Kidney-Type Glutaminase Inhibitor Hexylselen Selectively Kills Cancer Cells via a Three-Pronged Mechanism

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ABSTRACT: Tumor metabolism has been deeply investigated for cancer therapeutics. Here, we demonstrate that glutamine deficiency alone could not completely inhibit cancer cell growth and that many potent kidney-type glutaminase (KGA) inhibitors did not show satisfying *in vivo* efficacy. The potent KGA allosteric inhibitor, CB-839, resulted in up to 80% growth inhibition of all tested cell lines, whereas Hexylselen (CPD-3B), a KGA/glutamate dehydrogenase (GDH) inhibitor, showed essentially no toxicity to normal cells up to a 10 μ M concentration and could completely inhibit the growth of many aggressive cell lines. Further analyses showed that CPD-3B targets not only KGA and GDH but also thioredoxin reductase (TrxR) and amidotransferase (GatCAB), which results in corresponding regulation of Akt/Erk/caspase-9 signaling pathways. In an aggressive liver cancer xenograft model, CPD-3B significantly reduced tumor size, caused massive tumor tissue damage, and prolonged survival rate. These provide important information for furthering the drug design of an effective anticancer KGA allosteric inhibitor.

KEYWORDS: glutaminase allosteric inhibitor, GatCAB, EZMTT assay, biomolecular interaction assay, proteomic analysis, hexylselen

INTRODUCTION

Metabolic change in response to oncogenic growth-factor signaling is a core hallmark of cancer.¹ In normal cells, glucose feeds the TCA cycle to produce ATP in mitochondria. In comparison, proliferating cancer cells have an ADP:ATP ratio that is high enough to sustain necessary glycolytic flux; the Warburg effect enhances glycolysis 200 times over normal cells. However, the majority of the glucose carbon is converted to lactate for excretion, even under oxygen rich conditions. Consequently, cancer cells have developed glutamine-dependence; glutamine is converted via glutamate to α -ketoglutarate to feed into the TCA cycle. Dramatically increased glycolysis and glutaminolysis are essential for cancer cell growth, because cancer cells have high demand for biosynthesis of ATP, NAD(P)H, glutathione, lipid, protein, nucleotide and other biomaterials.^{2,3} Therefore, scientists have hypothesized that blocking glutaminolysis will starve cancerous cells to death.⁴

The predominant glutaminolysis pathway in most cancer cells is through formation of glutamate (Glu) by the rate-limiting glutaminase (isomers: KGA/GAC) and then, to α -ketoglutarate (α -KG) by GDH or by aminotransferase in the presence of other amino acids.² Another alternative pathway for Gln metabolism is first transamination to α -ketoglutar-

amate (KGM) followed by hydrolysis of KGM to α -ketoglutarate by an amidase,⁵ and finally into the TCA cycle. The relative contribution of each pathway varies among different cell types and tissues.

However, clinical applications of glutaminase inhibitors listed in Figure 1 have been met with major obstacles and generally poor outcomes. The active site KGA inhibitors, Acivicin and DON,⁶ showed high drug toxicity, perhaps, due to the lack of selectivity against liver type glutaminase (LGA) that is important for liver function. Compound 968 was reported as an allosteric inhibitor of GAC (a KGA isoform), but perhaps due to its poor solubility, its *in vivo* efficacy is poor.⁷

BPTES is a known allosteric glutaminase inhibitor with an IC_{50} of 0.1–3 μ M in the KGA assays, and its binding site has been defined by an X-ray cocrystal structure with GAC, but has poor solubility (0.01 μ M).⁸ BPTES derivatives such as COMPOUND 6,⁹ Thiazolidine-2,4-dione,¹⁰ and UPGL-00004¹¹ showed potent inhibition of KGA, but relatively poor efficacy in cell-based assays (incomplete inhibition).

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Figure 1. Various KGA inhibitors and chemical synthesis of a biotinylated CPD-3B derivative.

Table 1. Glutamine Metabolism Inhibitors Showed Difference in Growth Inhibition of Cancer Cell Lines

	CPD-3B		Ebselen		CB-839	
	IC ₅₀ (μM)	maximal inhibition (%)	IC ₅₀ (μM)	maximal inhibition (%)	IC ₅₀ (μM)	maximal inhibition (%)
human KGA	0.1 ± 0.03	100 ± 2	>10	no inhibition	0.026 ± 0.01	100 ± 2
human GDH	0.8 ± 0.06	100 ± 2	2.4 ± 0.01	100 ± 2	>10	0 ± 2
human TrxR	2 ± 0.05	100 ± 3	>10	30 ± 3	>10	13 ± 2
A549	0.65 ± 0.12	100 ± 2	>10	21 ± 5	0.023 ± 0.02	74 ± 7
HCT116	1.2 ± 0.18	100 ± 2	>10	19 ± 3	0.11 ± 0.03	88 ± 3
Caki-1	1.02 ± 0.27	100 ± 2	6 ± 0.45	80 ± 4	0.075 ± 0.02	87 ± 4
U251	1.45 ± 0.11	100 ± 2	>10	8 ± 2	0.07 ± 0.01	70 ± 3
SW1990	2.22 ± 0.21	100 ± 2	>10	23 ± 2	0.232 ± 0.05	67 ± 3
PC12	1.8 ± 0.25	100 ± 2	>10	15 ± 4	>10	30 ± 2
H22	0.92 ± 0.26	100 ± 2	>10	19 ± 1	>10	36 ± 5
HL7702	>10	20 ± 2	>10	9 ± 3	>10	26 ± 4

CB-839¹² is the most potent allosteric KGA inhibitor published with an IC₅₀ value near 20–30 nM and was reported to inhibit a “triple negative” breast cancer cell line, but only *in vitro*. At high dosage (200 mg/kg oral), CB-839 achieved only 50% tumor growth suppression in an *in vivo* xenograft model, although it has shown synergy with Paclitaxel and Rapamycin¹³ in reducing tumor growth. CB-839 is a successful compound in stage II clinical investigation for triple negative breast cancer therapeutics. However, it remains to be investigated whether the limited efficacy is the result of a bypass through an alternative pathway involving aminotransferase⁵ or through improved glycolytic flux.¹³

In addition, Ebselen was initially reported as a very potent nM level allosteric KGA inhibitor,¹⁴ but lacks significant anticancer activity in cell based assay.¹⁵ However, more detailed analysis at the enzyme level showed that Ebselen is not a potent inhibitor of KGA, but a potent GDH inhibitor.^{16,17} High concentration (100 μM) is needed for Ebselen to bind to the tetramer interface and inactivate KGA,¹⁷ although at this concentration, a biotinylated Ebselen derivative was shown to bind to 461Cys containing proteins in Hela cells.¹⁹

