Enhanced Antitumor Activity of 3-Bromopyruvate in Combination with Rapamycin In Vivo and In Vitro

Qi Zhang1,2, Jing Pan1,2, Ronald A. Lubet3, Steven M. Komas4, Balaraman Kalyanaraman4, Yian Wang1,2, and Ming You1,2

Abstract

3-Bromopyruvate (3-BrPA) is an alkylating agent and a well-known inhibitor of energy metabolism. Rapamycin is an inhibitor of the serine/threonine protein kinase mTOR. Both 3-BrPA and rapamycin show chemopreventive efficacy in mouse models of lung cancer. Aerosol delivery of therapeutic drugs for lung cancer has been reported to be an effective route of delivery with little systemic distribution in humans. In this study, 3-BrPA and rapamycin were evaluated in combination for their preventive effects against lung cancer in mice by aerosol treatment, revealing a synergistic ability as measured by tumor multiplicity and tumor load compared treatment with either single-agent alone. No evidence of liver toxicity was detected by monitoring serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes. To understand the mechanism in vitro experiments were performed using human non–small cell lung cancer (NSCLC) cell lines. 3-BrPA and rapamycin also synergistically inhibited cell proliferation. Rapamycin alone blocked the mTOR signaling pathway, whereas 3-BrPA did not potentiate this effect. Given the known role of 3-BrPA as an inhibitor of glycolysis, we investigated mitochondrial bioenergetics changes in vitro in 3-BrPA–treated NSCLC cells. 3-BrPA significantly decreased glycolytic activity, which may be due to adenosine triphosphate (ATP) depletion and decreased expression of GAPDH. Our results demonstrate that rapamycin enhanced the antitumor efficacy of 3-BrPA, and that dual inhibition of mTOR signaling and glycolysis may be an effective therapeutic strategy for lung cancer chemoprevention.

Cancer Prev Res; 8(4); 318–26. © 2015 AACR.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States (1). Lung cancer also has an extremely high mortality rate, with up to 85% of newly diagnosed patients dying within 5 years (2). Given these dismal statistics, potential the most effective strategy to address the rising disease burden will be rational strategies, that when properly targeted can prevent disease development. Individuals at risk for lung cancer should enter into a chemoprevention trial based on selected biologic features that both help define the heterogeneity of those at elevated risk and suggest their response to a particular agent or agents (3). Lung cancer development is associated with a number of molecular abnormalities. Targeting specific molecular mutations that are unique to lung tumor cells is potential development in the treatment of lung cancer with potential applicability to prevention strategies as well.

One of the most promising targets identified in general is mTOR, which is found to be activated in a substantial number of lung cancer cases as well (4). mTOR is a major regulator of cell growth, proliferation, and differentiation, cell metabolism and proliferation, and it was also found that mTOR was a major positive regulator of the Warburg effect, not only in cancer cells but also in benign tumor cells and even in premature senescent primary cells. A higher rate of glycolysis occurred in the cells with augmented mTOR signaling, and these cells could be more vulnerable to the suppression of either glycolysis or mTOR.

mTOR-mediated aerobic glycolysis may play an essential role in tumorigenesis (5). Therefore, an important strategy to affect cancer cell energy metabolism is to target the regulatory mechanisms that affect the expression or functions of protein molecules that are involved in metabolism, combining mTOR inhibitors with glycolysis inhibitors may have better therapeutic outcomes (6).

Ideally, chemopreventive agents should be efficacious at doses that elicit little or no toxicity (7). Using chemopreventive agents in combination may enable reduced dosages, and potentially a reduction in toxicities. Combination chemoprevention is especially important for lung cancer because this disease is induced by a complex mixture of tobacco carcinogens and toxicants with different modes of action (8). The combination of specific molecular target inhibitors is especially appealing because such an approach can theoretically improve clinical efficacy with minimal cumulative toxicity (9). Therapies targeted against unique molecular events in cancer have significantly diminished side effects.
compared with other broader cytotoxic therapies; therefore, combining multiple therapies that target tumor-specific molecular aberrations may have additive or synergistic effects with a reduced side effect profile compared with broadly targeted agents.

