Regulation of the Proliferation of Colon Cancer Cells by Compounds that Affect Glycolysis, Including 3-Bromopyruvate, 2-Deoxyglucose and Biguanides

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Abstract. *In previous studies performed by our group, we observed that 2-deoxyglucose blocked the acidification of the medium used for culture of colon cancer cells caused by incubation with biguanides and it had an additive inhibitory effect on growth. In the present work, we found that 3-bromopyruvate can also prevent the lowering of pH caused by biguanide treatment. 3-Bromopyruvate inhibited colonic cancer cell proliferation, but the effect was not always additive to that of biguanides and an additive effect was more notable in combined treatment with 3-bromopyruvate and 2-deoxyglucose. The induction of alkaline phosphatase activity by butyrate was not consistently affected by combination with other agents that modified glucose metabolism. The drug combinations that were examined inhibited proliferation of wild-type and p53-null cells and affected colonic cancer lines with different growth rates.*

In earlier work, our group has found that incubation of colonic cancer cells with 2-deoxyglucose had an additive inhibitory effect on proliferation when used with the biguanides metformin and phenformin, and blocked the acidification caused by these biguanides (1). A number of mechanisms have been suggested for the antitumor effects of 3-bromopyruvate (2-5). Prominent among these are inhibitory effects on glycolysis, particularly those exerted at the levels of hexokinase and glyceraldehyde 3-phosphate dehydrogenase. Following our work with 2-deoxyglucose, we have sought to examine the following questions. Firstly, does 3-bromopyruvate block the acidification of the medium caused by incubation of colonic cancer cells with metformin or phenformin and does such an effect correlate closely with a decrease in glucose metabolism? Secondly, are there additive inhibitory effects on proliferation when treatment with 3-bromopyruvate is combined with metformin or phenformin? Thirdly, is the differentiating effect of butyrate on colonic cancer cells influenced by 3-bromopyruvate? Fourthly, do *p53* null HCT116 colonic cancer cells differ from wild-type cells in their response to 3-bromopyruvate as judged by rates of glucose metabolism and proliferation? Fifthly, are additive effects seen when colonic cancer cells are co-incubated with 2-deoxyglucose and 3-bromopyruvate?

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Materials and Methods

Cells and determination of cell proliferation. SW1116, HCT116, HT29, and Caco-2 human colon cancer cells were obtained from the American Type Culture Collection, Rockville, MD, USA, and were incubated at 37˚C in RPMI-1640 medium with 5% fetal calf serum. Of these cell lines, the HCT116 cells exhibited the most rapid proliferation, and the slowest growth was seen with the SW1116 cells. HCT116 *p53* null cells were described by Bunz *et al.* (6). Cell proliferation was generally monitored by the increase in protein. In studies with 96-well plates, the procedure involved staining with sulforhodamine-B, essentially as described by Vichai and Kirtikara (7). Cells were routinely allowed to attach to tissue culture dishes or 96-well plates for 24 h before changing the medium and incubating for a further 72 h before determining the impact of the compounds under study on medium pH, glucose concentration, and cell proliferation, as judged by protein mass.

Reagents. 3-Bromopyruvate, butyrate, 2-deoxyglucose, metformin and phenformin were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Enzyme assays. A total of 1.0 or 1.5×10^6 cells were incubated with 10 mL RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 h and unless stated otherwise the cells were incubated for 72 h before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris pH 8.0. The protein concentration of the extract was determined using the