To enhance the potency, dimeric selen derivatives were synthesized¹⁶ based on the information from KGA/BPTES crystal structure and the Ebselen chemical structure. The dimers with 5–6 atom bridges in the middle of the structure were shown to be true KGA inhibitors with IC₅₀ around 100 nM for CPD-3B, but not those with 0–4 atom bridges. In addition, CPD-3B showed dual KGA/GDH activity, complete inhibition of many cancer cells, and low toxicity to the normal cells.¹⁶

To better understand the potency and efficacy issues with the KGA allosteric inhibitors, we investigated cell growth under selective conditions: in glucose-deficient media to inhibit glycolysis, in glutamine-deficient media to inhibit glutaminolysis, and in the presence of KGA inhibitors such as CPD-3B (a dual inhibitor) or CB-839 (allosteric KGA inhibitor) to block various pathways involved in glutaminolysis. The cell growth was monitored continuously for 5 days by measuring the cellular NAD(P)H levels using the EZMTT cell viability reagent^{16,15} which is a nontoxic version of the MTT reagent. Biotinylated CPD-3B derivative (Figure 1) was synthesized to identify potential protein targets for CPD-3B by biomolecular interaction analyses and proteomic analysis. We discovered that glutamine deficiency reduced cancer cell

Figure 2. Time-dependent growth of cancer cell lines and normal cell line (HL7702) after 5 days of treatment under 3 selected conditions: glucose-deficient media, glutamine-deficient media, and presence of glutamine metabolism inhibitors. Glucose deficiency nearly completely inhibited the growth of A549, HCT-116, Caki-1, U251, H22, and SW1990 cancer cells and HL7702 normal cells. Glutamine deficiency greatly inhibited the growth of A549, U251, and Caki-1, but the cells were not dead. CB839 showed 70% growth inhibition of A549 but not H22 cancer cells, and CPD-3B showed high efficacy. Comparisons of cell growth between treated cells and no cell controls were performed by unpaired *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

growth tremendously, but not completely. CPD-3B causes cancer cell death by mainly targeting KGA, but also through inhibition of GDH, TrxR and GatCAB enzymes to some extent. Thus, it blocked glutaminolysis, inhibited Akt and Erk mediated growth factor signaling pathways, and stimulated caspase-9 initiated apoptosis and cell death. Importantly, the cell-based assay translated well into significant *in vivo* efficacy in causing tumor tissue damage and size reduction.

RESULTS AND DISCUSSION

Dual Inhibitor (CPD-3B) Showed Higher Efficacy than Its KGA Allosteric Inhibitor Counterpart (CB839). CB-839 is an allosteric inhibitor of KGA (IC_{50} 26–300 nM) and was shown to inhibit various glutamine-dependent cancer cell lines.¹² The IC_{50} values reported were measured using the end point Cell-Titer-Glo cell viability assay which lysed the cells and measured the cellular ATP level as an indication of cell viability. However, the IC_{50} only represents the potency, and the efficacy is measured by the maximal percentage of inhibition. Since different types of cells have different levels of glutamine dependence, we were curious to know how much glutamine dependence effected the efficacy of CB-839 in cell-based assays.

To investigate the efficacy, we compared the inhibition of human KGA, GDH and TrxR enzymes by CPD-3B, CB-839 and Ebselen. Complete inhibition of KGA enzyme by CB-839 and CPD-3B was observed, and in addition, CPD-3B showed complete inhibition of GDH and TrxR enzymes. However, when we monitored the growth of cancer cell lines after CB-839 treatment using a nontoxic EZMTT viability test reagent, CB-839 provided only partial inhibition of many cell lines as shown in Table 1 and Figure 2. For example, CB-839 inhibited the known glutamine-dependent A549 cancer cell line¹² with high potency (IC_{50} 20 nM) but with limited efficacy (maximal growth inhibition 79%); the discounted efficacy in the cell-

based assay might predict weak efficacy in *in vivo* tumor models.

In comparison, under the same conditions, the KGA/GDH inhibitor, CPD-3B, achieved good potency and high efficacy in inhibiting all tested cancer cell lines (Table 1) with low toxicity in the normal cell line (HL7702), whereas the GDH inhibitor (Ebselen) showed essentially no inhibition of most cancer cell lines (Table 1).

Our next question was why both the KGA allosteric inhibitor (CB-839) and the GDH inhibitor (Ebselen) did not show full efficacy in comparison to CPD-3B, a modest KGA inhibitor? Our hypothesis is that KGA inhibition alone may not completely stop the cell growth. However, it remains to be investigated, if blocking the alternative pathways in glutaminolysis is adequate to completely inhibit cancer cell growth. This was tested by monitoring cell growth after treatment under glutamine-deficient conditions, which is similar to the blockage of glutamine metabolism.

Growth Measurement Demonstrated That Glutamine-Deficiency Greatly Reduced Cell Growth but Did Not Completely Inhibit Cancer Cell Growth. The EZMTT reagent¹⁵ showed essentially no toxicity as a detection reagent and was used to track the cancer cell growth, after a 5 day treatment in glucose-deficient or glutamine-deficient media. As shown in Figure 2, after a 5-day starvation in glucose-deficient media followed by EZMTT viability detection for 24 h, the growth of all cell lines except for the PC12 cell was nearly completely inhibited in the absence of glucose. Also, under the same conditions, the growth of normal cells (HL7702) was also significantly inhibited, which may indicate potentially adverse drug toxicity by a glycolysis inhibitor.

Surprisingly, in the absence of glutamine, growth of A549, Caki-1, and U251 cancer cell lines was significantly inhibited, but the cells were not dead; during the 24 h measurement by EZMTT viability detection method, statistically significant

Figure 3. ROS production in A549 cancer cells or in HL7702 normal cells after incubation in glucose-deficient medium, glutamine-deficient medium, rich medium, or rich medium containing H₂O₂, CPD-3B, BPTES, or Ebselen. (a) Time course of DCF Fluorescence in A549 cells after treatment with glucose or glutamine-deficient medium. (b) Time course of DCF Fluorescence in HL7702 cells after treatment with glucose or glutamine-deficient medium; (c) DCF Fluorescence in A549 cells after treatment with glutamine metabolism inhibitors. (d) Cellular NADPH level with or without 10 μM CPD-3B treatment for 12 h. (e) Mitochondrial redox system including NADPH, TrxR, Trx, and many disulfide-bond-containing proteins of cellular redox system. Comparisons of ROS induction between treated and untreated conditions were performed by unpaired *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

time-dependent growth was observed after a 5-day starvation (Figure 2).

