Targeting Metabolism in Brain Tumors

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INTRODUCTION:

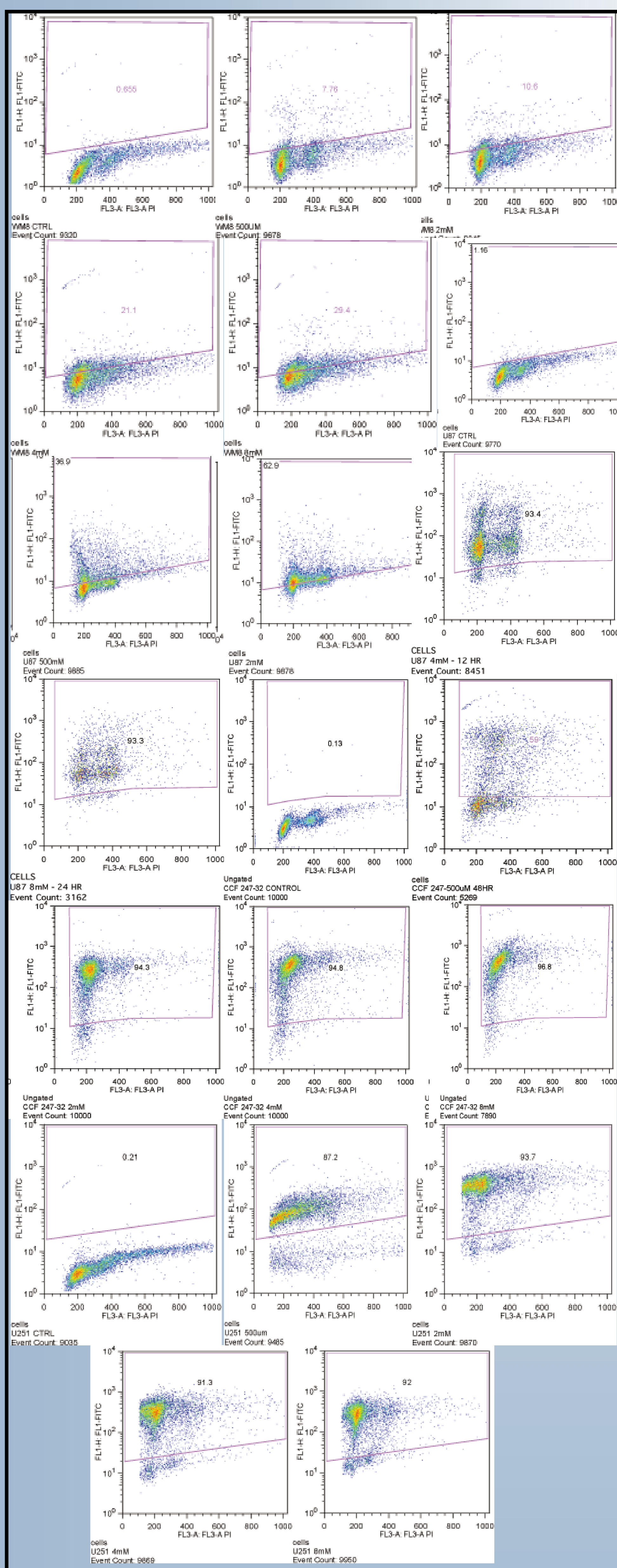
According to Otto Warburg, irreversible cellular respiratory damage causes cancer cells to depend entirely upon aerobic glycolysis [1]. This is a hallmark of most cancers including malignant brain tumors. Brain tumor cells are known to have dysregulated glycolysis and cellular respiration. He attributed this enhanced glycolytic activity to metabolic alterations in the mitochondrial respiratory chain. This is considered the most fundamental alteration in malignant transformation or the origin of cancer cells. Among the possible mechanisms, mitochondrial malfunction and hypoxia in the tumor micro-environment are considered the two major factors contributing to the Warburg effect. We propose that it is possible to control the growth/progression of malignant brain cancer through changes in the metabolic environment.

1) Selak MA *Cancer Cell.* 2005;7:77–85. doi: 10.1016/j.ccr.2004.11.022.