Rapamycin, a natural product derived from Strepomyces hygroscopicus, is the first defined inhibitor of mTOR (10). Rapamycin and its derivatives exhibit significant growth inhibition of breast cancer, prostate cancer, leukemia, melanoma, renal cell cancer, and lung cancer in vivo and in vitro (11). Our previous study has demonstrated that rapamycin can inhibit mouse lung tumor progression in a benzo(a)pyrene-induced mouse lung tumor carcinogenesis model when given by oral gavage (12).

3-Bromopyruvate (3-BrPA), an alkylating agent and a well-known glycolysis inhibitor, has been proposed as a specific anticancer agent. In a previous study, we demonstrated that 3-BrPA is a potent lung cancer chemopreventive agent (13). Inhalable medications have been available for many years for treating lung diseases such as asthma and chronic obstructive pulmonary disease (14). In comparison with systemic means of administration, drugs can be delivered directly to the target tissue resulting in better efficacy at lower doses resulting in decreased toxicity (15). Aerosol delivery of therapeutic drugs for lung cancer in humans has been reported to be an effective route of delivery with little systemic distribution of the therapeutic agents (16, 17). In our previous studies, we demonstrated that aerosolized 3-BrPA was capable of decreasing tumorigenesis in benzo(a)pyrene-treated A/J mice (13, 18–21). Using the aerosol delivery approach, we successfully reduced the side effect such as liver toxicity associated with systemic administration of 3-BrPA. The particle size of the target agent is a crucial consideration in determining the suitability of aerosol delivery strategies. Smaller particles are more likely to be deposited deep in the airway and lung tissue. Because mice have a much smaller respiratory tract than human, the optimal particle size for mice inhalation studies is <0.3 μm. With our Collison atomizer, the mass median diameter was less than 0.2 μm, which is favorable for aerosolized drug delivery in this mouse model.

In the present study, we evaluated the effect of mTOR inhibitor rapamycin and 3-BrPA on benzo(a)pyrene [B(a)P]-induced lung tumorigenesis. We found that aerosolized rapamycin enhances the antitumor efficacy of aerosolized 3-BrPA without adverse effects. Our data support further investigation of the combination of 3-BrPA and rapamycin as potential lung cancer chemopreventive agents.

Materials and Methods

Reagents

3-BrPA, Benzo(a)pyrene [B(a)P], 99% pure, tricaprylin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Cell Signaling Technology. Rapamycin was purchased from LC laboratories. B(a)P was prepared immediately before use in animal bioassays. CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega. For Western blotting analysis, antibodies were purchased from Cell Signaling Technology.

Cell lines

Human lung non–small cell lung cancer (NSCLC) cell lines H1299 and H23 were purchased from the ATCC, but not authenticated by the authors. Both cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Chemopreventive studies

Female A/J mice at 6 weeks of age were obtained from The Jackson Laboratory. Animals were housed with wood chip bedding in environmentally controlled, clean-air room with a 12-hour light–dark cycle and a relative humidity of 50%. Drinking water and diet were supplied ad libitum. The study was approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Mouse body weight was recorded weekly.

The protocol of experiment is shown in Fig. 1A. Mice were given a single i.p. injection of B(a)P at 100 mg/kg body weight in 0.2 mL of tricaprylin. Mice were then randomized into 4 groups with 12 mice per group: (i) the vehicle control group (DMSO:ethanol = 20:80); (ii) the aerosolized rapamycin group (2 mg/mL); (iii) the aerosolized 3-BrPA group (5 mg/mL); (iv) the aerosolized 3-BrPA (5 mg/mL) + rapamycin (2 mg/mL) group. The dose of 3-BrPA and rapamycin administered was guided by our previous study (13) and our unpublished data. Powdered rapamycin and 3-BrPA were dissolved in a 20% DMSO:ethanol solution. These solutions were prepared fresh every day. Solution formulations were atomized into droplets by atomizer. Aerosol flow was then passed through two scrubbers with activated carbon to remove ethanol and DMSO. The resulting dry aerosol flow with only the desired chemicals was then introduced into the nose-only exposure chamber from the top inlet. Effluent aerosol was discharged from an opening at the bottom of the chamber. The size distribution of the aerosol was determined by Scanning Mobility Particle Sizer spectrometer, which includes an electrostatic classifier (TSI model 3080), a differential mobility analyzer (TSI model 3081), and a condensation particle counter (TSI model 3025). Geometric median diameter, mass median aerodynamic diameter (MMAD), and geometric SD were obtained.