Interestingly, the level of glutamine-dependence showed good correlation with the efficacy of CB-839 (Table 1 and Figure 2); CB-839 achieved approximately 90% maximal inhibition for Caki-1, 70% maximal inhibition for U251, A549, HCT116 and SW1990, and weak inhibition for PC12 and H22, but essentially no inhibition for HL7702 normal cells whose growth was very mildly affected.

Taken together, KGA allosteric inhibitors that block the main glutaminolysis pathway in glutamine-dependent cancer cell lines only achieve limited efficacy. CPD-3B showed full efficacy in most cancer cell lines indicating that it could have biological targets other than KGA/GDH. Investigating its relevant targets was of interest as the compound showed high efficacy and low toxicity.

Glucose Deficiency, Glutamine Deficiency, or CPD-3B Treatment Increased ROS Level in Cancer Cells. Because CPD-3B showed relative strong inhibition of TrxR which is important to maintain thio-homeostasis, we measured its effect on NAD(P)H level and ROS level.

Reactive oxygen species (ROS) are generated by decreasing the cellular NADPH, which is important for TrxR/Trx system as shown in Figure 3e; the TrxR/Trx system includes NADPH, TrxR, Trx and many redox proteins that are regulated by Trx through the disulfide bond formation.^{20,21} Depleting NADPH will result in blocking the mitochondrial electron transport chain (ECT), disrupting cellular redox system and generating excess ROS that damages DNA and other cellular

components.¹² Cancer cells lower ROS level by increasing NAD(P)H level or glutathione (GSH; glutamate-glycine-Cysteine) levels.²⁰

The total cellular level of NADH and NADPH are readily compared using the EZMTT reagent, and a dramatic decrease in EZMTT signal strength after 10 μM CPD-3B treatment indicated that less NAD(P)H existed in the cells, demonstrating that glucose or glutamine starvation greatly lowered NADPH production. In addition, because the growth of A549 cells was inhibited significantly in both glucose-deficient and glutamine-deficient media, A549 cells were used to test the ROS level. As shown in Figure 3, a large amount of ROS was detected in A549 cells in the presence of the oxidant H₂O₂, but not in rich media. However, glucose deficiency caused ROS levels to increase after 4 h incubation, and glutamine deficiency took more time to increase ROS levels, peaking after 8 h incubation. These results show that both glucose deficiency and glutamine deficiency result in increased levels of cellular ROS.

In normal HL7702 cells, the ROS level increased slightly in glucose-deficient media, but not in glutamine-deficient media. This is consistent with early observations that growth of the normal cells was reduced in glucose-deficient media, but was not affected in glutamine-deficient media or in the presence of KGA allosteric inhibitors such as CB-839, BPTES and CPD-3B etc. (Table 1). The fact that the normal cells are essentially unaffected by glutamine-deficiency explains the low toxicity of the KGA allosteric inhibitors.

Figure 4. CPD-3B shut down mitochondrial function by inhibiting several key enzymes. (a) CPD-3B showed dose-dependent disruption of MMP; Comparisons of JC-1 assay results between treated and untreated conditions were performed by unpaired *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (b) Sequence of GatB: the red sequence could potentially covalently link with CPD-3B; (c–g) Biomolecular interaction assay showing that the purified KGA, GDH, TrxR and GatCAB proteins could bind to the biotinylated CPD-3B derivative; (h–l) Biomolecular interaction assay showing that the purified KGA and GatCAB proteins could bind to the biotinylated-BPTES derivative; (m) GatB (PDB code: 3KFU) (gray ribbon); the potential CPD-3B binding site is colored in red ribbon (21–59 (TKIFCSCSTSFGEPSNSNTCPVCLGLPGALPVLNK) and cyan ribbon (340–354 (TSVTWLCVELLGR); Purple ball represents Zn^{2+} ; blue curve represents tRNA. (n) Binding site on GatB (upper-left) LC trace showing the missing peptide (t_R 78.93 min) after CPD-3B treatment; (upper-right) MS analysis of the red peak (t_R 78.93 min) showing a sequence TSVTWLCVELLGR (marked in red in panel b); (lower-left) LC trace showing the missing peptide (t_R 80.36 min) after CPD-3B treatment; (lower-right) MS analysis of the peak (t_R 80.36 min) showing a sequence TKIFCSCSTSFGEPSNSNTCPVCLGLPGALPVLNK (marked in red in panel m).

Furthermore, treatment of A549 cells with KGA inhibitors (BPTES, CPD-3B) showed significant increase of ROS levels whereas the GDH inhibitor (Ebselen) did not. This is consistent with the previous observation that glutamine deficiency induced ROS overproduction and reduced cancer cell growth. In addition, we measured NADPH levels in H22 cells with or without CPD-3B treatment. As shown in Figure 3d, 12 h treatment with 10 μ M CPD-3B reduced cellular NADP(H) level by approximately 50%.

CPD-3B-Treated Cells Showed Dose-Dependent Disruption of Mitochondrial Membrane Potential (MMP). MMP is a measure of mitochondrial function and is dependent on the TCA cycle. Completely blocking glutaminolysis is expected to result in shutting down the TCA cycle. In A549 cells as shown in Figure 4a, the KGA allosteric inhibitor (BPTES) and the GDH inhibitor (Ebselen) did not show statistically significant effects in blocking MMP, whereas CPD-3B showed good efficacy in disruption of MMP that is even stronger than the effects of glucose or glutamine starvation conditions. This indicates that CPD-3B might have more

protein targets other than KGA and GDH. Therefore, we utilized the biomolecular interaction assay and proteomics to look for potential CPD-3B binding proteins.

