In Vitro Effects of Boron-Containing Compounds upon Glioblastoma Cells (44196)

SARAH F. BAILEY,* GEORGE W. KABALKA,† AND JOSEPH E. FUHR*,[1]

Departments of Pathology and Radiology,† University of Tennessee Medical Center, Knoxville, Tennessee 37920-6999*

Abstract. **Boron-neutron capture therapy (BNCT) is currently under investigation as a novel therapeutic modality for glioblastoma. This study was undertaken to determine whether boron-containing compounds 4-borono-2-fluoro-D,L-phenylalanine (FBPA) and FBPA-fructose have direct effects upon kinetics of A172, a glioblastoma cell line. Flow cytometry analyzed cell-cycle distribution and S-phase kinetics (bromo deoxyuridine [BUdR] incorporation). BUdR incorporation was increased during a 1-hr pulse after 24-hr or 72-hr exposure of cells to varying concentrations of FBPA or FBPA-fructose. Results suggest that boron-containing compounds may effect cell kinetics apart from neutron activation, and this effect should be further evaluated for potential impact upon tumor responsiveness to BNCT.** [P.S.E.B.M. 1997, Vol 216]

Boron-neutron capture therapy is a binary methodology for the treatment of certain malignancies (1). Efficacy of treatment is dependent upon uptake, and even localization of an administered boron-containing compound within the targeted tissue followed by neutron beam irradiation of the tissue with consequent activation of the boron nucleus. Melanoma (2) and glioblastomas (3) are the two principal malignancies for which this treatment approach has been tested. This study was designed to evaluate *in vitro* whether two boron-containing compounds exert any influence on cell kinetics independent of this neutron beam exposure. This study was considered particularly appropriate in light of reports that uptake of boron-containing compounds was elevated in tissues with increased levels of DNA synthesis as measured by [^{3}H]thymidine uptake (4).

Materials and Methods

A172 is a human glioblastoma cell line obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in this laboratory at 37°C as monolayers grown in modified Dulbecco's medium, supplemented with penicillin, streptomycin, gentamicin, bicarbonate, and 1% nonessential amino acids. Regular media also contained 8% heat-inactivated fetal calf serum. For assay of 4-borono-2-fluoro-D,L-phenylalanine (FBPA) (Fig. 1) and FBPA-fructose activity, cells were transferred to Dulbecco's modified Eagle's medium, which is phenol red free. This was done to eliminate the weak estrogenic effect associated with phenol red (9).

For each assay, cells in the indicated media were plated in six-well plates at a seeding concentration of 1×10^5 cell/ml. Test agents FBPA and FBPA-fructose were prepared as previously described (5).

At designated times, cell-cycle distribution was assayed by harvesting cells and preparing nuclear extracts for analysis. As previously described (6), cells were disrupted by means of a detergent solution (0.6% non-idet P40, 0.1% albumin in 0.9% NaCl). Extracts were treated with RNase (100 mg/ml) for 10 min at room temperature. Thereafter, DNA was stained with propidium iodide (50 μg/ml) for 20 min at 4°C.

Cell-cycle distribution was determined on a Becton-Dickinson FacScan flow cytometer (Mountain View, CA). DNA histograms were generated from events analyzed as right angle fluorescence area versus right angle fluorescence peak. In this manner doublets and debris were reduced or eliminated from the processed histogram. Proliferation index was determined manually and represented the number of cells in G_2 and S divided by the total number of cells gated.

Bromo Deoxyuridine Labeling After cells were cultured in the presence or absence of the test agents for the times indicated they were pulsed for 60 min with 0.2 m*M* bromo deoxyuridine (BUdR). Cells were then harvested and resuspended in 70% ethanol and held at −20°C at least 15 hr. Cells were then washed with phosphate-buffered saline

This work was partially supported by an award from the Department of Energy.
[1] To whom requests for reprints should be addressed at University of Tennessee Medical Center, 1924 Alcoa Highway, Knoxville, TN 37920-6999.

Received February 6, 1997. [P.S.E.B.M. 1997, Vol 216]
Accepted July 8, 1997.

0037-9727/97/2163-452$10.50/0

(PBS) and resuspended for 30 min at 37°C in PBS containing 0.1% bovine serum albumin and RNase, followed by 10 min with cells resuspended in 0.1 *M* HCl and 0.5% Triton X-100. Cells were boiled for 10 min and than held at 4°C for 10 min. After which cells were resuspended in PBS and 0.5% Triton X-100, then 100 μl of anti-BUdR fluorescein conjugate (Becton-Dickinson) was added. After being washed, cells were resuspended in the presence of propidium iodide as indicated above.

