Glycolytic Enzyme Inhibitors Affect Pancreatic Cancer Survival by Modulating Its Signaling and Energetics

VIKAS BHARDWAJ, NISHA RIZVI, MARIA B. LAI, JAMES C.K. LAI and ALOK BHUSHAN

Department of Biomedical and Pharmaceutical Sciences, ISU Biomedical Research Institute, College of Pharmacy, Idaho State University, Pocatello, ID, U.S.A.

Abstract. *Background and Aim: The importance of glycolysis in cancer cells is well documented. The effects of inhibiting glycolysis using metabolic inhibitors iodoacetate (IAA), an inhibitor of GAPDHase, and 3-bromopyruvate (3BP), an inhibitor of hexokinase-II, on survival and signaling of pancreatic cancer cells (Panc-1) were investigated. Materials and Methods: Cellular survival was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Lactate dehydrogenase (LDH) assay was used to analyze the induced necrosis and protein levels were evaluated using Western blot analysis. Results: The results show that the inhibitors lowered cellular survival and increased cellular necrosis. Mitogenic signaling pathways were affected by 3BP but not by IAA. Conclusion: We conclude that there may be a cross-talk between signaling pathways and glycolysis in regulating pancreatic cancer cell survival and signaling. Thus, a combination of agents that inhibit both energy production and cell signaling may provide a novel and effective approach to target pancreatic cancer effectively.*

Pancreatic cancer is one of the most morbid forms of cancer with a low mean 5-year survival rate of about 4-5% (1, 2). With around 33,680 deaths in 2008, pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States (USA). Due to lack of effective therapeutic options, the survival rate has not improved significantly over the last two decades (3). Cigarette smoking, chronic pancreatitis, age, diet and occupational exposure are some of the risk factors of this form of cancer (4, 5)). The exocrine form of pancreatic cancer characterized by mutations in the K-ras gene at codon 12, 13 or 16 is responsible for around 90-95% of all reported cases (6, 7). *K-ras* mutation is one of the earliest mutations observed in pancreatic adenocarcinomas (8). In addition to K-ras, expression of growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGFα), fibroblast growth factor (FGF) and growth factor receptors such as EGF receptor 2 (erbB-2) and TGF receptor (TGFR) β 1-3 are also augmented in pancreatic cancer cells (9). Certain tumor suppressor genes such as *p16, p53* and deleted in pancreatic cancer locus 4 (*DPC4*) are found to be either mutated or down-regulated in pancreatic cancer (7, 8). Activation of K-ras results in proliferation, differentiation, cell cycle progression and inhibition of apoptosis of the cancer cells mediated by downstream proteins such as extracellular receptor kinase (ERK) and protein kinase B (PKB or Akt) (10).

In 1926, Otto Warburg hypothesized that cancer cells rely on glycolysis for their energy production. According to this hypothesis, cancer cells derive around half of their energy from the glycolytic pathway and the tricarboxylic acid (TCA) cycle activity is suppressed (11). Various investigators have tested this hypothesis and found that glycolysis is up-regulated and oxidative phosphorylation is decreased in cancer cells (12). The up-regulation of glycolysis can be viewed as a mechanism to increase energy production by the cancer cells *via* this pathway. This phenotype acquired by the cancer cells is known as the glycolytic phenotype. Mitogenic signals, a hypoxic environment and mutations of glycolytic or TCA cycle enzymes have been suggested to be responsible for these biochemical adaptations (13-15). Glycolytic enzymes such as hexokinase II (HK II), glyceraldehyde-3-phosphate dehydrogenase (GAPDHase), aldolase, phosphofructokinase (PFK), enolase and pyruvate kinase have been shown to be up-regulated in various cancer forms (13). Glycolytic enzymes such as hexokinase and GAPDHase perform additional function of regulating apoptosis within the cell (16). Hexokinase inhibits apoptosis by modulating apoptotic proteins such as Bax, Bad and Bak (16). Additionally, glycolytic enzymes have a multifaceted role in cancer progression (16).