All groups were treated for 8 minutes per day, 5 days a week for a total duration of 24 weeks. Mice were euthanized by CO2 asphyxiation. Lungs of each mouse were fixed in Tellyesniczky’s solution (90% ethanol, 5% glacial acetic acid, and 5% formalin) overnight and then stored in 70% ethanol. The fixed lungs were evaluated under a dissecting microscope to obtain surface tumor count and individual tumor diameter. Tumor volume was calculated on the basis of the following formula: V = 4π/3r3. The total tumor volume in each mouse was calculated from the sum of all tumor volumes. Tumor load was determined by averaging the total tumor volume of each mouse in each group. Serum was collected and the levels of alanine transaminase (ALT) and aspartate transaminase (AST) were determined on the basis of validated laboratory protocols using an Ortho Clinical Diagnostik Vitros Fusion 5.1 analyzer.

Histopathology analysis

Fixed lung samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). H&E-stained slides were scanned with the NanoZoomer HT slide scanner (Hamamatsu Photonics), and virtual slides were analyzed and quantified. Immunohistochemistry was performed on lung tissue sections using specific antibodies to detect the localization and to quantify the levels of the positive staining. Briefly, five lungs from each group were analyzed to evaluate activated caspase-3, Ki-67, and GAPDH expression in lung tissues. Cell proliferation was assessed using primary

Published OnlineFirst February 2, 2015; DOI: 10.1158/1940-6207.CAPR-14-0142
monoclonal antibody against Ki-67 (1:400 dilutions; Labvision, Sp6). Cells undergoing apoptotic changes were detected using activated caspase-3 (BioCare). All slides were deparaffinized in xylene and rehydrated in gradients of ethanol. Microwave antigen retrieval was carried out for 20 minutes in citrate buffer, pH 5.0 – 6.0. Primary antibody was diluted in DaVinci Green (BioCare) and incubated at 4°C overnight. Secondary antibody diluted in phosphate buffered saline tween-20 (PBST) and SA-HRP (1:800) was then applied.

Cell viability assay

The cell growth inhibition of 3-BrPA, rapamycin, or their combination was assessed using the MTT method, according to standard protocols. Briefly, human lung cancer cell lines, H1299 and H23, were obtained from the ATCC and were maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C with 5% CO2. Cells were seeded onto 96-well tissue culture plates at 10,000 cells per well. Twenty-four hours after seeding, cells were exposed to different concentrations of 3-BrPA, rapamycin, or their combination as indicated for 24 or 48 hours, whereas that of the control group was replaced with fresh medium. The 3-BrPA solutions were prepared in RPMI-1640 medium and then they were adjusted to pH 7.4 with sodium hydroxide. The solutions were sterilized with 0.2 μmol/L filter unit (Millipore). Rapamycin was dissolved into DMSO and then diluted in RPMI-1640 medium (final concentration of DMSO is less than 0.1%). MTT (0.5 mg/mL) was added after the exposure period. After 4-hour incubation with MTT, the formazan crystals were dissolved in DMSO and the absorbance was measured at 595 and 655 nm by Infinite M200 Pro plate reader (Tecan). All assays were performed in triplicate.

Antibodies and Western blotting

H1299 and H23 cells in a 6-well tissue culture dish at 60% confluence were treated with 3-BrPA, rapamycin, or combination compared with control groups. Asterisk, *P < 0.05; **, *P < 0.01; ***, *P < 0.001, compared with the solvent control group. C, differential serum ALT and AST level in liver toxicity experiment and chemopreventive experiment. Serum ALT and AST level in treated mice after 24 weeks of aerosol versus aerosol treatment.
Chemoprevention by Aerosolized 3-Bromopyruvate and Rapamycin