METHODS:

Commercial Glioblastoma (GBM) cell lines U87, U251, LN229, and CCF 247; a primary line obtained from our brain tumor tissue bank, were used for in vitro studies. Normal human astrocytes (HA) were also evaluated in vitro and obtained from normal brain from epilepsy surgery patients. Cells were treated with 3-Bromopyruvate (3-BrPA), which is a lactate/pyruvate analog which selectively depletes ATP and inhibits Hexokinase-2 dependent aerobic metabolism. We evaluated the effects of 3-BrPA treatment in a dose dependent manner with use of TUNEL (DNA Flow Cytometric Analysis Kit, Roche Diagnostic Corp) and flow cytometry (Becton Dickinson FACScan, San Jose, Ca.). Caspase activity was measured directly from the 96-well plate using the Apo-ONE Homogeneous Caspase-3 assay according to the manufacturer's protocol (Promega, Madison, WI).
Institutional animal facility approval was obtained for in vivo studies. U87 and LN229 cells were implanted in the flank of a total of 12 athymic nude rats. Ten days later, rats were treated with either 3-BrPA or Phosphate Buffer Saline (PBS) by direct tumor injection every forty eight hours for two weeks. Tumors were measured every forty eight hours and tumor volume was calculated.

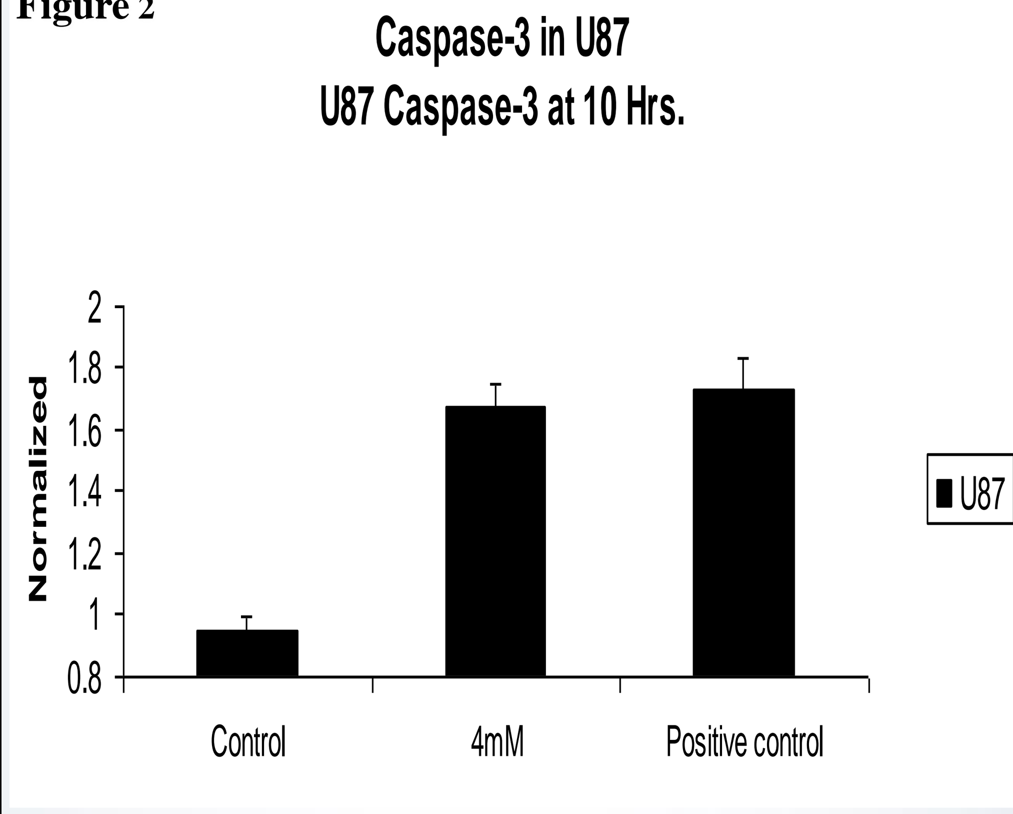
Figure 1



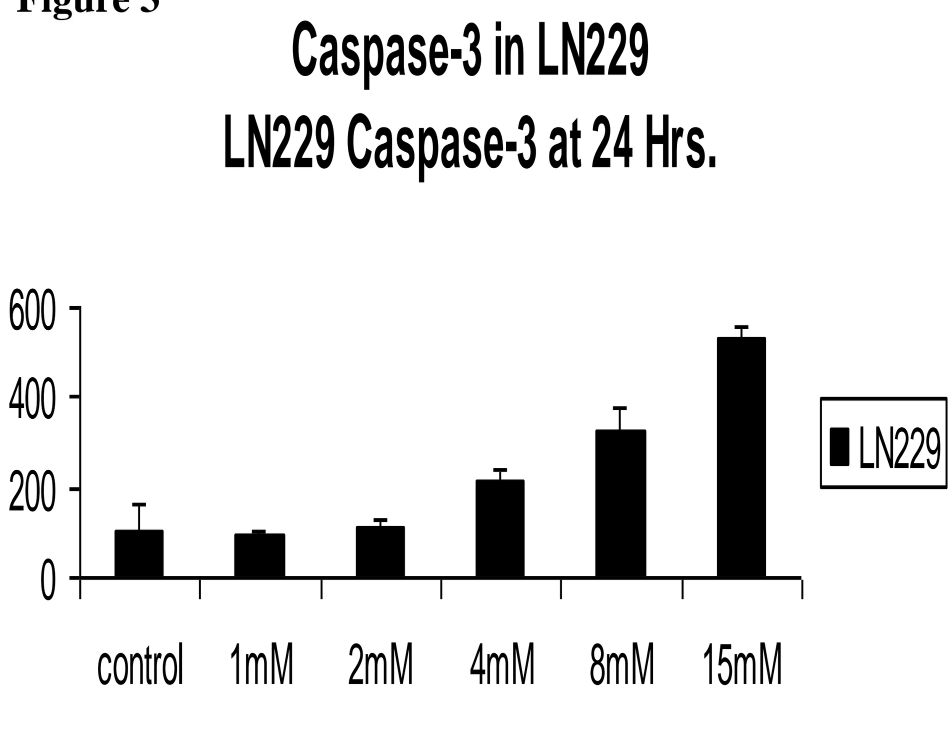