In light of the considerations above, there is clearly the possibility that inhibition of glycolysis will result in decreased proliferation of cancer cells and hence reduce

Correspondence to: Alok Bhushan, Department of Biomedical and Pharmaceutical Sciences, ISU Biomedical Research Institute, College of Pharmacy, Idaho State University, 921, South 8th Ave., Pocatello, Idaho 83209-8334, U.S.A. e-mail: abhushan@otc.isu.edu

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tumor progression. Glycolytic inhibitors such as 3-bromopyruvate (3BP, a selective inhibitor of hexokinase II) and 2-deoxyglucose (a glucose analog) have been tested *in vivo* and *in vitro* and have been found to be effective in decreasing the proliferation of various forms of cancer. 3BP is currently in preclinical trials and has shown promising results (13).

Iodoacetate (IAA) selectively inhibits GAPDHase, however, its anticancer properties have not been fully investigated. We hypothesize that 3BP and IAA differentially inhibit pancreatic cancer cell survival by modulating energetics as well as the signaling mechanisms involved in cancer cell proliferation. To test this hypothesis, we performed a dose- and time-related cell survival assay upon treatment of Panc-1 cells with these inhibitors to analyze the cytotoxic effect of these inhibitors. Lactate dehydrogenase (LDH) release assay (marker of necrosis) was carried out to identify the mechanism of cell death. Western blot analyses were performed to determine the effects of 3BP and IAA on the signaling pathways responsible for cellular proliferation and anti-apoptotic effect.

Materials and Methods

Pancreatic cancer cell line (Panc-1) was obtained from the American Type Tissue Collection, Manassas, VA, USA. RPMI-1640, fetal bovine serum (FBS) and trypsin-EDTA were purchased from Atlanta Biologicals, GA, USA. Glycolytic inhibitors IAA and 3BP and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich, MO, USA. Protease inhibitor cocktail tablets and antibodies (K-Ras, Erk 1, phosphor-Erk 1, beta-actin, Akt, phospho-Akt) were purchased from Santa Cruz Biotechnology, CA, USA. Mamalian target of rapamycin (mTOR) and phosphor-mTOR antibodies were obtained from Cell Signaling Technology, Danvers, MA, USA.

Cell culture. Panc-1 cells were cultured in RPMI-1640 supplemented with 10% fetal FBS at 37˚C (5% CO_2) in an incubator (Nuaire, CO_2 Air Jacketed Incubator).

Cell survival assay. The MTT assay was used to determine the effect of 3BP and IAA on pancreatic cancer cell survival. A total of 2,500 cells were seeded per well in a 96-well plate and incubated for 48 hours with different concentrations of 3BP (0.05-1000 μM) and IAA (0.05-1000 μM). After incubation, 20 μl MTT (5 mg/ml in phosphate-buffered saline; PBS) reagents were added to each well. After incubation with MTT for 4 hours, the medium was removed, the formazan product was solubilzed in 100 μl dimethyl sulfoxide (DMSO) and the optical density was read in a BioTek plate reader at 570 nm. For control, cells were treated with 20 μl PBS. The experiments were performed in triplicates and analyzed for statistical significance.