**Figure 2.**
A, synergistic cytotoxic effect of 3-BrPA and rapamycin in human lung cancer cell lines. H1299 and H23 cells were treated with 50 μmol/L (H) 3-BrPA with rapamycin or their combination for 48 hours. B, effect of aerosolized 3-BrPA, rapamycin, or their combination on caspase-3 staining in the B(a)P-induced lung tumorigenesis model. Lungs harvested from mice on the 26 weeks in B(a)P study (n = 5 mice/group) were stained using specific antibodies as detailed in Materials and Methods. Representative picture from immunohistochemistry for cleaved caspase-3 (a, solvent control group; b, aerosol 5 mg/mL 3-BrPA group, c, aerosol 2 mg/mL rapamycin group; d, combination group) e, apoptosis index as determined by caspase-3; ***, P < 0.001, the 3-BrPA group versus the combination group.

inhibitor cocktails (Pierce). Lysates were separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane and blotted with primary antibodies against mTOR, P-mTOR, p-p70S6K, 4E-BP-1 and were visualized using the ECL Western Blotting Analysis System.

Measurements of oxygen consumption rate and extracellular acidification rate (extracellular flux assay)

The bioenergetics function of H1299 and H23 cells in response to 3-BrPA, rapamycin, or their combination was determined using a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Seahorse Bioscience). A total of 4 × 10^4 H23 or 2 × 10^4 H1299 cells were seeded in the 96-well Seahorse tissue culture plates in RPMI-1640 medium for 24 hours before beginning the XF-96 assay. One hour before the start of the experiment, cells were washed and changed to unbuffered assay medium adjusted to pH 7.4. After establishing the baseline oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), 3-BrPA, rapamycin, or combination were administered through an automated pneumatic injection port of XF96. The changes in OCR and ECAR were monitored for 4 hours. The resulting effects on OCR and ECAR are shown as a percentage of the baseline measurement for each treatment. (Measurements are reported in pmol/min for oxygen consumption and mpH/min for ECAR.)

Measurement of adenosine triphosphate level and GAPDH expression

For adenosine triphosphate (ATP) assays, H1299 and H23 cells seeded at 1 × 10^4 per well in 96-well plates were seeded in complete media for 24 hours, and subsequently treated with rapamycin, 3-BrPA, or the combination of rapamycin and 3-BrPA for various periods of time, from 0 to 180 minutes. Relative ATP levels were determined using a luciferase-based assay per the manufacturer’s instructions (Promega). Relative GAPDH levels were assessed by Western blotting.

Statistical analysis

The data on tumor multiplicity, tumor load, were analyzed by one-way ANOVA; *, P < 0.05; **, P < 0.01; ††, P < 0.001. For the synergistic activity, data were analyzed using CalcuSyn software.
( Biosoft) to determine whether the combination of rapamycin and 3-BrPA was synergistic. When CI = 1, effects are additive; when CI < 1.0, effects are synergistic.

Results

Inhibitory effect of 3-BrPA, rapamycin, and combination on B(a)P-induced lung tumor multiplicity and tumor load in A/J mice

The aerodynamic typical particle size distribution of nebulized 3-BrPA and rapamycin was as follows: The geometric median diameter was 0.046 and 0.077 μm and geometric SD was 1.8. The mass median diameter of 3-BrPA and rapamycin are approximately 0.2 μm.

In this study, we determined the effect of combination 3-BrPA and rapamycin treatment on B(a)P-induced lung tumorigenesis. Mice were treated with aerosolized 3-BrPA, rapamycin, or a combination of the two agents 5 days per week, beginning 2 weeks after B(a)P injection, and continuing for 24 weeks. Lung tumor incidence was 100% in each group. All lung nodules were measured as lung adenomas (Supplementary Fig. S1). B(a)P-induced an average of 7.4 ± 1.0 tumors per mouse in the control group, and tumor load was 3.7 ± 0.6 mm³. Mice treated with 3-BrPA or rapamycin alone showed a significant decrease in both tumor multiplicity (42% inhibition, 4.3 ± 0.6 tumors; 48% inhibition, 3.8 ± 0.8 tumors) and tumor load (62% inhibition, 1.4 ± 0.5 mm³; 64% inhibition, 1.3 ± 0.3 mm³) when compared with the control group (Fig. 1B). The combination treatment further decreased tumor multiplicity (64% inhibition, 2.7 ± 0.7 tumors) and tumor load (84% inhibition, 0.6 ± 0.4 mm³). There was no significant body weight difference between vehicle control group and treatment groups (data not shown).

