustained autophagic flux in absence of glutamine. Enhanced autophagy induction was observed in PC3 and DU145 cells, as well (Supplementary Fig. S2B). 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 drug-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-Ma, an inhibitor of initiation steps of autophagy, and evaluated cell viability upon 3-BrPA treatment. In line with the prosurvival 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 chemopotentiation, as the percentage of dead cells was almost halved (Fig. 2E) and inhibited autophagosomes formation, as shown by the significant decrease of LC3-II levels (Supplementary Fig. S2C). This finding was substantiated by experiments carried out in PC3 and DU145 cells, as well (Supplementary Fig. S2D). To confirm the role of autophagy in cell demise, we silenced the expression of Atg5, an essential protein for autophagosomes formation. siRNA-mediated knockdown of Atg5 decreased cell death extent upon 3-BrPA addition (Fig. 2F and Supplementary Fig. S2E) and reduced autophagy (Supplementary Fig. S2F), 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 chemopotentiation 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 Supplementary Fig. S3A). To address this issue, we measured intracellular ATP content (Fig. 3A) and extracellular lactate levels (Supplementary Fig. S3B) 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, the cell-permeable form of the glycolytic intermediate D-glucose (2-DG; Supplementary Fig. S3E), a glycolytic inhibitor more efficient in reducing lactate levels than 3-BrPA (Supplementary Fig. S3D), suggests that 3-BrPA chemopotentiation was not dependent on glycolysis block in our experimental conditions. Next, we challenged HeLa cells with 3-BrPA upon pharmacologic inhibition of specific glutaminolytic enzymes (scheme of Supplementary Fig. S3A). Inhibition of glutaminolysis did not chemopotentiate 3-BrPA (Supplementary Fig. S3F). Furthermore, supplementation of glutamine-free medium with glutamate, the first metabolite generated from oxidative deamination of glutamine, did not prevent 3-BrPA cytotoxicity (Supplementary Fig. S3A). Inhibition of glutaminolysis did not chemopotentiate 3-BrPA treatment. 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Next, we challenged HeLa cells with 3-BrPA upon pharmacologic inhibition of specific glutaminolytic enzymes (scheme of Supplementary Fig. S3A). Inhibition of glutaminolysis did not chemopotentiate 3-BrPA (Supplementary Fig. S3F). Furthermore, supplementation of glutamine-free medium with glutamate, the first metabolite generated from oxidative deamination of glutamine, did not prevent 3-BrPA cytotoxicity (Supplementary Fig. S3A). Inhibition of glutaminolysis did not chemopotentiate 3-BrPA treatment. In line with the ability of glutamine to sustain ATP synthesis and lactate generation, glutamine-deprived 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. 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Fig. S3F), suggesting that reduction of glutaminolytic flux was not responsible for 3-BrPA chemopotentiation. It has been previously reported that 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 scavengers NAC or DMTU prevented 3-BrPA–mediated cell death (Fig. 3D), induction of autophagy (Fig. 3E), protein carbonylation (Supplementary Fig. S4A), and ATP loss (Supplementary Fig. S4B), 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 O₂ 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 (Supplementary Fig. S5A and B), the subunit responsible for succinate oxidation and electron 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 (Supplementary Fig. S5E) upon 3-BrPA treatment. Moreover, they were almost insensitive to 3-BrPA–induced cytotoxicity as shown by the lower cell death extent (Fig. 3H) and autophagic activity (Supplementary Fig. S5E) with respect to their controls. Moreover, in line with their lower sensitivity to the drug (Supplementary Fig. S5E), we found that PCEC were characterized by a lower complex II activity with respect to tumor counterparts (Supplementary Fig. S5E), thereby substantiating the role of complex II as pivotal target underlying 3-BrPA cytotoxicity.