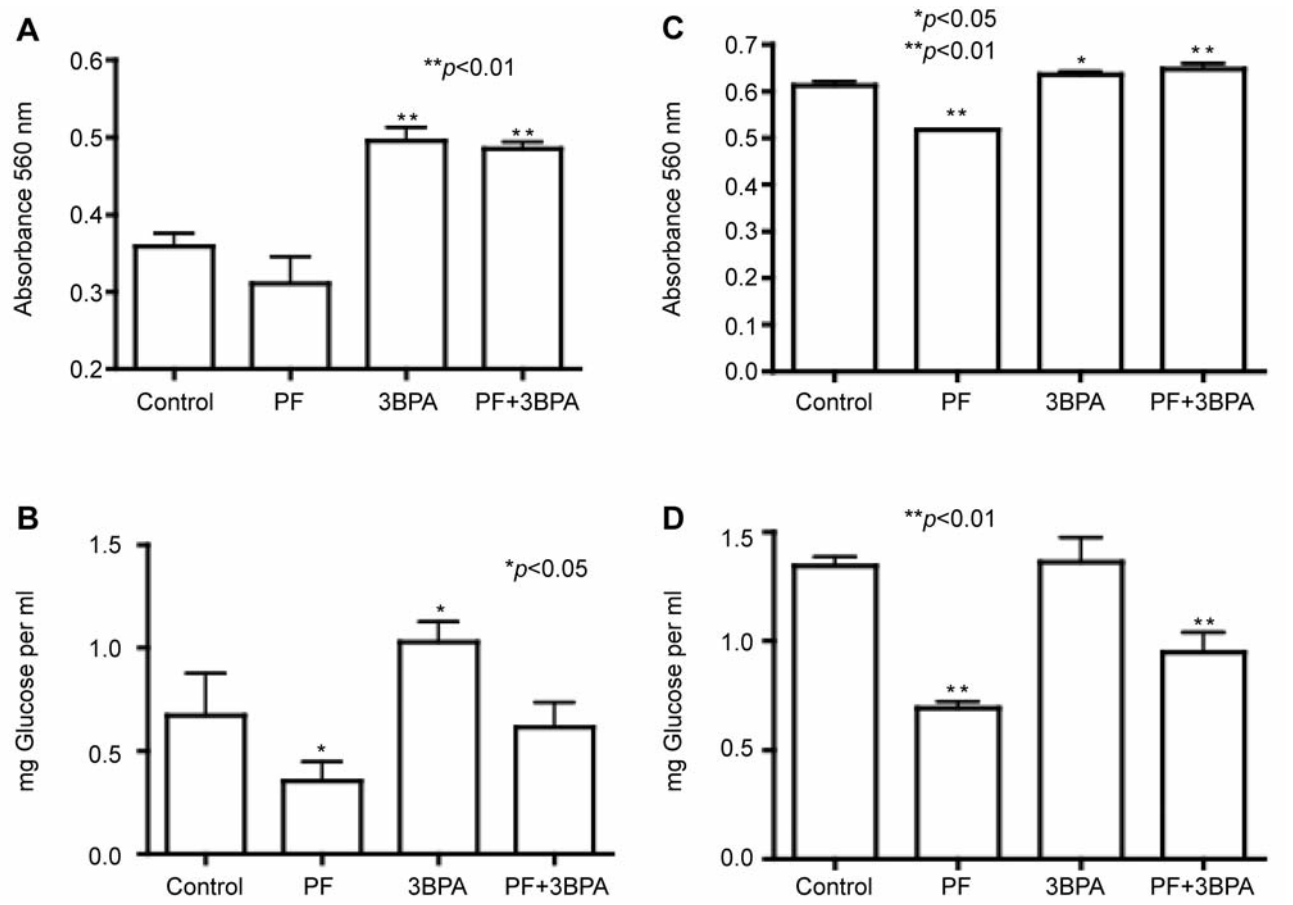

Figure 1. *Effects of a 72-h incubation of Caco-2 (A, B) and SW1116 (C, D) cells with phenformin (PF; 25 μM) and 3-bromopyruvate (3BPA; 25 μM) on absorbance of phenol red in the medium at 560 nm (A, C) and final glucose concentration in the medium (B, D). One million cells were plated in 10 ml medium. Means and standard deviations are presented for three incubations.*

BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Alkaline phosphatase was assayed at 37˚C, as previously described (8) using para-nitrophenyl phosphate as substrate. Formation of the product was monitored by the change in absorbance at 410 nm.

pH determination. pH determination with an electrode has been found previously to correlate well with changes in the light absorbance at 560 nm reflecting changes in the pH indicator, phenol red, where a higher absorbance reflects a higher pH (1). The latter method was found particularly convenient for work with 96 well plates and was used routinely in the present work.