CPD-3B Derivative Binds to Key Mitochondrial Enzymes with Good Affinity. To look for potential targets that are responsible for shutting down mitochondrial function, we chemically synthesized biotinylated CPD-3B, as shown in (Figure 4c–g). Biomolecular interaction assay showed that CPD-3B binds to GDH (K_D 77 nM, $R^2 = 0.92$), KGA (K_D 3.5 nM, $R^2 = 0.96$), GatCAB (K_D 5.4 nM, $R^2 = 0.86$), TrxR (K_D 0.5 nM, $R^2 = 0.86$), but not to GST proteins. The K_D was calculated by global kinetic fitting to obtain k_{off}/k_{on} and the accuracy of the fit was shown as R^2 . Rat liver TrxR was reported to be inhibited by Ebselen,²⁴ which is the parent molecule for CPD-3B.¹⁶ Aminotransferase was reported as an important alternative path involved in glutaminolysis.⁵ Among these proteins, the binding affinity of GatCAB to the biotinylated CPD-3B has a K_D of 5.4 nM and might be a novel protein target for CPD-3B. Under the same conditions, the biotinylated BPTES (Figure 4) showed significant binding

Figure 5. CPD-3B -caused cancer cell necrosis in H22 cancer cells. (a) Percentage of Sub-G1 type of cells with or without 10 μM CPD-3B treatment. (b) FACS analysis showed that CPD-3B resulted in dose-dependent cancer cell necrosis. Comparisons of the necrosis level between treated and untreated conditions were performed by unpaired *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (c) Inhibition of H22 cancer cells by CPD-3B, CB839, and Ebselen; drug combination test showed that CB839 and Ebselen synergistically inhibited H22 (d), HCT116 (e), and A549 (f) cancer cells. In addition, time-dependent cell growth showed that Ebselen provided additional inhibition that could not be achieved by CB839 in H22 (g), HCT116 (h), and A549 (i) cancer cells.

interaction with KGA (K_D 43 nM, $R^2 = 0.87$) and GatCAB (K_D 54 nM, $R^2 = 0.91$).

As shown previously, Ebselen can react with the thio-group of the cysteine residue in a targeted protein and “label” its target.¹⁶ To identify exactly which subunit in the GatCAB protein complex binds to CPD-3B, we did mass spectrometric analysis of the GatCAB protein after coincubation with 1 μM CPD-3B overnight. On the basis of the LC-MS data shown in (Figure 4o), CPD-3B was shown to bind to the GatB subunit at the cysteine residues of two peptides: KIFCSCSTSFGE-SPNSNTCPVCLGLPGALPVLNKEVVK peptide which is located near the Zn^{2+} at the catalytic site and TSVTWLCV-ELLGR peptide that is in the vicinity of acyl-tRNA (Figure 4m) and may block the delivery of the amino acid portion to the active site of GatB or GatA.²² Since both regions are important functional domains, CPD-3B is likely blocking the glutaminase activity of GatCAB.

GatB is important for acyl-tRNA (Glu-tRNA^{Gln}; Asp-tRNA^{Asn}) binding and delivering the acyl-tRNA to the glutaminase site of GatA subunit; GatB is responsible for the conversion between glutamine and glutamate²² or between asparagine and aspartate. Therefore, CPD-3B is also likely to inhibit the alternative glutamine metabolism pathways mediated by amidotransferases (e.g., GatCAB) which are reported to be essential for mammalian mitochondrial function.²³

Furthermore, inhibition of Ebselen in Rat liver TrxR1 has been reported.²⁴ Therefore, we cloned and measured the inhibition of human TrxR1 enzymatic activity (Table 1). Human TrxR1 is a central component in thioredoxin system that is responsible for forming reduced disulfide bonds of many

cellular proteins and defense against oxidative damage; therefore, inhibition of TrxR is tied with the increased level of ROS. In addition, the mitochondrial Trx/TrxR system is also important for mitochondrial thio-homeostasis. However, because the TrxR inhibitor (Ebselen) showed no effect on MMP, it is less likely that TrxR inhibition alone can cause cell death. Interestingly, Hexylselen (CPD-3B) showed 10-fold stronger TrxR activity than Ebselen, and much better efficacy in inhibiting cancer cells than CB-839; this indicated that inhibition of KGA is likely to play the predominant role in disrupting the MMP, and TrxR is necessary for complete cancer cell inhibition.

CPD-3B Results in Cancer Cell Death. Previously, we discovered that Ebselen could link with the thio group of cysteine; at 1 μM low concentration, Ebselen selectively inhibited GDH and TrxR enzymes.¹⁷ However, at 100 μM high concentration, it cross-links nonspecifically to the thio groups of many proteins and inactivate the enzymes by destabilization.^{17,19} Therefore, we carefully controlled the Ebselen concentration under 10 μM .

Figure 5a,b demonstrated that CPD-3B treatment caused more DNA fragmentation and increased the amount of sub-G1 phase cells in a dose-dependent manner, which is an indication of cell death. For further validation that the high efficacy of CPD-3B comes from additional inhibition of KGA, we did combination test of CB839 and Ebselen, because Ebselen is the parent compound of CPD-3B.

Interestingly, when the less glutamine-dependent cancer cell line H22 was used in growth inhibition assay (Figure 5c), CPD-3B showed complete growth inhibition, whereas CB-839 (KGA inhibitor) and Ebselen showed very weak inhibition

Figure 6. Western analysis of H22 cells after CPD3B or CB839 treatment for 12 h. Tubulin, actin, and GAPDH were used as internal standards. Relative changes in protein level were quantified. Comparisons of the expression level between treated and untreated conditions were performed by unpaired *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

(<10% growth inhibition at 10 μ M). When we mixed CB-839 with Ebselen at a concentration below 10 μ M, strong synergistic effects were observed in inhibiting H22 cancer cells. As shown in Figure 5d, 0–12 μ M CB839 or 0–10 μ M Ebselen alone does not inhibit the growth of H22 cancer cells; when 6–12 μ M CB-839 mixed with 10 μ M Ebselen, greater than 50% growth inhibition was observed. Further, the drug combination was tested in Gln-dependent cell lines (HCT116 and A549). As shown in Figure 5e,f, CB-839 is potent but its maximal % inhibition reached a steady state at 60%, but in the presence of Ebselen, the maximal % inhibition increased significantly. As shown in Figure 5g–i, the drug combination dramatically reduced the time-dependent cancer cell growth. Taken together, CPD-3B demonstrated impressive efficacy in blocking mitochondria function by inhibiting KGA, GatCAB, and TrxR/GDH (Figure 4a; Table 1).

To further investigate the effect of CPD-3B on glutaminolysis and glycolysis, we evaluated its effect on key cancer signaling pathways in H22 cancer cells.