Our previous study has shown that aerosol delivery of 3-BrPA has the potential advantage of achieving high concentrations at the target site without hepatotoxicity. In this study, we evaluated mice for both pneumonitis and hepatotoxicity by H&E staining. We found no histologic evidence of hepatotoxicity (data not shown) and pneumonitis (Supplementary Fig. S2) in any treated group. We also measured serum ALT and AST levels in the mice after treatment with 3-BrPA, rapamycin, or combination by aerosol (Fig. 1C). The results revealed no significant differences between the aerosol control groups and aerosolized 3-BrPA, rapamycin, or combination groups. Aerosolized 3-BrPA, rapamycin, or combination showed no obvious side effects in this study.

Rapamycin increased the inhibitory effect of 3-BrPA on the viability of human lung cancer cells and induced cell apoptosis in vivo

In our previous study, we found 3-BrPA elicited significant effects on the viability of multiple human NSCLC cell lines in a dose- and time-dependent manner (13). In the present study, we investigated the effect of rapamycin on cell viability in H1299 and H23 cells. Similar to our previous findings, H1299 and H23 cells varied in their susceptibility to the toxic effects of 3-BrPA with H23 cells being more sensitive. The differential sensitivity could be a result of a different genetic and mutational background between these cell lines. Both H1299 cells and H23 cells harbor p53 mutations. However, H23 cells also have KRAS, LKB1, and PTEN mutations. Combining rapamycin treatment with 50 μmol/L 3-BrPA results in decreased cell proliferation, compared to 3-BrPA treatment alone. This combination effect showed dose dependency in both cell lines (Fig. 2A). These results showed that rapamycin can enhance the inhibitory effect of 3-BrPA on cell proliferation in vitro.

On the basis of these in vitro results, we sought to evaluate the efficacy of combination treatment with 3-BrPA and rapamycin in vivo. Tumors from animals treated with these agents were evaluated using immunohistochemical staining to evaluate the expression of proliferative markers (Ki-67) as well as markers of apoptosis. As we reported previously, we did not observe significant changes on Ki-67 in 3-BrPA-treated mice (data not shown). However, the addition of rapamycin to 3-BrPA treatment results in increased expression of cleaved caspase-3, compared with treatment with either agent alone, suggesting that the addition of rapamycin promotes apoptosis in mouse lung tumors in vivo (Fig. 2B).

Effect of rapamycin and 3-BrPA on phosphorylation of molecules downstream of the mTOR pathway

The effects of rapamycin, 3-BrPA and the combination on the mTOR pathway were investigated. As expected, treatment with rapamycin alone suppressed the phosphorylation of mTOR, which was also reflected in decreased phosphorylation of two downstream molecular targets of the mTOR pathway, p70S6k and 4E-BP-1. 3-BrPA had no significant effect on these targets, and combination of rapamycin and 3-BrPA did not cause further decrease on any of these molecular targets (Fig. 3). These results indicate that suppression of the mTOR pathway by rapamycin was not enhanced by the addition of 3-BrPA.

Effects of 3-BrPA, rapamycin, and combination on bioenergetic function in H1299 and H23 cells

3-BrPA is a glycolysis inhibitor, presumably altering the bioenergetic state of the cell. To investigate the effect of 3-BrPA on the bioenergetic state of tumor cells, alone or in combination with rapamycin, we analyzed ECAR, which is a surrogate marker for glycolysis. Baseline ECAR was measured as shown in Figure 3.

![Figure 3.](Image)

Figure 3. Effect of 3-BrPA and rapamycin on phosphorylation of mTOR and its downstream targets. H1299 and H23 cell lysates were treated with 1 μmol/L rapamycin or 50 μmol/L 3-BrPA for 24 hours. For combination, cells were incubated with rapamycin and 3-BrPA or rapamycin at the same time for 24 hours. β-Actin was used as a loading control.
Supplementary Fig. S3. H23 cells have a significantly greater ECAR than H1299 cells, indicating that H23 cells are more glycolytic, which may partially explain the greater sensitivity of H23 cells to 3-BrPA. After baseline measurements were taken, rapamycin or 3-BrPA was added to the media. 3-BrPA decreased ECAR, whereas rapamycin itself did not change the ECAR level. Interestingly, the combination of 3-BrPA with rapamycin decreased the ECAR level further. In many cell lines, cells are capable of compensating for lack of glycolytic activity by increasing oxidative metabolism, which is reflected in an increased OCR. Interestingly, when H1299 and H23 were treated with both 3-BrPA and rapamycin, they were incapable of increasing their OCR in response to treatment (Fig. 4A and B).