Chemopotentiation of 3-BrPA depends 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 (MCT) and Na⁺-coupled (SMCT) MCTs. To discriminate which subset could be involved in 3-BrPA internalization and cytotoxicity, we measured the viability of 3-BrPA-treated HeLa cells coinubated...
alternatively with α-C HC, a specific pharmacologic inhibitor of MCTs, and ibuprofen, a blocker of SMCTs. α-C HC, but not ibuprofen, rescued HeLa cells from 3-BrPA–induced cell death upon glutamine starvation (Supplementary Fig. S6A), suggesting that MCT-dependent 3-BrPA uptake could be operative in our experimental conditions. α-C HC 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 (Supplementary Fig. S6B) displayed higher levels of MCT-1, with respect to their glutamine-fed and GPNA-untreated counterparts. Glutamine deprivation–induced MCT-1 upregulation was also observed in PC3 and DU145 cells (Supplementary Fig. S6C) and was coupled to the enhanced localization of the transporter in the plasma membrane (Fig. 4C). As documented in literature (27), MCT-1 upregulation was not due to a stimulation of MCT-1 transcription. Indeed, no significant differences in MCT-1 mRNA levels were measured in HeLa cells, regardless of glutamine supplementation (Supplementary Fig. S6E). 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 CHX 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 upregulated levels of MCT-1 could facilitate the drug uptake, we stably overexpressed MCT-1 (pCI-MCT-1; Supplementary Fig. S7A). pCI-MCT-1 displayed a significant decrease of extracellular 3-BrPA levels with respect to their mock counterparts, indicating that MCT-1 upregulation strongly stimulated drug internalization (Fig. 4E). Interestingly, MCT-1 overexpression was sufficient to increase 3-BrPA toxicity, as shown by cell death (Fig. 4F), ATP drop (Supplementary Fig. S7B), and protein carboxylation (Supplementary Fig. S7C) with respect to mock counterparts. In agreement with the enhanced sensitivity of pCI-MCT-1 cells to the drug, siRNA-mediated MCT-1 silencing (Supplementary Fig. S7D) almost completely abolished 3-BrPA chemopotentiation (Fig. 4G). These results were supported by the reduction of protein carbonyls content (Supplementary Fig. S7E), the maintenance of ATP levels (Supplementary Fig. S7F), and inhibition of autophagic flux (Supplementary Fig. S7G). The link between MCT-1 upregulation 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 upregulation. To this aim, we evaluated the cytotoxicity of 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 chemopotentiated upon glutamine deprivation (Supplementary Fig. S7H) or inhibition of its uptake (Supplementary Fig. S7I) as well as by MCT-1 overexpression (Supplementary Fig. S7J), 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 chemopotentiation

Glutamine synthetase, 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 predict 3-BrPA chemopotentiation only in glutamine-addicted tumors, expressing low levels of glutamine synthetase. To validate this assumption, we compared glutamine synthetase activity among different tumor cells. MCF-7 and T-47D, 2 breast cancer cell lines, are characterized by a higher glutamine synthetase 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 3 carcinoma cells (Supplementary Fig. S8A). Moreover, MCF-7 and T-47D become sensitive to 3-BrPA only upon glutamine synthetase inhibition, achieved either by incubating cells with MSO, the specific pharmacologic inhibitor of glutamine synthetase (Fig. 5B) or overexpressing a dominant-negative form of this enzyme (DN-GS; Supplementary Fig. S8B and C). In accordance with these results, MCT-1 upregulation was observed only in glutamine-free medium upon inhibition of glutamine synthetase activity (Fig. 5C and Supplementary Fig. S8D). Overall, these results identified the molecular strategy exploited by cancer cells to circumvent 3-BrPA chemopotentiation.

Discussion

Targeting metabolic adaptations of cancer cells has the potential to chemopotentiate antitumor drugs (2, 9, 31). In line with this concept, we show 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 (Supplementary Fig. S9). Although 3-BrPA has antitumor effect on multiple cancer cell types, no trials attesting its clinical use are so far documentable, probably because of the putative side effects resulting from its alkylation properties. Therefore, in line with recent investigations focused on identifying strategies to chemopotentiate 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 show 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 in 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 show 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 chemopotentiation 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 indicates 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 upregulate MCT-1 levels, thereby circumventing 3-BrPA chemopotentiation. We substantiate that the independence on exogenous glutamine of these cell types is due to a high glutamine synthetase activity, which, ensuring a high intracellular glutamine content, restrains them from upregulating MCT-1 and chemopotentiating 3-BrPA. Besides providing a mechanistic explanation of resistance to 3-BrPA chemopotentiation, our findings elucidate also the metabolic rationale for the assessment of the glutamine synthetase levels in tumors as
a valuable tool in predicting response to the combined therapy described in this study. Indeed, MSO, the only specific glutamine synthetase inhibitor, is a toxic compound. Therefore, 3-bromopyruvate chemopotentiation may not be applicable to tumors that are capable of synthesizing glutamine for as long as a safe procedure is designed to inhibit glutamine synthetase 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) glutamine synthetase, 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 upregulation 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 upregulation 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 DCA could be chemopotentiated in vivo by capitalizing on MCT-1 stabilization. Therefore, this study represents a proof-of-concept that anticancer effects can be enhanced by combining monocarboxylate-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, because 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 chemopotentiation, thereby driving the process of clinical translation of this drug for glutamine-addicted malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Cardaci, M.R. Cirio lo Development of methodology: S. Cardaci, S. Rizza Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rizza, G. Filomeni, M. Paci Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Cardaci, G. Filomeni, G. Rotilio Writing, review, and/or revision of the manuscript: S. Cardaci, M.R. Cirio lo Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Bortocchi Study supervision: G. Rotilio, M.R. Cirio lo

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Glutamine Deprivation Induces 3-BrPA Chemopotentiuation


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Simone Cardaci, Salvatore Rizza, Giuseppe Filomeni, et al.


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