TUNEL Analysis Using 3-BrPA Escalation Dose in Human Glioblastoma (GBM) Cell Lines and White Matter (WM)
3-BrPA is an inhibitor of Hexokinase-2, which blocks the use of glucose as a source for the metabolism of energy. We were able to show >90% apoptosis in commercial GBM Cell lines U87, LN229, U251 and primary GBM cell line CCF 247 compared to WM which was only around 20%.

RESULTS:

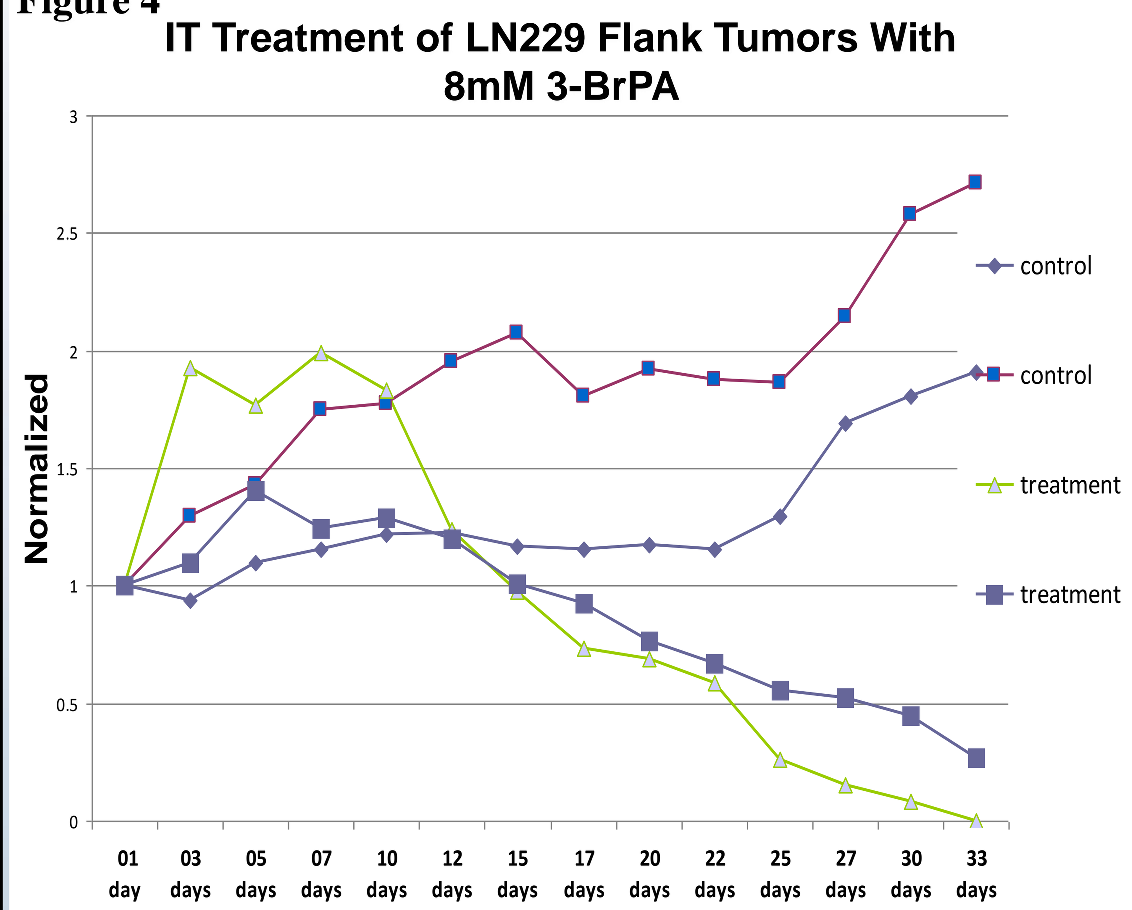
Cells were treated with 3-BrPA at doses ranging from 500 micromolar to 15 millimolar for 48 hours. TUNEL analysis showed evidence of apoptosis in more than 95% of GBM cells (all cell lines) following 3-BrPA treatment at any dose, compared to HA which showed 20% apoptosis (P<0.005).
To investigate the possibility of early apoptosis, we used the Caspase-3 assay in LN229 and U87. We concluded in this assay that by using either 4 or 8 millimolar 3-BrPA, we were able to show an increase of Caspase-3 in these cell lines compared to the controls (P<0.005).
Western blot analysis showed activation of BAD after the use of 3-BrPA in the glioma cell lines.
In vivo experiments showed decreased tumor size in treated rats over the course of injections. Tumor size increased in control group. After stopping treatment, tumors have continued to decrease in size in the treated animals and have not returned after three months.