Preparation of cell lysates. Cells (1.5×10^6) were seeded in a T-75 tissue culture flask with RPMI-1640 medium containing 10% (v/v) FBS. The cells were allowed to adhere to the bottom of the flask by incubating them at 37˚C for one hour. Cells were treated with three concentrations of each inhibitor: 2 ($<IC_{50}$), 3 (IC_{50}) and 10 μM ($>IC_{50}$) for IAA, and 17 ($<IC_{50}$), 20 (IC_{50}) and 40 μM ($>IC_{50}$) for 3BP. The treated cells were incubated for 48 hours at 37˚C and the cell lysates were then prepared (17). Briefly, the cells in the flask were washed three times with cold PBS and 600 μl of lysis solution (lysis buffer (1% (v/v) Triton X-100, 10 mM Tris base pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% (w/v) sodium azide, 0.5 μM phenyl methyl sulfonyl fluoride, 0.2 μg aprotinin, 0.4 μg leupeptin, 100 μg sodium orthovandate, and distilled water at pH 7.6) + protein cocktail solution) was added. The cells were collected by scraping and the mixture sonicated using a probe sonicator with six bursts. The mixture was then centrifuged at 10,000 × g for 10 minutes. The resultant supernatant was separated and diluted with 300 μl of Laemmli's buffer (3×). The Bradford assay was used to determine the protein concentration before addition of Lamelli's buffer and the samples normalized for protein concentration.

Western blot analysis. The lysates were subjected to Western blot analysis to determine the effect of the inhibitors on the expression of K-ras and phosphorylation of ERK1, Akt and mTOR. The analysis was performed as described elsewhere (17). Briefly, 25 μg protein were separated using a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membrane. The membrane was blocked with 5% milk and 3% bovine serum albumin (BSA) solution in tris-buffered saline tween-20 (TBST) for 4 hours. The membrane was rinsed three times with TBST followed by incubation with the primary antibody overnight. The membrane was then washed and treated with horseradish peroxidase-conjugated secondary antibody (1:8,000) with 5% milk in TBST. The immunoreactive proteins were visualized with chemiluminescence detection kit as described by Thermo Fisher Scientific, Rockford, IL. β-Actin was used as the loading control. The experiments were performed in triplicates and analyzed for statistical significance

Lactate dehydrogenase (LDH) release assay. Cells (1.5×10^6) were seeded onto each T-75 flask with culture medium and allowed to adhere to the bottom of the flask. The flasks were treated with different concentrations of IAA (2-20 μM) or 3BP (10-50 μM) for 48 hours at 37˚C. After the treatment, the medium was collected from each flask and stored at –80˚C. The cells were collected using 8 ml of 0.9% (w/v) NaCl and centrifuged. The supernatant was discarded and the pellet was stored at –80˚C.

LDH release from cells was used as an indicator of necrosis. The cell pellet and the collected samples of medium were tested for LDH activity according to the method described by Clark and Lai (18). For assaying LDH activity in the medium, Triton X-100 was not added and the volume was made up with water. The cells were homogenized using 1 ml 0.9% NaCl. Activity of LDH was determined kinetically by measuring the oxidation of NADH. The experiments were performed in triplicates and analyzed for statistical significance. The percentage of LDH release was calculated according to the formula below:

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium}}{\text{Total LDH activity}} \times 100$$

Total LDH activity is equal to LDH activity in medium + LDH activity in cells.

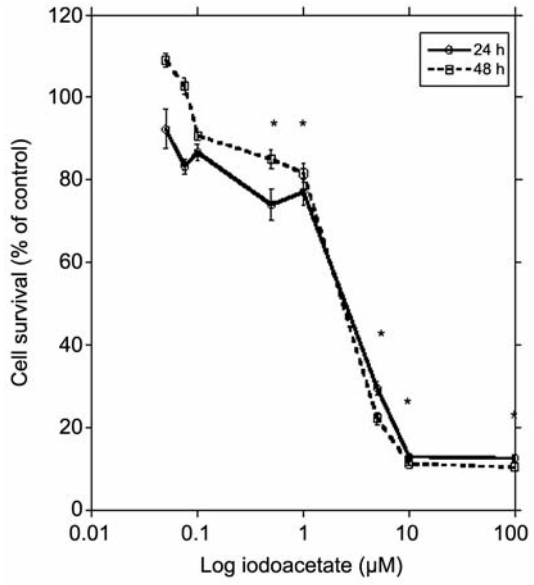

Figure 1. *2500 Panc-1 cells/well were seeded in 96-well plate and treated with IAA for 24 and 48 hours. Cell survival was analyzed using MTT assay. The graph shows that IAA reduce Panc-1 cell survival in a dose-dependent manner, reaching IC_{50} at around 3 μM. The cell inhibitory effect of IAA was not found to be time dependent. All data points correspond to mean survival±SEM for a minimum of 3 determinations. *p<0.01 compared to control.*