Results

Because the assay chosen to assess proliferation is labor intensive, each set of patterns presented is the result of individual experiments. Thus, although replicate assays were not performed coincidentally for each assay, the results presented represent typical results from multiple assays performed over several months.

Flow cytometry was chosen as the assay method because it offered the opportunity to measure multiple parameters on individual cells. Thus, in Figure 2 the middle images, or dot plots, represent the results of analysis of individual cells for both total DNA content per cell (*x* axis) and the amount of BUdR incorporated during a 1-hr pulse (*y* axis). Cells had been cultured 72 hr before the pulse in the absence (left column) or presence (right column) of FBPA. DNA is quantitated by fluorescence intensity due to the level of propidium-iodide binding. BUdR incorporation is quantitated by fluorescein-conjugated antibody binding to BUdR molecules incorporated into DNA. Simultaneous or multiparameter analysis of the two fluorescent signals is possible because of the different wavelengths of light emission from the two fluorochromes. Therefore, each dot in the middle panels represents an individual cell analyzed for dual fluorescence.

The upper images in Figure 2 represent histograms expressing the number of cells that contain a certain level of DNA per cell. The large peak in each of the top panels represents the number of cells in G_1 and G_0 of the cell cycle. Because there is proportionality between DNA content and fluorescence, the G_2/M phase of the cell cycle is double the fluorescence intensity of G_1/G_0. The trough between the two peaks represents those cells in S phase. No significant shift of cells between the stages of the cell cycle is apparent as the result of FBPA treatment.

It is obvious from the middle panels, however, that cells exposed to FBPA are displaced more from the origin on the *y* axis. In the control cells, it is particularly evident that

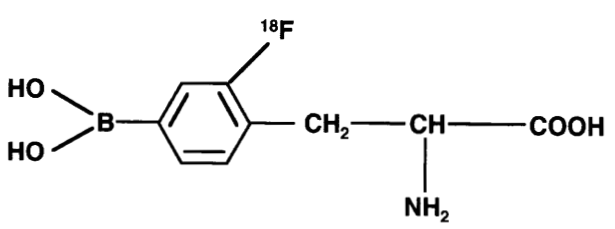

Figure 1. Structure of FBPA.

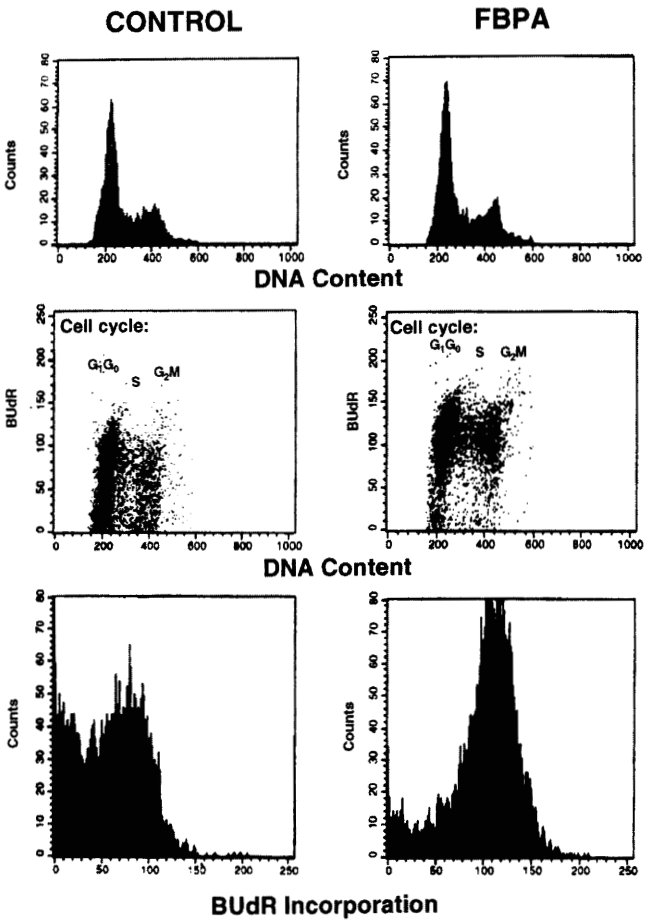

Figure 2. Effect of 72-hr exposure of glioblastoma cells *in vitro* to FBPA (10^{-8} *M*). The top histogram in each panel reflects the cell-cycle distribution as monitored by propidium iodide binding; the middle dot plot reflects the double labeling pattern for DNA on the *x* axis, and bromo deoxyuridine (BUdR) incorporation on the *y* axis; the lowest histogram reflects the distribution of cells incorporating BUdR.