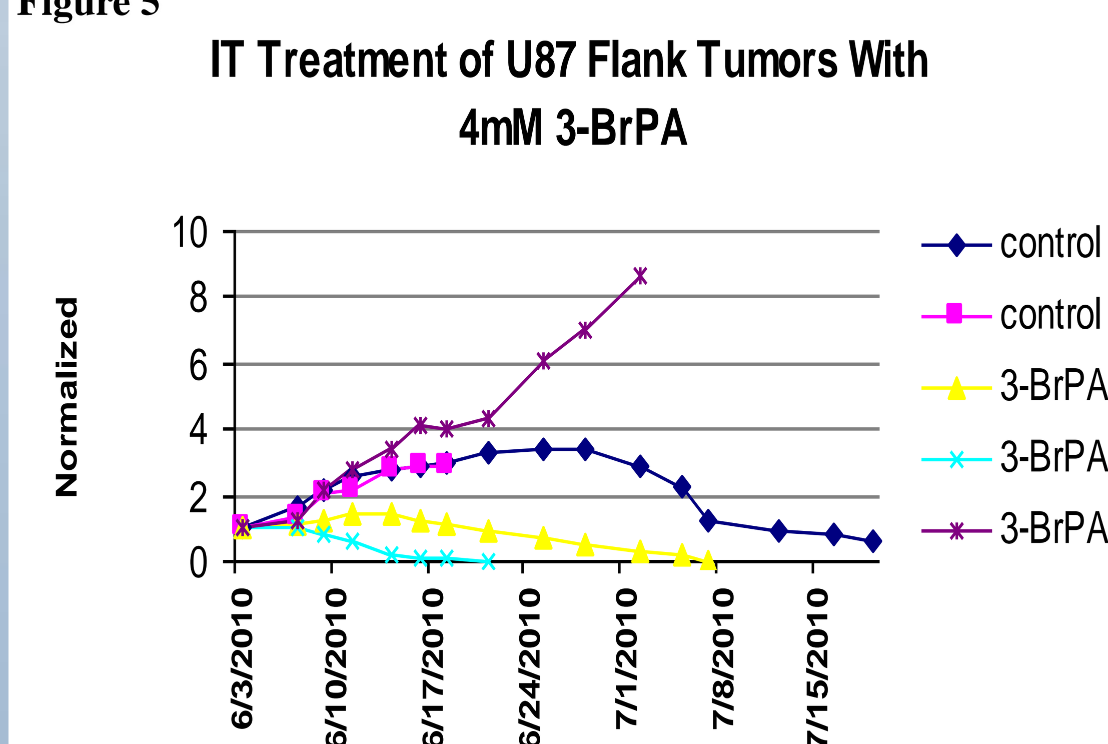
Caspase-3 elevation after 10 hours showing early apoptosis in U87 compared to the normalized control using 4 mM 3-BrPA.



Escalation dose response showing an increase of Caspase-3 at 24 hours in LN229 cells treated with 3-BrPA.



Animals were followed for 3 weeks in a randomized prospective analysis of an LN229 flank tumor rat model. Results are shown for local tumor injection of 8mM 3-BrPA in PBS or plain PBS every 3 days for a total of 7 injections.