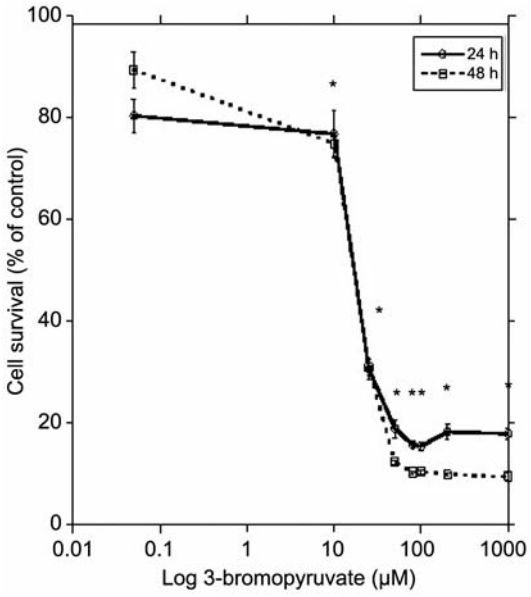

Figure 2. *2500 Panc-1 cells/well were seeded in 96-well plate and treated with varying concentrations of 3BP for 24 and 48 hours and cell survival was analyzed using MTT assay. The graph shows that 3BP reduces the Panc-1 cell survival in a dose-dependent manner reaching IC_{50} at around 20 μM. The cell inhibitory effect of 3BP was not found to be time dependent at lower doses but time differential effect is observed at concentration of 40 μM and above. All data points corresponds to mean survival±SEM for a minimum of 3 determinations. *p<0.01 compared to control.*

Cellular morphology. The morphology of Panc-1 cells upon treatment with inhibitor(s) for 48 hours at 37˚C as described above was compared to that in the untreated cells using bright field microscopy. Bright field images of cells were acquired using a Leica light microscope (Leica DM IRB, Bannockburn, IL, USA)} equipped with a digital camera (Leica DFC 300FX) at a final magnification of ×630.

Statistical analysis. Cell survival data were analyzed by one-way ANOVA (KaleidaGraph version 4.03, Reading, PA, USA). ANOVA was followed up with Tukey's HSD *post-hoc* test with alpha level of $p<0.01$ for all comparisons. The protein expression and LDH release data were analyzed using one-way ANOVA (KaleidaGraph 4.03), followed by Dunnett's *post-hoc* analysis with alpha level of $p<0.05$. KaleidaGraph 4.03 was also used to calculate standard error of mean and then to plot the graphs.

Results

3BP and IAA induced concentration-related decreases in survival of Panc-1 cells. 3BP and IAA reduced the survival of Panc-1 cells in a dose-related manner, IAA was more potent, with an IC_{50} ~3 μM compared to the IC_{50} of ~20 μM for 3BP (data not shown).

To investigate the time-dependent effect of the inhibitors, the cells were incubated for 24 or 48 hours with IAA or 3BP before adding the MTT reagent. The inhibitory effect of IAA was not time dependent (Figure 1). Similarly, the inhibitory effect of 3BP was not time-dependent at lower concentrations, but at 40 μM and above, a time-dependent effect was observed (Figure 2).