S-phase cells incorporated BUdR with varying levels of intensity, ranging from low intensity or low incorporation to moderately high. In the presence of FBPA, however, virtually the entire population of S-phase cells have moderate to high incorporation. Cells in G_1 and G_2 are also somewhat affected. Obviously, labeled cells in G_1 are actually in the earliest stages of S but have not produced enough DNA to be detected by flow cytometry as S-phase cells. Similarly, cells in G_2 were presumably in late S when the pulse began and were thus indistinguishable from G_2.

Total BUdR incorporation is reflected in the bottom pair of images. The level of BUdR per cell is now expressed on the *x* axis, and the total number of cells with any given level of incorporation is expressed as the *y* axis. Inspection indicates not only that there was more incorporation per cell in the presence of FBPA (right panel) as indicated by the shift to the right of the main population, but also that the number of relatively negative cells apparent in the control culture as the first peak from the origin is virtually eliminated in the FBPA-treated culture.

Figure 3 depicts the effect of 24-hr exposure of varying

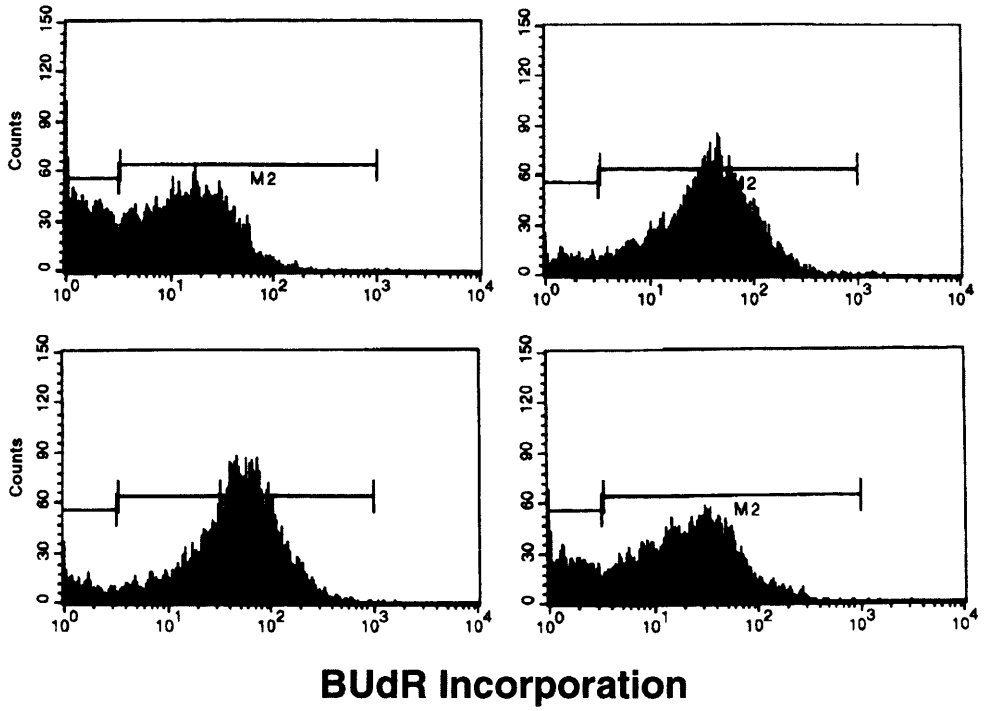

Figure 3. BUdR incorporation into glioblastoma cells incubated in the presence of varying concentrations of FBPA for 24 hr. (A) The distribution and magnitude of incorporation per cell in control conditions. (B–D) Histograms depicting incorporation in the presence of 10^{-6}, 10^{-8}, and 10^{-10} *M* FBPA, respectively.

concentrations of FBPA upon BUdR incorporation. These panels are comparable to the bottom images in Figure 2. Thus, the *x* axis represents the level of DNA synthetic activity, as measured by BUdR incorporation. The control culture Panel A again indicates the presence of two populations of cells with respect to DNA synthetic activity. At concentrations of 10^{-6} *M* and 10^{-8} *M* FBPA (Panels B and C), the number of BUdR-positive cells is enhanced 33% above the control. A more modest, 14% increase is noted at 10^{-10} *M* FBPA. In addition, the intensity of BUdR incorporation per cell is highest (shifted to the right) in the 10^{-8} *M* culture. At 10^{-10} *M*, the effect is greatly attenuated, as marked both by a drop of BUdR incorporation per cell (main peak is shifted to left) and by the reappearance of the relatively negative population which was seen in the control.