CPD-3B Effectively Reduced GAC Level, Activated Caspase-9-Mediated Signaling Pathways and Resulted in Decreasing the Cellular p-Akt (Thr375) and p-Erk Levels. As shown in Figure 6, CPD-3B treatment showed dose-dependent decrease of glutaminase and GAC. Therefore, CPD-3B is on target for glutaminase and showed better activity than CB-839. Further, 12 h CPD-3B treatment significantly decreased the level of pro-caspase-9, slightly decreased the pro-caspase-3 level, and increased the activated caspase-3 level. This is in agreement with the early JC-1 assay results that

showed disruption of mitochondrial function by CPD-3B (Figure 4a), since the damaged mitochondria is expected to release cytochrome c that would in turn initiate cleavage of the pro-enzyme (caspase-9) to activate caspase-9. The activated caspase-9 then cleaves executioner caspases, such as pro-caspase-3, to increase the level of activated caspase-3 that was reported to commit the cells to programmed cell death.²⁵

These results indicated that cell death is likely to be induced through loss of the mitochondrial function. As expected, CB839 treated H22 cancer cells showed no obvious changes as shown in Figure 6.

To further evaluate if the loss of the mitochondrial function and induction of ROS in cytosol will cause further cell death, we investigated the changes in Akt and/or Erk mediated signaling pathways²⁶ after 12 h CPD-3B treatment in H22 cancer cells. As shown in Figure 6, CPD-3B showed dose-dependent dephosphorylation of pAkt (Thr308), pErk-1, and pErk-2; pErk-1 and pErk-2 are key mediators of tumor growth factor induced signaling pathway, and pAkt promoting survival and growth in response to extracellular signals. Dephosphorylation of pAkt and pErk indicated the growth and survival paths are essential for cancer cells. Under the same conditions, the same concentration of CB-839 did not show a dramatic difference Figure 6.

Other key cancer biomarkers were also investigated. CPD-3B treatment showed dose-dependent decrease of p-NF-κB p65, c-Myc, and PARP at protein level, which leads to reduced glutaminolysis, inhibited cell growth and further cell death.²⁸ Also, the level of HIF 1α, the master transcriptional regulator

Figure 7. *In vivo* efficacy comparison in 4 different groups ($n = 10$ mice per group): CPD-3B significantly reduced tumor size, increased necrotic area within tumors, and prolonged survival rate. After injections of N.S. control, 10 mg/kg CB839, 10 mg/kg CPD-3B, and 0.25 mg/kg Act D ($n = 10$). (a) Pictures of end-point tumors after 10 day treatment. (b) Tumor weight. Comparisons of the tumor weights between untreated and treated groups were performed by unpaired t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (c) Body weight changes; (d) survival rate measurement after treatment with 10 mg/kg CB839, 10 mg/kg CPD-3B, or 10 mg/kg CPD-3B twice a day. (e) H&E staining of CPD-3B treated tumor slices; (f) H&E staining of untreated tumor control.

of cellular and developmental response to hypoxia,^{29,27} was increased, which is in agreement with the observed high ROS level shown in Figure 3.

Taken together, these cells signaling results support the mechanism that CPD-3B completely inhibited glutamine metabolism by targeting KGA/GDH/GatCAB and induced ROS by targeting TrxR. This effectively shut down cytosolic cancer signaling pathways and mitochondrial function, which can induce programmed cell death. Importantly, even though multiple targets are involved, CPD-3B retains low toxicity toward normal cells.

CPD-3B Significantly Reduced Tumor Size and Caused Tumor Damage in H22 Liver Cancer Xenograft Mouse Model. CPD-3B completely inhibits the growth of various cancer cells, but showed no inhibition of normal cells.¹⁶ To test if the efficacy and low toxicity translate in animal studies, we tested compounds in the H22 cancer cell xenograft model in young ICR mice. H22 is an aggressive cancer cell line that undergoes tumor metastasis in 15–20 days.³⁰ In our model, ICR mice died within 7–12 days after H22 cell inoculation. Therefore, we used the 10-day model to mainly evaluate tumor size reduction. Using the survival model, we treated the mice, until 3 out of 10 remained.

Figure 7a shows tumor sizes of all animals after 10-day subcutaneous injection at 10 mg/kg compound concentration. CPD-3B treatment resulted in significantly reduced tumor size in comparison with the control. Further quantification determined that the H22 tumor weight was significantly reduced by 60% Figure 7b, whereas at the same dosage CB-839 showed only minor reduction in comparison with the control group. The positive control Actinomycin D (Act D) achieved nearly complete tumor size reduction, but Act D is toxic and resulted in animal loss and weight loss. Unfortunately, limited by its solubility, CPD-3B was dosed at 20 mg/kg IP injections, and a single injection per day resulted

in 65% tumor size reduction, and twice a day reduced tumor size by 80%.

To further evaluate CPD-3B related tumor tissue damage, we performed H&E staining of the end-point tumor tissues. In the untreated control group (Figure 7e), the tumor cells were arranged in sheets with features of large cell size, large nuclei, high nucleus/cytoplasm ratio, and infiltrated with scattered lymphocytes. In the CPD-3B treated group (Figure 7e), tumor cells cannot be seen clearly under microscopic examination. The site of the original tumor has been replaced by fibrous hyperplasia, inflammatory cell infiltration, foam-like histiocytic reaction and scattered deformed nuclei. This is evidence typical of severe reaction following molecular targeted therapy.

Importantly, CPD-3B showed low toxicity, as demonstrated during the entire treatment. In addition, essentially no loss of body weight or animal death was observed Figure 7c. In a separate experiment measuring the survival rate (Figure 7d), CPD-3B treatment was statistically significant in prolonging survival rate. Treatment once a day prolonged survival by an extra 40%, and twice a day dosing by 180%. This is in good agreement with the cell growth inhibition results reported in Table 1.

DISCUSSION AND CONCLUSION

Efficacy, toxicity and drug resistance are critical factors in cancer therapeutics. General toxicity limited the utility of many cancer drugs, such as DNA chelators. KGA allosteric inhibitors attracted a lot of attention because of their low toxicity. A vast amount of biology supports targeting glutamine metabolism as a valid approach in cancer therapeutics.² However, clinical application of KGA inhibitors has shown limited efficacy even at high doses.