Glycolytic enzyme inhibitors differentially modulate pancreatic cancer cell signaling. To assess if glycolytic enzyme inhibitors modulate pancreatic cancer cell signaling, expression of signaling proteins was analyzed. 3BP reduced the expression of K-ras at 20 and 40 μM, whereas IAA had no effect on K-ras expression at the concentrations tested (Figure 3a). Furthermore, we analyzed the effect of these inhibitors on the phosphorylation of three important downstream signaling molecules of ras namely, Erk1, Akt and mTOR. As shown in Figure 3b, IAA and 3BP did not affect the phosphorylation of ERK1 but the phosphorylation of Akt and mTOR decreased significantly at 40 μM 3BP (Figure 3c, d). However, treatment of Panc-1 cells with IAA did not have any effect on phosphorylation of Akt and mTOR.

3BP and IAA induce cellular necrosis at higher concentrations. LDH release assay was performed to elucidate the cytotoxic effects of IAA and 3BP on Panc-1 cells. The LDH release assay indicates that necrosis was one of the cell death mechanisms induced by the glycolytic

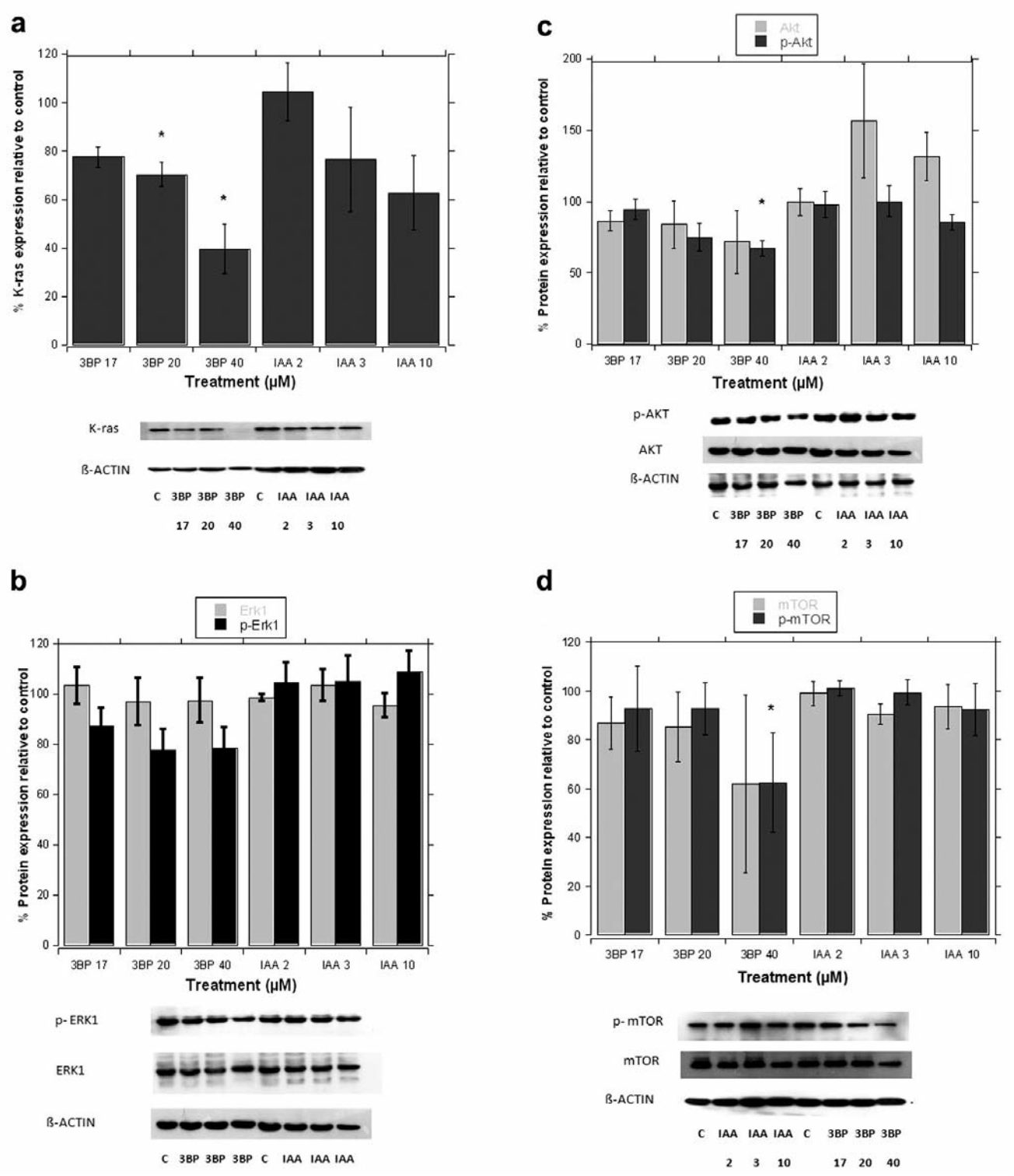

Figure 3. *Panc-1 cells were seeded in a T-75 culture flask and treated with IAA and 3BP at different concentrations. Lysates were prepared and the protein expression was analyzed using Western blot analysis. a, K-ras expression decreases