A similar analysis was done to assess the effect of an added fructose moiety to the basic FBPA molecule. The top panels of Figure 4 represent the cell-cycle distribution (DNA content per cell); the middle panels represent the results of simultaneous dual analysis of individual cells for DNA content (*x* axis) and BUdR incorporation (*y* axis, DNA synthetic activity); and the bottom panels reflect the overall level of BUdR incorporation.

As was noted in assays of FBPA (Fig. 2), FBPA-fructose–treated cells were characterized by an increased level of DNA synthetic activity or BUdR incorporation (Fig. 4). After the 24-hr exposure to FBPA-fructose, there was an increase in the number of cells actively incorporating BUdR, comparable to that observed with FBPA. Assay results paralleling those seen with FBPA (Fig. 2) indicated that the majority of the effect was noted in cells in S phase. The middle panel of Figure 4 indicates that S-phase cells more actively incorporated BUdR in the presence of FBPA-fructose. The histograms on the bottom panels of Figure 4 confirm the impression and show not only more cells actively incorporating BUdR but also the displacement to the right on the *x* axis indicating a higher rate of incorporation per cell.

Discussion

Kubota *et al.* (4) have previously reported that cellular accumulation of 4-borono-2-fluoro-D,L-phenylalanine was related to the level of DNA synthesis in B16 melanoma cells. Their study, which was performed *in vivo,* correlated FBPA uptake and thymidine incorporation. The largest amount of incorporation of FBPA was noted in S-phase melanocytes. Thus, it was reasonable to conclude that FBPA incorporation was related to the inherent DNA synthetic activity of the target cells. The results of Kubota *et al.* confirmed the earlier observations of Coderre *et al.* (7), who studied incorporation patterns in Harding-Passey melanoma cells carried sc in BALB/c mice. These authors had also found that increased levels of BPA incorporation were associated with elevated levels of [^{3}H]thymidine incorporation. It should also be noted that incorporation of the precursor molecule into melanin was only slightly associated with uptake (4). The present results, which must be considered preliminary, suggest that incorporation of the boron-containing compounds may directly effect the DNA syn-

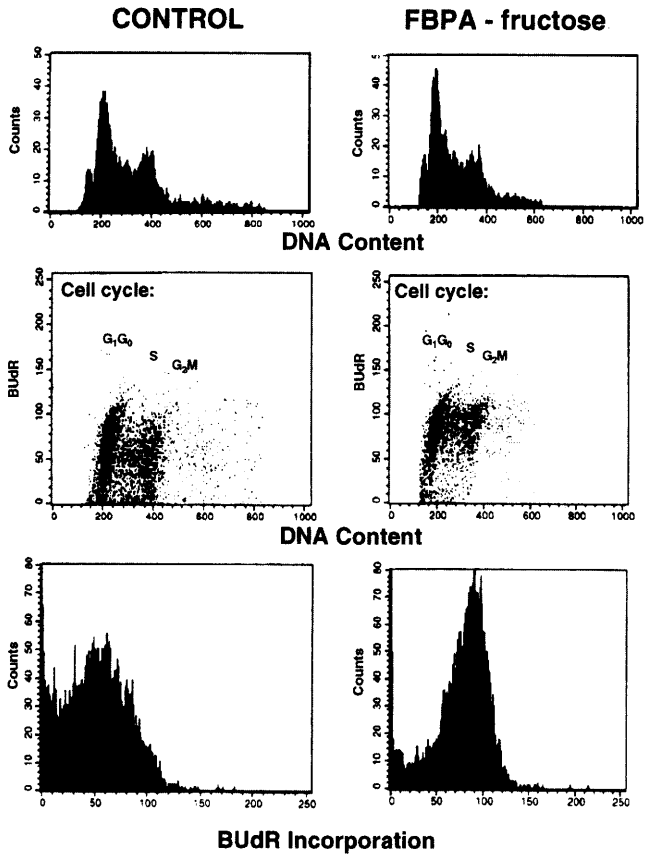

Figure 4. Effect of 24-hr exposure of glioblastoma cells *in vitro* to FBPA-fructose (10^{-9} *M*). The top histogram in each panel reflects the cell-cycle distribution as monitored by propidium iodide binding; the middle dot plot reflects the double labeling pattern for DNA on the *x* axis, and bromo deoxyuridine (BUdR) incorporation on the *y* axis; the lowest histogram reflects the distribution of cells incorporating BUdR.

thetic pathway of the target cells, and thus the previously reported increase of incorporation of FBPA in DNA synthesizing cells may actually reflect the action of FBPA upon the DNA synthetic pathway of the target cells.