Glucose assay. Glucose was assayed in the cell culture medium using GAGO-20 Kit from Sigma-Aldrich. This is a colorimetric procedure in which the oxidation of glucose is coupled with glucose oxidase and peroxidase to the oxidation of dianisidine.

Statistical evaluation. Statistical significance of the results was determined by a two-tailed Student's *t*-test or by Dunnett's test for multiple comparisons using the Instat program from GraphPad Software, Inc., La Jolla, CA, USA. A probability of less than 5% was considered significant and differences compared to the control are shown.

Results

Acidification of the medium seen when colonic cancer cells are incubated with biguanides was reduced by 3-bromopyruvate. This is illustrated for the combined actions of phenformin and 3-bromopyruvate on Caco-2 (Figure 1A) and SW1116 cells (Figure 1C).

Effects on acidification were paralleled by changes in glucose metabolism. Glucose metabolism in Caco-2 (Figure 1B) and SW1116 cells (Figure 1D) was increased by incubation with phenformin and the effect was diminished by co-incubation with 3-bromopyruvate. The data in Figure 2A and B for HT29 cells suggest that effects of phenformin and 3-bromopyruvate on pH paralleled those on glucose concentration in the medium. A similar conclusion can be drawn from the data in Figure 3A and B for HCT116 cells, with respect to both the wild-type and *p53*-null cells.

Differentiating effects of butyrate on colonic cancer cells were not greatly affected by co-incubation with 3-

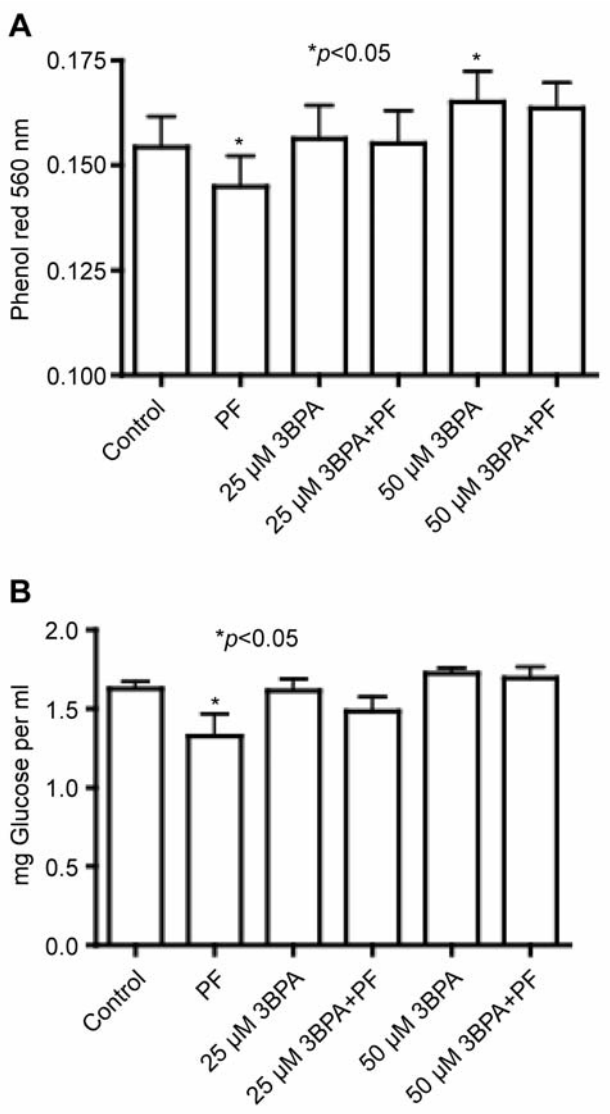

Figure 2. *Effects of a 72-hour incubation of HT29 cells with phenformin (PF; 25 μM) and 3-bromopyruvate (3BPA) on absorbance of phenol red in the medium at 560 nm (A) and final glucose concentration in the medium (B). 5,000 cells were plated in 0.2 ml medium in a 96-well plate. Means and standard deviations are presented for six or more incubations.*

bromopyruvate. A typical result is shown in Figure 4A. The protein values shown in Figure 4B suggest that the decreased proliferation with the drug combination was not significantly different from that seen with 3-bromopyruvate-alone.