significantly on treatment of cells with 20 and 40 μM BP. IAA does not have any effect on K-ras expression. b, Neither of the inhibitors were able to reduce the phosphorylation of Erk1 significantly. c, 3BP significantly reduced the phosphorylation of Akt at 40 μM concentration. IAA on the other hand had no effect on the same. d, 3BP significantly reduced the phosphorylation of mTOR at 40 μM concentration. IAA on the other hand had no effect on the same. The blots are representative and the data are represented as the mean±SEM for 3 determinations. β-Actin was used as loading control. *p<0.05 compared to control.*

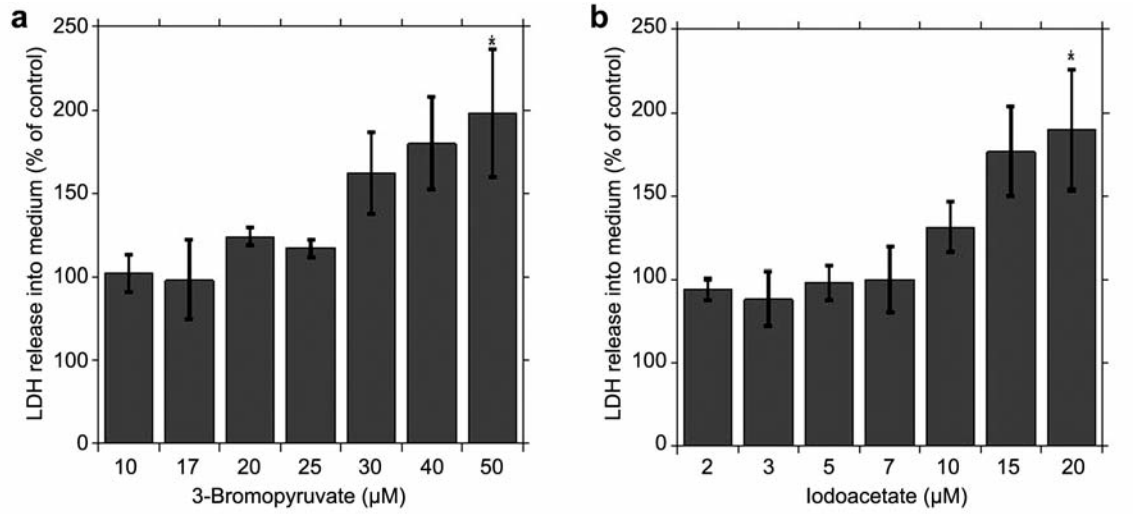

Figure 4. *2×10^6 cells were seeded in T-75 cell culture flasks. The cells were treated with different concentrations of 3BP (a) and IAA (b). After the incubation time, the media and the cells were collected separately and the LDH activity in them was analyzed. LDH activity corresponds to the level of LDH and from that percentage LDH released into the media was calculated. The graphs show that at higher treatment concentrations of 3BP and IAA there is a dose-dependent increase in the LDH release suggesting that at higher doses, the extent of necrosis increases. The data are represented as mean percentage LDH release into the medium±SEM of 3 determinations. *p<0.05 compared to control.*