Effects of 3-BrPA, rapamycin, and combination on ATP level and GAPDH expression

To clarify the role of glycolysis on ATP levels in lung cancer cells, we measured the intracellular levels of ATP upon treatment with 3-BrPA, rapamycin, and the combination of both agents. Rapamycin treatment did not significantly alter cellular ATP levels in either H1299 or H23 cells (Fig. 5A). In agreement with our previous study, 3-BrPA treatment caused a significant dose- and time-dependent decrease in cellular ATP levels first observed after 30 minutes of exposure to 3-BrPA. The combination resulted in even greater ATP depletion than treatment with 3-BrPA alone in both cell lines.

This result demonstrates that rapamycin can increase the ability of 3-BrPA to block energy metabolism in these cell lines. GAPDH is a key glycolytic enzyme. Several reports showed that 3-BrPA can suppress GAPDH protein levels. In our study, we found that rapamycin alone did not change GAPDH levels. 3-BrPA, as expected, decreased the expression of GAPDH. The combination of rapamycin and 3-BrPA suppressed GAPDH expression more than the single agents (Fig. 5B). Mouse lung tissue from both control and treated groups were also examined with GAPDH staining (Fig. 5C). We found that GAPDH expression decreased in the treated group (dark brown) compared with the control- and rapamycin-treated alone groups, which is in line with our in vitro finding.

Discussion

The PI3K-Akt-mTOR signaling pathway is commonly observed in lung cancer. Dereregulated PI3K-Akt-mTOR activity

Figure 4. Bioenergetic profile of lung cancer cell H1299 or H23 treated with 3-BrPA, rapamycin or their combination. 40,000 H23 or 20,000 H1299 cells were seeded in the 96-well Seahorse tissue culture plates in complete media for 24 hours before beginning the XF-96 assay. One hour before the start of the experiment, cells were washed and changed to unbuffered assay medium adjusted to pH 7.4. After establishing the baseline OCR and ECAR, 3-BrPA, rapamycin, or combination were administered through an automated pneumatic injection port of XF96. Data, the means ± SE (n = 4/treatment group). A and B, the changes in OCR and ECAR were monitored for 4 hours. The resulting effects on OCR and ECAR are shown as a percentage of the baseline measurement for each treatment. (Measurements are reported in pmol/min for oxygen consumption and pmH/min for ECAR.)
Figure 5.
Effect of 3-BrPA, rapamycin, and combination treatment on GAPDH expression and ATP level. A, effects of combination on relative ATP level in H1299 and H23 cells. Cells were from 0 to 180 minutes, ATP levels were monitored using a luciferase-based assay. B, GAPDH expression on 3-BrPA, rapamycin, and combination in H1299 and H23 cells. Right, quantification of Western blot analysis. Values are presented as mean ± SD. C, immunohistochemical staining shows a decrease in the staining GAPDH (brown). (a, solvent control group; b, aerosol 2 mg/mL rapamycin group; c, aerosol 5 mg/mL 3-BrPA group; d, combination group).
is known to contribute to lung cancer development and maintenance. In tobacco carcinogen-induced mouse lung tumorigenesis, activation of the mTOR pathway is an important and early event (22). These critical functions of mTOR have led to the development of a number of novel compounds that target the mTOR pathway, including rapamycin and its derivatives, these compound are revealed as potent anticancer agents in several cancer types, including lung cancer, in preclinical studies or clinical trials (23–25). These clinical trials suggest that mTOR inhibitors are well tolerated and may induce tumor regression, but they appear to only have limited antitumor effects (26, 27). Therefore, using an mTOR inhibitor in combination with another anticancer agent(s) to achieve more robust tumor inhibition would appear to be a viable strategy.