By tracking cell growth in glutamine-deficient or glucose-deficient media, we discovered that glucose-starvation greatly inhibited most cancer cells and normal cell growth, whereas

Figure 8. Mechanism of CPD-3B generated cancer cell death: targeting glutamine metabolism (KGA/GDH/GatCAB) played the predominant role in blocking cancer cell growth, and additional ROS stimulation by inhibition TrxR/NADPH caused death in metabolism blocked and weakened cancer cells but not in normal cells.

glutamine starvation does not affect the viability of normal cells, thus supporting the idea that KGA allosteric inhibitors have low toxicity. Interestingly, in all cancer cells tested, glutamine starvation can reduce the cell growth dramatically, but does not completely inhibit the cell growth and cause cell death. In addition, the observed glutamine dependence in a cancer cell line correlated well with the efficacy of the KGA allosteric inhibitor CB-839. Therefore, our conclusion is that the efficacy of KGA allosteric inhibitors is limited in cancer therapeutics, because the surviving cancer cells are expected to present problems in the future through gene mutations and drug resistance. To achieve high efficacy as cancer therapeutics, KGA allosteric inhibitors need additional drug combinations to block alternative pathways and to cause cancer cell death.

As shown in Figure 8, CPD-3B presents an interesting combination of drug targets: targeting KGA/GDH/GatCAB significantly blocked glutamine metabolism and targeting TrxR increased ROS in both mitochondria and cytosol and resulted in blocking the NADPH-driven redox chain and further disrupt thiol homeostasis. Complete blockage of mitochondrial glutamine metabolism and dramatically increased ROS level caused inhibition of cancer cell growth and cell death. Further, as indicated by signaling pathway analysis (Figure 6), we propose that CPD-3B primarily caused mitochondrial malfunction in cancer cells which resulted in activation of caspase-9 mediated programmed cell death. In addition, TrxR mediated ROS stimulation further activated HIF mediated response to hypoxia. Both increased programmed cell death, as manifested by the dephosphorylation of the key mediators p-Akt and p-Erk that inactivated the main cancer signaling pathways important for cancer cell survival and growth, respectively. This explains why CPD-3B showed good efficacy with various aggressive cancer cell lines that are less glutamine-dependent.

Furthermore, mechanistic study of CB-839, Ebselen and CPD-3B provided a clear picture about the role of glutamine metabolism in cancer therapeutics. As a potent KGA allosteric inhibitor, CB-839 could only achieve limited efficacy up to 80% of growth inhibition in cell-based assay. Ebselen reacts

with many enzymes at very high 100 μ M compound concentration,¹⁹ but showed essentially no cellular activity below 10 μ M compound concentration.^{16,15,17} Interestingly, great synergy was observed between CB839 and Ebselen in cell-based assays. When CPD-3B, an Ebselen derivative with additional KGA inhibitory activity, was tested at a concentration below 10 μ M, it demonstrated high efficacy in complete inhibition of cell growth and resulted in cell death. Translated in animal models, CPD-3B is more efficacious *in vivo* than CB-839. By targeting predominantly glutamine metabolism in mitochondria and the additional inhibition of cytosolic TrxR to stimulate ROS, CPD-3B achieved high *in vivo* efficacy by shutting down mitochondrial function, causing tumor cell death while increasing the survival rate of the animals.

The conclusion is that efficacy translates well between the EZMTT-based cell models and the xenograft animal models for KGA inhibitors. By incorporating additional TrxR, GatCAB, and GDH inhibitory functions to a KGA allosteric inhibitor, high efficacy could be achieved; this represents a novel approach in drug designing of an effect anticancer KGA allosteric inhibitor.

MATERIALS AND METHODS

Materials and Cell Lines. Chemicals were purchased from Aladdin (California, U.S.A). ¹HNMR was performed on a Varian Mercury 500 or 600 MHz NMR spectrometer. Low- and high-resolution mass spectra were obtained in the ESI mode. Compounds 1 and 2 (Figure 1) were prepared according to reported procedures.^{16,17} EZMTT detection reagents were from JNF Biotech Inc. (Hangzhou, CN). A549, T24, H22, HL7702, and other cell lines, cloning enzymes, and assay kits were purchased from Labpal (Shanghai, China). Proteins (KGA, GDH) were cloned and purified as we reported previously.^{16–18} *H. Pyroli* GatCAB protein was gift from Dieter Söll's lab (Yale University, New Haven, CT). The UV absorbance was measured by Flexstation 3 (Molecular Device, USA). The fluorescence was measured using MiniMaxPro fluorescent microscope (Molecular Device). RPMI 1640 medium was purchased from M&C Gene

Technology Inc. (Beijing, China). Trypsin and EDTA were purchased from Amresco (Solon, OH). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. (Zhejiang, China). Flow cytometry was performed using ACEA NovoCyteTM2060R at flow rate of 1 mL/min. ICR mice (SPF) were purchased from Zhejiang Institute of Medical Science (Hangzhou, CN), and were treated in compliance with ethical standards. The animal experiments were carried out at the animal facility of Zhejiang No. 1 Hospital, and permissions was obtained from Zhejiang Province Health Planning Committee of the subject of animal experiments with accreditation number of SYKX(Zhe)2013–0180.

Statistical Analysis. Statistical analysis of the samples between treated and untreated conditions were performed by unpaired *t* test in Excel: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *P*-values < 0.05 were considered statistically significant. All data are reported as the means $\pm$ the standard deviation (SD) unless otherwise stated.

Human TrxR1 Cloning and Protein Expression. Human TrxR1 (*Txnrd1* gene (GI: 7296), AA161–647) was PCR-amplified using the forward primer TATACATATGT-ATGACTATGACCTTATCATC and the reverse primer TATACTCGAGTTAGCAGCCAGCCTGGAGGAT. The purified PCR product was restriction-enzyme-digested and ligated into the pET 28a expression vector, then transformed into the *E. coli* DH5 α strain. After DNA sequencing, the plasmid was transformed into an *E. coli* BL-21 strain. The recombinant N-terminal His-tagged human TrxR1 protein was expressed after 1 mM IPTG induction for 3 h and purified using nickel affinity chromatography. The purified protein was analyzed by SDS-PAGE, which showed a single band with a molecular weight of 36 kDa, and further was stored in buffer (20 mM Tris-HCl, 1 mM DTT, 10% glycerol) at -80°C .

Human TrxR1 Activity Assay. A dilution series of inhibitors (0–13 μM ; 3-fold dilutions) were spotted in 96-well plates and incubated for 20 min with human TrxR1 (100 nM) in buffer (50 mM Hepes, 100 mM NaCl, 0.5 mM EDTA, 0.01% BSA, 0.003% Brij-35, 0.001% Tween 20, pH 7.5). To each well, a mixture of DTNB in ethanol (5 mM final) and NADPH (300 μM final) was added, and the reaction was carried out at room temperature for 1 h before the absorbance measurement at 412 nm.