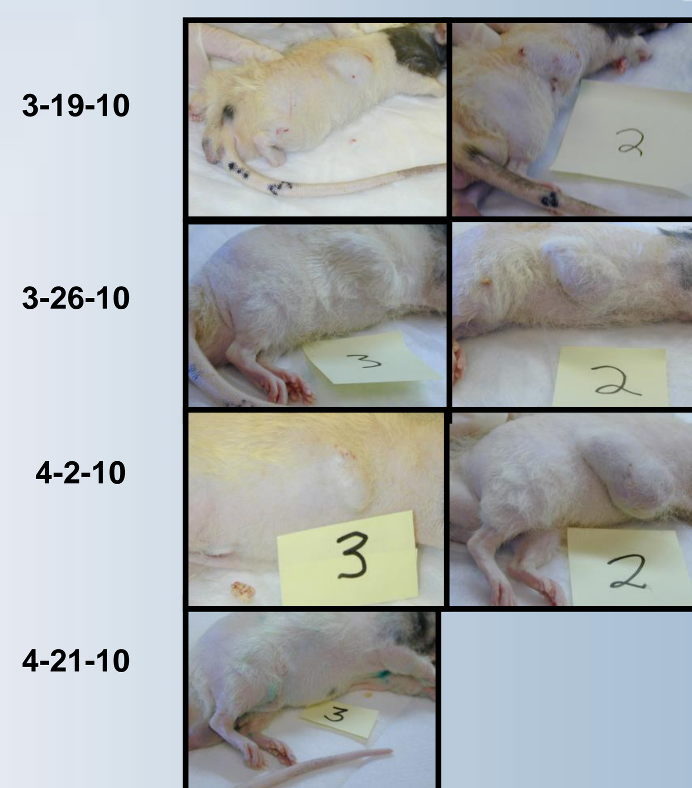
Animals were followed for 2 months in a randomized blinded prospective analysis of a U87 flank tumor rat model. Results are shown for local tumor injection of 4mM 3-BrPA in PBS or plain PBS every 3 days for a total of 7 injections.

RESULTS CONTINUED:

Figure 6

IN-VIVO EXPERIMENT

Rat # 3 Treated
Rat # 2Control



Animals were followed for 6 weeks over the course of treatment. The tumor in treated animal disappeared completely after 4 weeks of intra-tumoral injections.

DISCUSSION:

The initial question in this project was to determine if 3-BrPA was able to induce apoptosis (programmable cell death). To answer that question, we used a series of commercial Glioblastoma cell lines, U87, LN229, and U251 and a primary GBM cell line that we obtained from our brain tumor tissue bank collected from patients who had surgery here at the Cleveland Clinic; CCF 247.
We compared that with a white matter cell line obtained from normal brain from an Epilepsy patient. We began by looking for apoptosis using the TUNEL assay with the cell lines mentioned above using dose response to evaluate the percentage of cell death compared to the controls (see figure 1). In these experiments we were able to conclude that a dose of 4 millimolar to 8 millimolar was able to produce efficient apoptosis in more than 90% of the GBM cell lines. These doses affected around 20% of the while matter cells. To evaluate the effect of early apoptosis, we re-evaluated this phenomenon using the Caspase-3 assay in the two cell lines that we would subsequently use in our in vivo work; LN229 and U87 (figures 2 and 3). We concluded in this assay that by using 4 millimolar and 8millimolar 3-BrPA we were able to show an increase of Caspase-3 in U87 and LN229 respectively.
Previous papers showed that the maximum tolerated toxicity dose of 3-BrPA in rats is 15 millimolar. Based on this information and our conclusion of the dose effect of this drug in the cell lines, we proceeded to establish an in vivo rat flank tumor model to translate our results from in vitro to in vivo. We did two pilot experiments, one with LN229 and the other with U87. We collected $3x10^6$cells and implanted them using Matrigel into the right flank of each rat. We divided them into two groups, a control group which was treated with intra-tumoral injections of PBS for seven treatments every three days. The treatment group was injected with 3-BrPA in a PBS vehicle (figures 4, 5 and 6).
We collected protein from the tumors that resulted from the in vivo experiment and also from the cell lines, including cells treated with 4, 8 and 15 millimolar 3-BrPA. An increase of BAD apoptotic protein was present in western blot analysis (data not shown).

CONCLUSIONS:

Animals treated with 3-BrPA showed tumor regression without apparent toxicity, neurologic deficits or recurrence. These findings attest to the feasibility of completely destroying advanced, highly glycolytic brain tumors. We feel that the possibility of controlling the progression of brain tumors through changes in the metabolic environment should be studied in depth. We will continue to explore the potential impact this could have on treating brain tumors in both children and adults.

ACKNOWLEDGEMENTS:

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