The effects of 3-bromopyruvate on cell proliferation were similar in wild-type and *p53*-null HCT116 cells (Figure 3C) and did not show a notable additive effect when used in combination with phenformin. The data in Figure 5A for Caco-2 cells suggest that there might be some additivity but

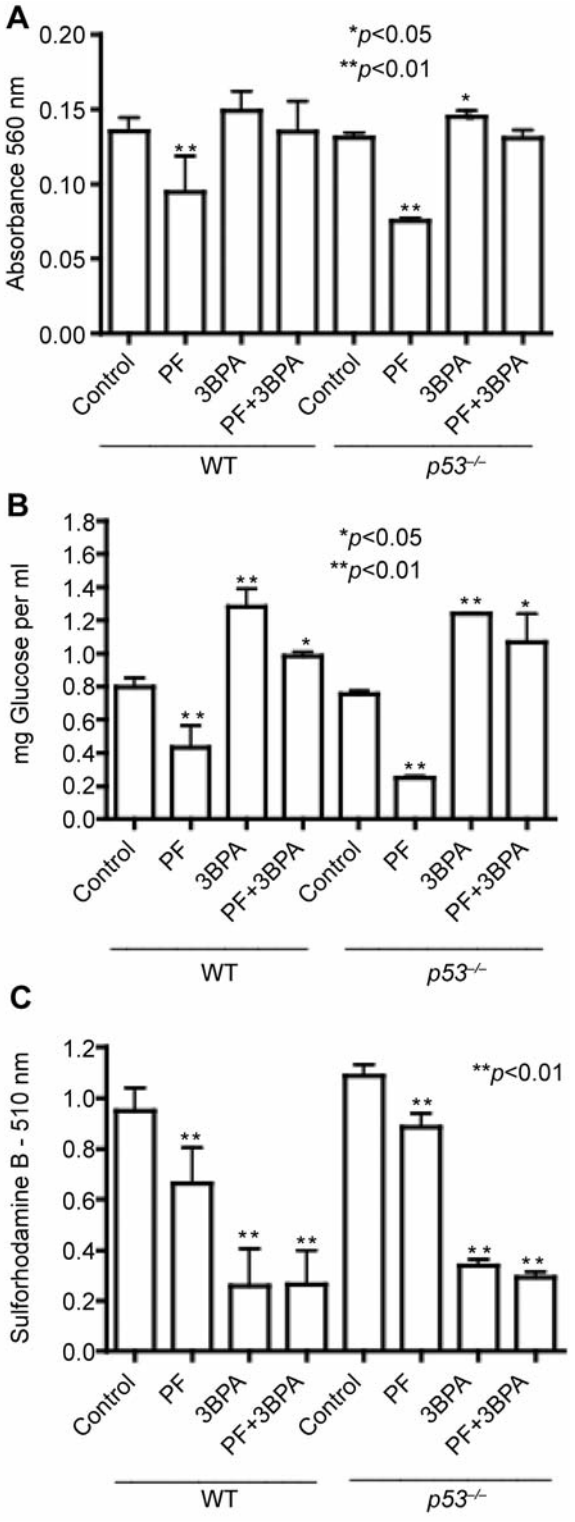

Figure 3. *Effects of a 72-hour incubation with phenformin (PF; 25 μM) and 3-bromopyruvate (3BPA; 25 μM) of HCT116 wild-type (WT) and p53-null cells on absorbance of phenol red in the medium at 560 nm (A), final glucose concentration in the medium (B) and staining with sulforhodamine B (C). 5,000 cells were plated in 0.2 ml medium in a 96-well plate. Means and standard deviations are presented for six or more incubations.*