Human GDH Activity Assay. GDH inhibition was tested using the optimized condition that we have reported previously.¹⁸ A dilution series of inhibitors (0–13.3 μM ; 3-fold dilution) were spotted in 96-well plates and incubated with human GDH (4 nM final) in buffer (50 mM Tris-Cl, 0.01% BSA, 0.003% Brij-35, 0.001% Tween 20, pH 8.0) for 0.5 h at 25°C . To each well, a mixture of glutamate (10 mM final), NADP⁺ (150 μM final) and the EZMTT detection agent (1 $\times$, final) was added, and the reaction was carried out at 25°C for 1 h before the absorbance measurement at 450 nm.

Human KGA/GDH Coupled Assay. The compound was premixed with human KGA protein (1 nM) in 100 μL of Hepes buffer A for 15 min. To the 96-well plate was added Gln (20 mM) in 100 μL Hepes buffer B, and the reaction was shaken at 25°C for 3 h. Glutamate formation was then measured in a GDH coupled assay by adding a mixture containing GDH (8 nM), NADP⁺ (20 μM), and the EZMTT detection reagent.¹⁵ After the solution was incubated at 25°C for 5 min, the absorbance at 450 nm was measured against the reference wavelength of 620 nm.

Cellular NADPH Quantification. H22 cells (10^7) in 30 mL dishes were treated with 10 μM CPD-3B in 0.1% DMSO, and the corresponding vehicle was used as control group. The experiments were carried out in duplicate. After 12 h of treatment, cells were counted and centrifuged to collect pellets. The pellets were frozen at -80°C for 2 h and then cooked at 95°C for 10 min (both NADPH and NADH were stable after 10 min of boiling at 95°C , as measured by EZMTT reagents). The cooked cell pellets were resuspended thoroughly in 1 mL of PBS, and the resulting solutions were divided into two tubes. One was treated with 10 mM α -KG and 20 nM *E. coli* GDH which uses only NADPH as the cofactor, and the treatment will deplete the cellular NADPH. The other was diluted to the same volume with PBS. After 30 min of treatment, EZMTT reagent was added to measure the total level of NAD(P)H. The cellular NADPH level was obtained by calculating the difference between the untreated sample and the one that was pretreated with α -KG and *E. coli* GDH and quantifying it based on NADPH standard curve.

Cell Proliferation Assay. Cells ($1-5 \times 10^3$ /well) were plated on a 96-well plate. After setting aside for 4 h for adherence, cells were treated with compounds for 5 days. Cell viability was measured using EZMTT method,¹⁵ and cell morphology was observed under microscope. Results are representative of at least two independent experiments in triplicates.

Glucose or Glutamine Dependence Assay. Cell proliferation was tracked using EZMTT assay. Briefly, cells ($1-5 \times 10^3$ /well) were plated on a 96-well plate. After setting aside for 4 h in rich media for adherence, cells were treated with compounds or washed with PBS and then treated with glucose-deficient or Gln-deficient media for 5 days. Then, NAD(P)H production in viable cells was measured using the EZMTT method after 1 h, 4 and 24 h incubation time. Results are representative of at least two independent experiments in triplicates.

ROS Assay. ROS level was measured in A549 cells (5000/well) grown at 37°C in a 5% CO_2 incubator, after treatment with 0.5% DMSO, compounds in 0.5% DMSO at 0.3 or 1 μM for 12 h, or glucose-deficient or Gln-deficient media for (4, 8, 20, and 72 h); H_2O_2 was included as a positive control. After treatment, the A549 cells were washed with the FBS-free medium, incubated with the DCFH-DA probe for 30 min, washed 3 times with the FBS-free medium, and then subjected to the DCF Fluorescence measurement at EX 485 nm, EM 535 nm by the Flexstation 3 plate reader.

JC-1 Assay. JC-1 assay was used to measure the mitochondrial membrane potential changes. A549 cells (5000/well) were seeded in a 96-well clear-bottomed black plate and grown at 37°C in a 5% CO_2 incubator for overnight. The cells were treated with compounds (0.3 or 1 μM) in 0.5% DMSO for 12 h or glucose-deficient or Gln-deficient media for 4, 8, 20, and 72 h. The CCCP-treated (100 μM for 30 min) group was used as positive control. Then, the cells were treated with JC-1 for 20 min, washed twice with cold staining buffer, and then RPMI1640 medium (100 μL) was added. The disruption of the mitochondrial membrane potential in each cell culture was determined based on the fluorescence ratio of the green fluorescence measured at EX 490 nm and EM 530 nm versus the red fluorescence at EX 525 nm and EM 590 nm.

Chemical Synthesis of Biotinylated CPD-3B Derivative. The chemical synthesis of compound 1 (a CPD-3B derivative) was accomplished according to previously

described methods.^{16,17} To a 10 mL round-bottomed flask were added compound 1 (58 mg, 0.11 mmol), compound 2 (49 mg, 0.167 mmol), K₂CO₃ (31 mg, 0.22 mmol), and DMF (1 mL), and the mixture stirred at 80 °C for overnight. The mixture was then treated with water (5 mL) to precipitate the product which was then filtered, washed with water and ether, and purified by silica gel column chromatography (DCM/MeOH = 20:1) to give compound 3 (a biotinylated CPD-3B derivative) as light yellow solid (63 mg, 78%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.90 (d, *J* = 8.5 Hz, 1H), 7.88 (d, *J* = 8.5 Hz, 1H), 7.29 (d, *J* = 2.5 Hz, 1H), 7.28 (d, *J* = 2.5 Hz, 1H), 7.23 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.22 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.45 (s, 1H), 6.36 (s, 1H), 4.34–4.24 (m, 1H), 4.17–4.10 (m, 1H), 4.02 (t, *J* = 6.0 Hz, 2H), 3.82 (s, 3H), 3.69 (t, *J* = 6.0 Hz, 4H), 3.15–3.08 (m, 1H), 2.82 (dd, *J* = 12.5, 5.0 Hz, 1H), 2.58 (d, *J* = 12.5 Hz, 1H), 1.77–1.69 (m, 2H), 1.67–1.55 (m, 5H), 1.52–1.40 (s, 4H), 1.37–1.29 (s, 5H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 166.1, 162.8, 158.2, 157.6, 129.8, 129.7, 129.1, 126.8, 120.9, 120.5, 110.4, 109.8, 67.9, 61.1, 59.3, 55.5, 55.5, 43.4, 29.9, 28.4, 28.4, 28.3, 25.8, 25.6. LRMS (ESI) *m/z* 761.1 [M + Na]⁺. HRMS (ESI) *m/z* calcd for C₃₁H₃₈N₄O₅NaSSe₂ [M + Na]⁺ 761.0791. Found: 761.0786.