Although this study does not lend itself to defining the pathways involved, it is possible to speculate that the apparent increase in DNA synthetic activity reported now *in vitro* and in previous *in vivo* studies may merely reflect an increase in uptake of the precursor molecule, BUdR or [^{3}H]thymidine, with a consequent increase in labeling. A similar result might be obtained if the intracellular level of thymidine was reduced, thus allowing the precursor a more favorable competitive position. If cell growth was actually increased in the target cells then it would seem reasonable to expect to see an increase in the percentage of cells in S phase. As we show in our figures, this was not a consistent finding. Hessels *et al.* (8), however, have reported that flow cytometry–based determination of cell-cycle distribution is a relatively insensitive marker of cell growth. It is also interesting to note that in the work of Kubota *et al.* (4) the labeling index, or the percentage of cells incorporating precursor, was not effected. In contrast, in their autoradiographic study the number of grains per cell was greatly enhanced in cells containing FBPA. This observation is confirmed by our results, which show more BUdR incorporation in S-phase cells treated with FBPA than in control S-phase cells. The effect is also not likely to be promoted by the phenylalanine moiety of the molecule inasmuch as the amino acid is a basic ingredient of the culture media at a concentration several orders of magnitude higher than the concentrations of FBPA used in this study.

The significance of these *in vitro* observations relative to *in vivo* conditions is more difficult to assess. In our *in vitro* studies, the cells were continuously exposed in some instances for as long as 72 hr to micromolar quantities of FBPA. In contrast, *in vivo* studies have utilized generally a single bolus injection with a peak incorporation into tumor occurring between 4 and 6 hr (4). Tumor tissue has been reported to concentrate FBPA relative to blood or other tissues, and the level of uptake has been shown to be proportional to the amount injected (4). In the studies of Coderre *et al.* (7), the tumor concentration of FBPA ranged between 9 and 33 μg/g tumor. *In vitro* concentrations would have represented only approximately 1% of those *in vivo* concentrations but of course would have been continuously present.

In conclusion, *in vitro* studies confirm that in the presence of FBPA there is an apparent increase in DNA synthetic activity per cell. Further studies will be needed to determine the mechanism and possible implications of these observations.

1. Barth RF, Soloway AH, Fairchild RG, Brugger RM. Boron neutron capture therapy for cancer. Cancer **70:**2995–3007, 1992.
2. Ishiwata K, Ido T, Honda C, Kawamura M, Ichihashi M, Mishima Y. 4-Borono-2 (19F) fluoro-D,L-phenylalanine: A possible tracer for melanoma diagnosis with PET. Nucl Med Biol **19:**311–318, 1992.
3. Hatanaka H. Clinical experience of boron-neutron capture therapy for gliomas: A comparison with conventional chemo-immuno-radiotherapy. In: Hatanaka H, Ed. Boron neutron capture therapy for tumors. Niigata, Japan: Nishimura Co., pp349–78, 1986.
4. Kubota T, Yamada S, Ishiwata K, Tada M, Ido T, Kubota K. Cellular accumulation of 18F-labelled boronophenylalanine depending on DNA synthesis and melanin incorporation: A double-tracer microautoradiographic study of B16 melanomas *in vivo*. Br J Cancer **67:**701–705, 1993.
5. Reddy NK, Kabalka GW, Longford CPD. 4 (10B) borono-2-(18F) fluoro-l-phenylalanine-fructose complex for use in timing boron neutron capture therapy (BNCT). J Labelled Cmpd Radiopharm **37:**599, 1995.
6. Fuhr JE, Kattine AA, Nelson HS. Evaluation of *in vivo* breast fine needle aspirates by flow cytometry. An efficacy study. J Natl Cancer Inst **89:**1272–1276, 1992.
7. Coderre JA, Glass JD, Fairchild RG Roy U, Cohen S, Fand I. Selective targeting of boronophenylaline to melanoma in Balb/C mice for neutron capture therapy. Cancer Res **47:**6377–6383, 1987.
8. Hessels J, Kingma AW, Muskiet FAJ, Sarhan S, Seiler N. Growth inhibition of two solid tumors in mice, caused by polyamine depletion, is not attended by alterations in cell-cycle phase distribution. Int J Cancer **48:**697–703, 1991.
9. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. Proc Nat Acad Sci U S A **83:**2500, 1986.