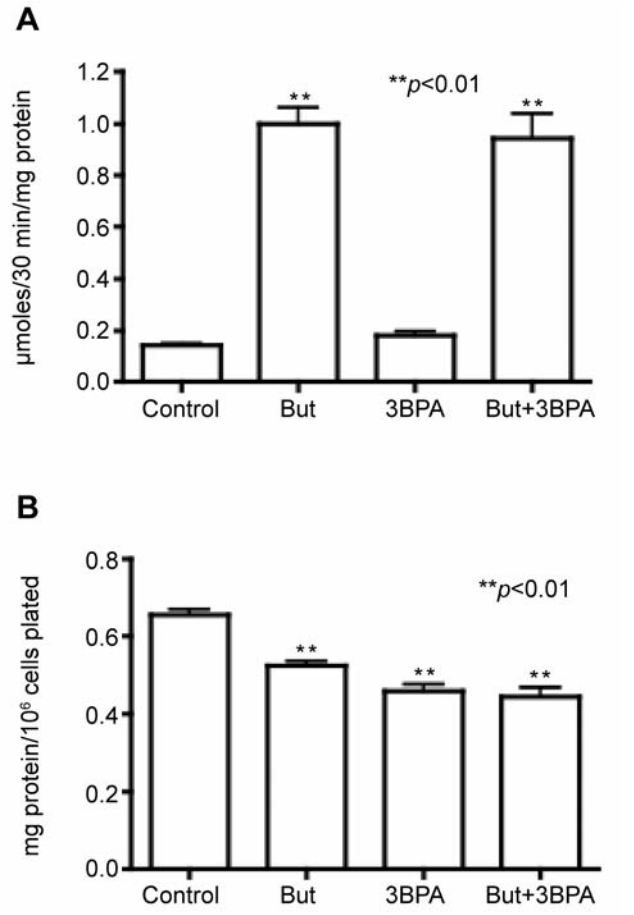

Figure 4. *Effects of a 72-h incubation with butyrate (But; 1 mM) and 3-bromopyruvate (3BPA; 37.5 µM) of Caco-2 cells on alkaline phosphatase activity (A) and protein yield (B). 10^6 cells were plated in 10 ml medium. Means and standard deviations are presented for 3 incubations.*

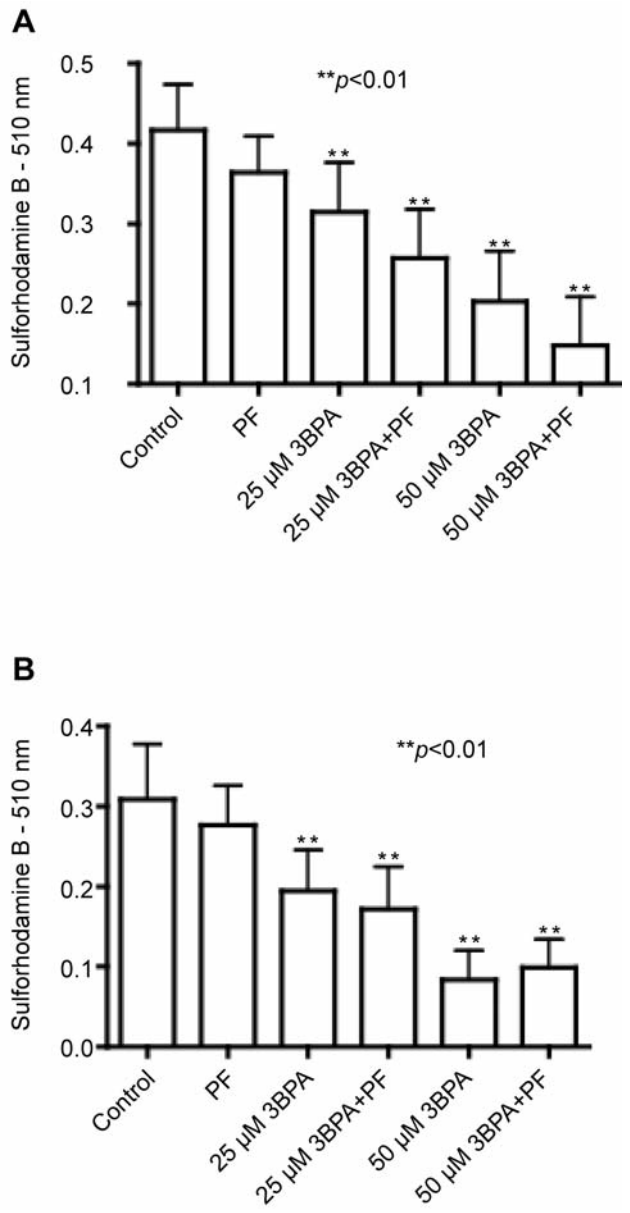

Figure 5. *Effects of a 72-hour incubation with phenformin (PF) and 3-bromopyruvate (3BPA) of Caco-2 cells (A) and HT29 cells (B) on proliferation monitored by staining with sulforhodamine B. 5.000 cells were plated in 0.2 ml medium in a 96-well plate. Means and standard deviations are presented for 6 or more incubations.*