3-BrPA is a novel inhibitor of glycolysis with efficacy in a variety of preclinical models (28). In our previous study, immunohistochemical studies indicated that cleaved caspase-3 staining was increased in lung tumor tissues from 3-BrPA-treated mice, consistent with a proapoptotic effect. Taken together, our previous data suggest that 3-BrPA can promote apoptosis within mouse lung tumors, consistent with the changes in mitochondrial potential shown here. Recent reports demonstrated combination of an mTOR inhibitor along with a glycolysis inhibitor could be useful for the treatment of human prostate cancer, leukemia, or neuroblastoma cells. Sun and colleagues (5) demonstrated that the combination of an mTOR inhibitor and a glycolysis inhibitor decreased the proliferation of Tsc2−/−MEFs cells in vitro and blocked tumor development following xenograft of human PTEN-deficient cancer cells in nude mice. Xu and colleagues (29) showed that the combination of an mTOR inhibitor with a glycolysis inhibitor synergistically suppressed glucose uptake and severely depleted cellular ATP pools in lymphoma and leukemia cells, leading to significant enhancement of cell killing. To our knowledge, this is the first study that demonstrated rapamycin and 3-BrPA showed enhanced chemopreventive efficacy in mouse lung cancer, and it is urgent to continue investigation of the combinatorial effects of these two agents.

Using a postinitiation protocol, we examined the effect of combining rapamycin and 3-BrPA on lung adenoma prevention in A/J mice. As expected, significantly increased inhibition of tumor multiplicity and tumor load were observed using this combination compared with either agent alone. One potential drawback of using 3-BrPA is the liver toxicity. In the present study, we did not find any hepatic toxicity as measured by serum AST and ALT levels.

Our studies demonstrate that combination of rapamycin and 3-BrPA produces cytotoxic effects in lung cancer cell lines. Rapamycin suppressed the phosphorylation of mTOR in the cells and also suppressed the phosphorylation of molecules downstream of mTOR, including P70 S6K and 4E-BP-1. In contrast, inhibition of the glycolytic pathway significantly blocked ATP generation in lung cancer cells, which are highly dependent on glycolysis for energy supply, and produced a cytotoxic effect. Because the mTOR pathway plays an important role in promoting cellular nutrient uptake, cell growth and cell survival, combination of mTOR and glycolysis inhibition seems to be an attractive mechanism-based strategy to severely impair cancer cell energy metabolism and effectively kill malignant cells. Indeed, our data showed that combination of rapamycin and 3-BrPA synergistically decreased ECAR and cellular ATP levels, leading to synergistic cell killing. In summary, our findings have demonstrated the synergistic effects of glycolysis inhibition and mTOR inhibition in vitro and in vivo. The evidence for the importance of the glycolytic pathway in lung tumor cell growth and for the demonstrated efficacy of the combination of 3-BrPA and rapamycin in these studies provides a strong biologic and clinical rationale for further investigation of the role of inhibition of glycolysis in the treatment of lung cancer.

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

Authors’ Contributions
Conception and design: Q. Zhang, Y. Wang, M. You
Development of methodology: Q. Zhang, J. Pan, B. Kalyanaraman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q. Zhang, J. Pan, S.M. Korman, Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Zhang, J. Pan, S.M. Korman
Writing, review, and/or revision of the manuscript: Q. Zhang, J. Pan, R.A. Lubet, Y. Wang, M. You
Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): Y. Wang, M. You
Study supervision: M. You

Grant Support
This work was supported in part by NIH grants: R01CA139959, R01CA134433, R01CA175360, N01CN201200013(7), N01CN201200015(1), (to Y.W. and M.Y.) and R01 CA152810 (to B.K.), this work was also supported by the MCW Cancer Center Bioenergetics shared resource and Advancing Healthier Wisconsin.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 29, 2014; revised January 16, 2015; accepted January 22, 2015; published OnlineFirst February 2, 2015.

References
Enhanced Antitumor Activity of 3-Bromopyruvate in Combination with Rapamycin \textit{In Vivo} and \textit{In Vitro}

Qi Zhang, Jing Pan, Ronald A. Lubet, et al.