Biomolecular Interaction Assay Using BLI. Biomolecular interaction analyses between the biotinylated compound and various purified proteins were performed using a ForteBio A2 instrument with Streptavidin (SA) biosensors. For example, SA biosensors were loaded with the biotinylated CPD-3B derivative by dipping the sensor into sample plate wells containing compound 3 in PBS buffer for 5 min. During the binding assay, 3-coated sensor was first dipped into PBST buffer (PBS + 0.02% tween 20) for 2 min to obtain the baseline signal, then into a protein sample for 5 min to measure the association rate, and finally into the PBST buffer again for 3–5 min to obtain the dissociation rate. A series dilution of proteins were tested in duplicate to obtain dose response. The bindings to the biotinylated BPTES were tested under the same condition.

Proteomic Analysis. CPD-3B was incubated with GatCAB, and TrxR1 proteins (final concentration 0.3 mg/mL) in buffer A (20 μL) at room temperature overnight. Protein digestion was carried out according to a previous study.¹⁷ Briefly, protein (10 μg) was suspended in ABC solution, trypsin digested, and then acidified with 10% TFA for desalting. The solution was desalted using a C18 column. The desalted peptide (2 μL) was injected to online nanoflow liquid chromatography via the easy-nano LC system using a 3 μm C18 HPLC column (75 μm × 15 cm). The column was developed using gradient elution with mobile components ACN/FA in water.

Data were acquired with an Orbitrap Q-Exactive mass spectrometer in a data-dependent manner, with automatic switching between MS and MS/MS scans using the top-20 method. Raw data were processed using the Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific) with Sequest HT (An updated High-Through version of SEQUEST) search engine against a forward-decoy approach. The database search results were processed by Percolator to improve peptide-spectrum matches and enforce a peptide level *q*-value threshold of ≤0.01, and two or more peptides were required for protein identification.

FACS Analysis. Cancer cells (10⁵/well) were seeded in 6-well plate, grown at 37 °C in a 5% CO₂ incubator overnight, and then treated with CPD-3B (1–10 μM) for 12 h. After

removing the medium, cells were trypsinized, isolated by centrifugation for 5 min at 1000 × *g*, washed with cold PBS, and fixed with cold 70% ethanol (1 mL) for 12 h. The fixed cells were then washed with cold PBS, treated with PI working solution (20× PI staining solution, 50× RNase A; 0.5 mL) at 37 °C for 30 min, and analyzed by flow cytometry ACEANovoCytTM 2060R. PI fluorescence associated with cells was measured using FL2 channel at EX 535 nm and EM 615 nm and calculated. For each sample, 2 × 10⁴ events were acquired, and analysis was carried out by triplicate determination on at least three separate experiments. The percentage of fluorescent cells and their fluorescent strength were quantified.

Western Analysis. A series of dilutions (0, 1, 10, and 100 μM) of compounds were incubated with cells for 12 h. Cells were collected and lysed by NP-40 buffer with protease inhibitors. Proteins were quantified use Bradford assay, and 100 μg samples were loaded on to SDS-PAGE gel for Western analysis. Then, the samples were analyzed by SDS-PAGE gel, followed by immunostaining using antibody against key signaling pathways: Akt (60 kDa), Phospho-Akt (Ser473) (60 kDa), Phospho-Akt (Thr308) (60 kDa), Erk1 (44 kDa), Erk2 (42 kDa), Phospho-Erk1/2 (44/42 kDa), Stat3 (92 kDa), Phospho-Stat3 (88 kDa), Bcl-2 (28 kDa), Phospho-Bcl-2 (Ser70), Caspase-3 (35kDa), Caspase-3 (active; 19/17 kDa), Caspase-9 (47/37 kDa), Caspase-8 (57 kDa); HIF-1α (120 kDa), NF-κB p65 (65 kDa), Phospho NF-κB p65 (Ser536), LC3B (16/14 kDa), p21 (21 kDa), Glutaminase (KGA, 73 kDa; GAC, 65 kDa), c-Myc (49 kDa), GAPDH (36 kDa), PARP (116 kDa). At least two independent experiments in triplicate were carried out for each sample.

Xenograft Animal Model. ICR male mice (around 20g in weight; 5–7 week old) were divided into 10 mice per group. The diluted liver cancer H22 ascites tumor cells (5.0 × 10⁶/mL; 0.2 mL) were transplanted subcutaneously into the right armpit of each mouse. After 24 h, the transplanted mice were treated by subcutaneous (SC) or intraperitoneal (IP) injection (1 mg/mL; 0.2 mL for SC or 1 mL for IP) on a daily basis or twice a day for 10 consecutive days. In survival rate measurement, mice were dosed every day until 3 mice were left in each group. The drugs were formulated with 5% ethanol, 5% Tween80, 10% PEG400, and 3% F68 in PBS buffer solution. Animal death was observed after 8 days in the no-drug-treated blank control. Animals were euthanized humanely to collect tumors, blood, tissues, and measure the body weight.

Hematoxylin and Eosin Staining of the Embedded Tumor Tissue. The tumors were fixed in 4% paraformaldehyde for 24 h, stepwise dehydrated in ethanol, and embedded in paraffin. Sections (4 μm) were dissected and loaded on to a glass slide. The sections were then deparaffinated in dimethylbenzene, ethanol, and DI water, followed by hematoxylin and eosin (H&E) staining, washes with water ethanol and xylene, and permanent mounting with resinene, according to assay kit instructions from Beyotime Institute of Biotechnology (Beijing, China). H&E stained tumor slices were analyzed for tumor cellularity.

Microscope Image Acquisition. Images of ROS were acquired and processed using microscopy machine OLYMPUS IX73, charge-coupled device (CCD) camera (Point Gray Research CMLN-13S2M-CS), mercury lamp (OLYMPUS U-RFL-T), Metamorph software, and FITC channel. The ocular was 10×, and the objective lense was 4×. Images of H&E staining were acquired and processed using OLYMPUS CX41,

CCD camera HTC1600, WT-1000GM software, and bright-field channel. The ocular was 10×, and the objective lens was 4× or 10×.

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Notes

The authors declare no competing financial interest.

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