the results in Figure 5B for HT29 cells do not reinforce that possibility. These results contrasted with the evidence for an additive effect of biguanides and 2-deoxyglucose that we had previously seen in studies that did not include HCT116 cells (1). The data in Figure 6A indicate that an additive effect on proliferation of HCT116 cells can be seen with metformin and 2-deoxyglucose. The same indication was observed with SW1116 cells (Figure 6B) using sulforhodamine B staining in contrast to the tetrazolium salt reduction assay used in a previous study (1).

An additive inhibitory effect on cell proliferation was observed with combined treatment with 3-bromopyruvate and 2-deoxyglucose. The data in Figure 7A, B and C for HCT116, HT29 and Caco-2 cells, respectively, suggest some additive effects but that is not clear for the more slowly-growing SW1116 cells (Figure 7D).

Discussion

Since the early studies of Warburg (9, 10), inhibition of glycolysis has occured as a promising target in cancer chemotherapy. However, progress has been slowed by limited specificity of inhibitors of glycolysis. Support for the Warburg effect has been provided by the utility of the uptake of 2-

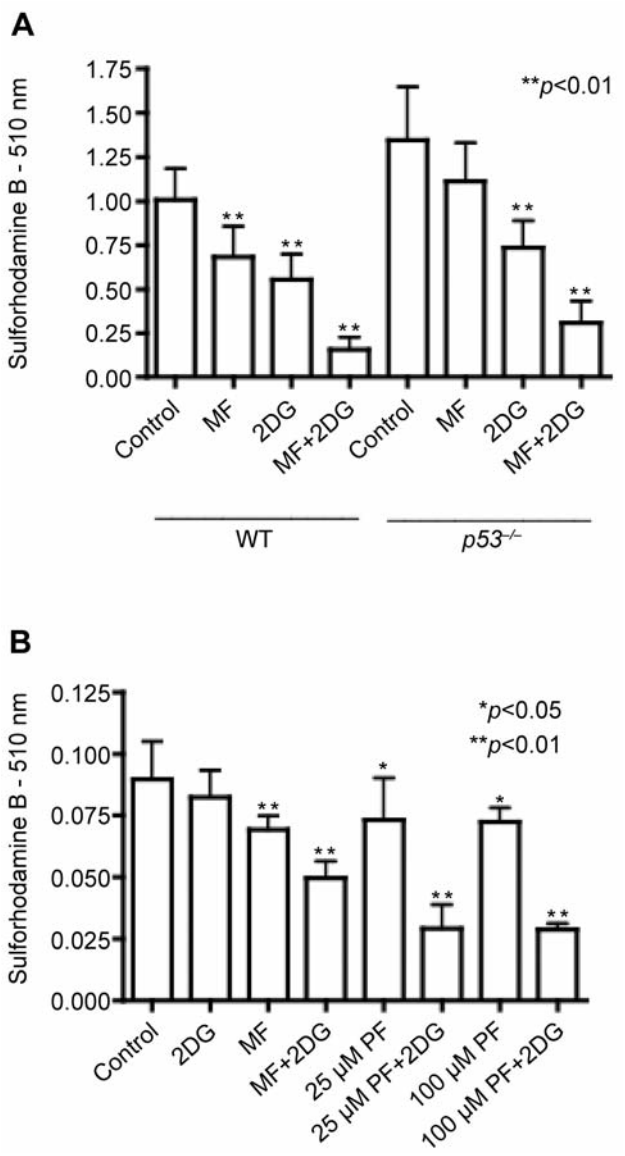

Figure 6. *Effects of a 72-h incubation with metformin (MF), phenformin (PF) and 2-deoxyglucose (2DG; 1 mM) of HCT116 wild-type (WT) and p53-null cells (A) and SW1116 cells (B) on proliferation monitored by staining with sulforhodamine B. 5,000 cells were plated in 0.2 ml medium in a 96-well plate. Means and standard deviations are presented for 6 or more incubations.*