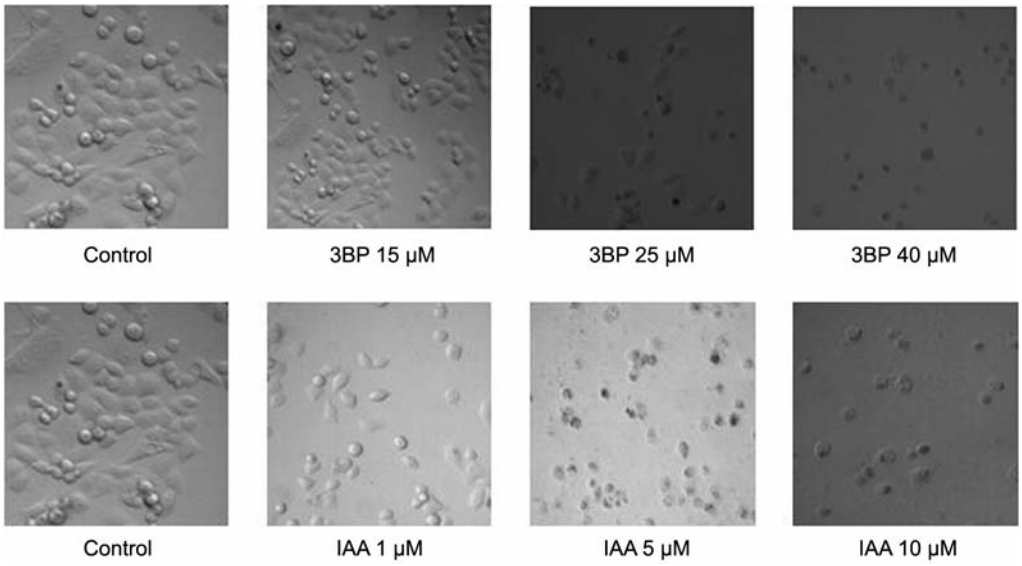

Figure 5. *The change in the morphology of Panc-1 cells was analyzed using bright field microscopy. We observed that with increasing concentrations, there was a decrease in the number of live cell, which supports our MTT data. The cytoplasm of the treated cells is opaque with prominent nuclei.*

inhibitors (Figure 4). LDH release was elevated when the cells were treated with higher concentrations of the inhibitors, suggesting an increase in necrosis under these conditions. The release of LDH into the medium of treated as compared to control cells was significantly higher at a concentration of 50 μM for 3BP and 20 μM for IAA. At these concentrations, more than 90% of total cellular LDH was released into the medium (data not shown).

Cellular morphology. As shown in Figure 5, the morphology of the Panc-1 cells changed on treatment with the inhibitors. As the concentration increase, the plasma membrane lost its integrity and the cytoplasm became opaque at higher treatment concentrations. The number of cells in each field also decreased noticeably. These observations correlated with the results of the MTT assay, where the number of surviving cells decreased with an increase in the inhibitor concentration.

Discussion

In this report, we focused on targeting the metabolic adaptations occurring in cancer cells to reduce their growth and survival. In the 1920s, the Warburg hypothesis, which discusses the metabolic alterations which occur inside the tumor cells to adapt to its microenvironment was proposed. One of these many metabolic alterations is an increased dependence of cancer cells on glycolysis for their energy requirements (11, 12). In this study, we analyzed the effect of two glycolytic inhibitors namely, IAA and 3BP on the survival and signaling of pancreatic cancer cells. Our study is unique in two respects, firstly, no other study has investigated the effect of these two inhibitors on pancreatic cancer cells and secondly, it is the first study to carry out a comparative analysis regarding the efficiency of the two inhibitors in inhibiting cancer cell survival. The results suggest that these inhibitors differentially inhibit the survival of pancreatic cancer cells (Panc-1), with IAA being more potent than 3BP. Necrosis was found to be the major cell death mechanism induced by these inhibitors at higher doses. Pancreatic cancer cell signaling was observed to be affected by 3BP treatment.