deoxyglucose in monitoring the localization of tumors. Furthermore, 2-deoxyglucose is an inhibitor of glycolysis. Another inhibitor of glycolysis that has been the subject of considerable investigation is 3-bromopyruvate (2, 3, 11, 12). At first consideration, biguanides such as metformin and phenformin would seem to act counter to such an approach because they increase glucose utilization. Nevertheless, there is evidence that a combination of 2-deoxyglucose and biguanides can have additive inhibitory effects on the proliferation of cancer cells (1, 13, 14). In the present investigation we have extended our studies on the combinations of these compounds and have included determinations of glucose utilization as well as cell proliferation.

In previous work, we observed that 2-deoxyglucose prevented the decrease in the pH of the incubation medium of cultured colonic cancer cells caused by treatment with metformin and phenformin (1). In the present study, a similar action to that of 2-deoxyglucose was seen with 3-bromopyruvate. Reduced glucose metabolism paralleled the change in pH. Glucose metabolism and change in pH was greater in more rapidly dividing colonic cancer cells.

In addition to inhibition of glycolysis at the levels of hexokinase and glyceraldehyde-3-phosphate dehydrogenase, 3-bromopyruvate has been reported to inhibit mitochondrial oxidative phosphorylation at the level of complex II of the electron transport chain (4). Since 3-bromopyruvate has the potential to interfere with tetrazolium reduction assays in the evaluation of cytotoxicity (15), we switched from that assay procedure in the present work to protein staining with sulforhodamine-B in monitoring effects on cell proliferation (16). The sulforhodamine-B assay did not provide the anomalous results observed previously, when SW1116 cells incubated with metformin were tested with a tetrazolium salt reduction assay and gave an apparent increase in activity (1). We have confirmed the additive inhibitory effects of 2-deoxyglucose and biguanides and extended the observation to wild-type and *p53*-null HCT116 cells. 3-Bromopyruvate inhibited colonic cancer cell proliferation but additive effects with biguanides were less notable than with combinations of 2-deoxyglucose and biguanides. A notable feature was an additive inhibitory effect on cell proliferation in combined treatment with 2-deoxyglucose and 3-bromopyruvate.

Induction of alkaline phosphatase activity in colonic cancer cells is commonly used as a marker of differentiation. Butyrate is effective in inducing alkaline phosphatase activity in many but not all colon cancer cell lines. This action can be seen with 1 mM butyrate in three out of the four colonic cancer cell lines studied: *i.e.* in Caco-2, HT29 and SW1116, but not HCT116 cells. Additive effects with butyrate and other compounds are sometimes seen (8). The induction of alkaline phosphatase activity by butyrate was not consistently affected by combination with the other agents studied that also reduce glucose metabolism namely 2-deoxyglucose and 3-bromopyruvate. Studies on the action of butyrate on cancer cells have mainly focused on its inhibitory effect on histone deacetylases but additional effects have been suggested (17).

There is evidence that glucose metabolism in cancer cells can be increased by loss of the *p53* tumor suppressor gene product (18). Ben Sahra *et al*. (13) reported that the