3BP and IAA are two enzyme inhibitors acting at different levels of the glycolytic pathway. 3BP is an inhibitor of hexokinase II, which converts glucose into glucose-6-phosphate (G6P) (19, 20). G6P is converted to glyceraldehyde 3-phosphate by the action of the pentose phosphate shunt known to be up-regulated in various forms of cancer. High levels of G6P inhibit the activity of enzyme hexokinase by feedback inhibition thus preventing the influx of glucose into the glycolytic pathway. Hence, by up-regulating pentose phosphate shunt, cancer cells prevent G6P induced inhibition on hexokinase (13). 3BP, which is also suggested to inhibit cancer cell survival by alkylating DNA, has shown to be effective in both *in vivo* and *in vitro* studies (13, 20 and 21).

IAA is a glycolytic enzyme inhibitor acting on GAPDHase, which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate (20). GAPDHase has been shown to be involved in cellular motility and transcriptional regulation along with its role in glycolysis (16). The expression of GAPDHase has also been found to be elevated in pancreatic cancer cells (22, 23). The anticancer properties of IAA have not been fully elucidated.

Our results demonstrate that while 3BP did not affect the phosphorylation of ERK, it did decrease the phosphorylation and consequent activation of AKT. mTOR, which is a downstream regulator of Akt, was also found to be reduced by 40 μM 3BP. IAA however, did not alter the phosphorylation of EKR, Akt, or mTOR. These results suggest that 3BP reduces pancreatic cancer cell survival by modulating energetics (glycolysis) as well as signaling. IAA, however, did not show any effect on pancreatic cancer signaling suggesting that inhibition of GAPDHase affects cancer cell survival by altering its energetics and perhaps not its signaling machinery.

In addition to the afore mentioned proteins, we were also intrigued to study the effect of the inhibitors on the K-ras protein. This interest arose from the literature suggesting that 90-95% of reported pancreatic cancer cases show a mutation in the gene encoding for it. We observed that 3BP reduced the expression of total K-ras protein in Panc-1 cells, which can be viewed as one of the mechanisms responsible for reduced survival of pancreatic cancer cells.

Our next aim was to investigate the cell death mechanism induced by the inhibitors. Previously, 3BP has been shown to stimulate apoptosis at low concentrations, but has a more prominent necrotizing effect at higher concentrations (24). Our results, using LDH release as a marker of necrosis, confirms previous studies. We observed a dose-related increase in the release of LDH into the medium after treatment with both glycolytic inhibitors (Figure 4a, b). Given that cell death *via* necrosis is triggered by energy failure, our results suggest that these inhibitors are able to block the energy production pathways in the cells and thus induce cellular necrosis.

Our study is the first to evaluate the effect of IAA and 3BP on pancreatic cancer cell survival, signaling and energetics. Our study suggests that these inhibitors act at multiple levels to reduce the survival of pancreatic cancer cells. Firstly, they prevent the activation of anti-apoptotic proteins such as Akt and mTOR and secondly, they induce energy failure in the cells leading to cell death *via* necrosis. The multiple target approach of these inhibitors might be especially useful in pancreatic cancer due to its highly aggressive nature and lack of effective therapies for its treatment. This study can be viewed as a platform for establishing the anticancer properties and evaluation of toxicological properties of IAA and 3BP as proof of concept drugs that block glycolysis for effective treatment of pancreatic cancer, either alone or in combination.

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Conflict of Interest

The Authors have no conflict of interest to declare.

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