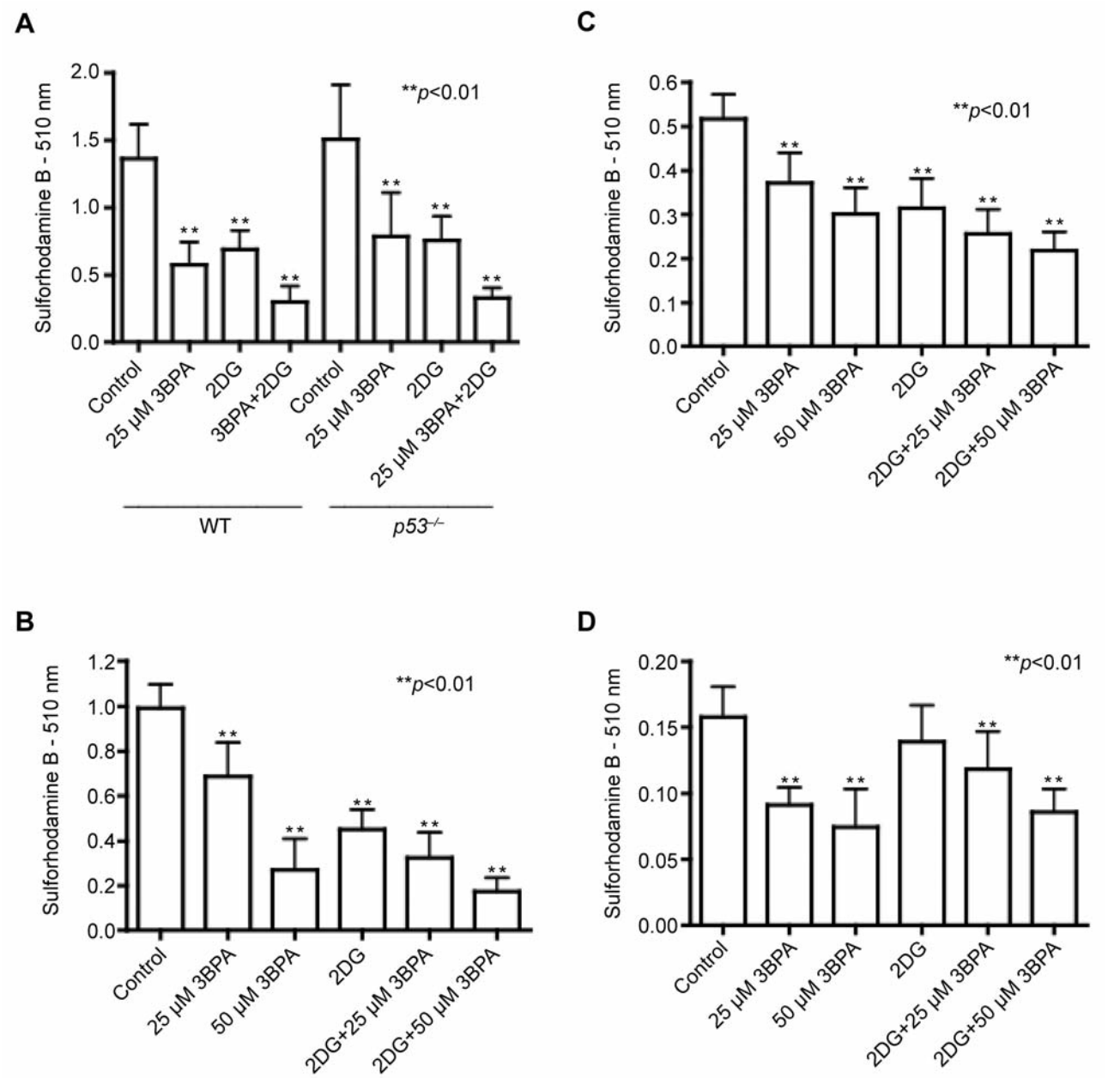

Figure 7. *Effects of a 72-hour incubation with 3-bromopyruvate (3BPA) and 2-deoxyglucose (2DG; 1 mM) of HCT116 wild-type (WT) and p53-null cells (A), HT29 cells (B), Caco-2 cells (C) and SW1116 cells (D) on proliferation monitored by staining with sulforhodamine B. 5,000 cells were plated in 0.2 ml medium in a 96-well plate. Means and standard deviations are presented for six or more incubations.*

combination of metformin and 2-deoxyglucose induced *p53*-dependent apoptosis in prostate cancer cells. The drug combinations that were studied in the present work inhibited proliferation in wild-type and *p53*-null HCT116 colonic cancer cells to a similar degree and affected colonic cancer cell lines with different growth rates. Enzymes in glycolysis have been suggested as potential targets in cancer chemotherapy (19). The observations made in the present work suggest that combinations of 2-deoxyglucose and 3-bromopyruvate merit further investigation as cancer chemotherapeutic agents.

Acknowledgements

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