3-Bromopyruvate as inhibitor of tumour cell energy metabolism and chemopotentiator of platinum drugs

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ABSTRACT

Tumour cells depend on aerobic glycolysis for adenosine triphosphate (ATP) production, making energy metabolism an interesting therapeutic target. 3-Bromopyruvate (BP) has been shown by others to inhibit hexokinase and eradicate mouse hepatocarcinomas. We report that similar to the glycolysis inhibitor 2-deoxyglucose (DG), BP rapidly decreased cellular ATP within hours, but unlike DG, BP concomitantly induced mitochondrial depolarization without affecting levels of reducing equivalents. Over 24 h, and at equitoxic doses, DG reduced glucose consumption more than did BP. The observed BP-induced loss of ATP is therefore largely due to mitochondrial effects. Cell death induced over 24 h by BP, but not DG, was blocked by N-acetylcysteine, indicating involvement of reactive oxygen species. BP-induced cytotoxicity was independent of p53. When combined with cisplatin or oxaliplatin, BP led to massive cell death. The anti-proliferative effects of low-dose platinum were strikingly potentiated also in resistant p53-deficient cells. Together with the reported lack of toxicity, this indicates the potential of BP as a clinical chemopotentiating agent.

1. Introduction

In normal cells, adenosine triphosphate (ATP) production is to at least 90% provided by mitochondrial oxidative phosphorylation, while tumour cells are to approximately 50% dependent on cytoplasmic, aerobic glycolysis (Shaw, 2006; Matoba et al., 2006), a tumour-specific feature usually termed the Warburg effect. The switch from oxidative phosphorylation to glycolysis is connected to tumour-specific alterations in the expression and/or modification of proteins involved in the electron transport chain in mitochondria, leading to decreased efficiency of the oxidative phosphorylation process (Plas and Thompson, 2002; Harper et al., 2002; Shaw, 2006; Kim and Dang, 2006; Matoba et al., 2006). This would ultimately lead to lower ATP yields, but aerobic glycolysis compensates the tumour cell for the loss of mitochondrial ATP by providing ATP independently of oxidative phosphorylation. The bioenergetic switch to glycolysis likely confers various growth advantages on the tumour cell: not only does it support growth also under hypoxia, but it helps meet the increased requirements for energy as well as building blocks for macromolecule syntheses. The increased glycolytic activity thus provides the tumour cell with higher levels of NADH and acetyl-CoA to fuel the citric acid cycle and to support fatty acid synthesis. Because oxidative phosphorylation is seldom or never completely down-regulated, tumour cells may also up-regulate the use of energy-rich fatty acids as fuel; this pathway

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eventually feeds into oxidative phosphorylation. Furthermore, via glucose-6-phosphate and the pentose phosphate pathway, glycolysis provides the NADPH required for NADPH-dependent biosyntheses, as well as precursors for purine (nucleotide) synthesis. Excess pyruvate produced by tumour cell glycolysis is converted into lactic acid which is transported out of the cell, and which by lowering the pH contributes to the microenvironmental changes seen in and around tumours in vivo (Stubbs et al., 2000).

In accordance with increased glucose dependence, tumour cells generally show increased cellular uptake of glucose and increased expression of glycolysis-related genes such as glucose transporters, hexokinase and glyceraldehyde-3-phosphate dehydrogenase (Cuevza et al., 2002; Harper et al., 2002; Shaw, 2006; Kim and Dang, 2006; Pedersen, 2007). Altered energy metabolism has been shown to correlate with tumour progression (Harper et al., 2002), and a bioenergetic index based on expression of such proteins has been shown to have prognostic value for several types of carcinoma (Cuevza et al., 2002; Isidoro et al., 2005).

Based on the reasoning that tumour progression and drug resistance might both correlate with altered energy metabolism, these and similar findings have inspired endeavours to pharmacologically target tumour cell energy metabolism. Hexokinase/glycolysis has been targeted experimentally using 2-deoxyglucose (DG) and 3-bromopyruvate (BP). DG is a glucose analogue which is easily taken up by tumour cells via glucose transporters and is then phosphorylated by hexokinase, but it is not further metabolized in the glycolytic process. DG will thus titrate endogenous glucose and thereby blocks glycolysis. Based on the increased glucose uptake specifically in tumour cells, the radiolabelled form of DG, $^{18}$F-deoxyglucose (18-FDG), is used for tumour imaging by positron emission tomography (PET). In the context of the present work, DG at higher concentrations has been shown to sensitize glioma cells to radiation (Mohanti et al., 1996), and potentiated adriamycin- and paclitaxel effects in an in vivo model (Maschek et al., 2004).

The alkylating agent 3-bromopyruvate (BP) inhibits hexokinase and also has effects on mitochondria (Ko et al., 2001; Pedersen, 2007). Being a lactate analogue, it is likely taken up by cells via lactate transporters which are overexpressed in tumour cells (Pedersen, 2007). BP at 100–300 μM is reported to rapidly induce cell death in cultured tumour cells, and to be cytotoxic also under hypoxic conditions and in the absence of p53 (Xu et al., 2005). BP has furthermore been shown to be effective, and indeed cytotoxic, as single agent against hepatic tumours in animals (Ko et al., 2001, 2004). For BP, the reported K_i of glycolysis/hexokinase inhibition is 2.4 mM, while for DG it is 15.5 mM (Ko et al., 2001), suggesting that BP is a more efficient glycolysis inhibitor than DG.

We have earlier found that DG may potentiate cell death induced by several chemotherapeutic agents, in particular cisplatin (manuscript under revision). Here, we have studied and compared effects of DG and BP on energy metabolism and on proliferation, and we have also examined BP as potentiator of the anti-proliferative effects of cisplatin and oxaliplatin, a third-generation derivative.

2. Results and discussion

2.1. Effects of 2-deoxyglucose and 3-bromopyruvate on ATP and reducing equivalents

HCT116 colon carcinoma cells were treated for 5 h with 2-deoxyglucose (DG) or 3-bromopyruvate (BP). With DG, both, 20 and 40 mM led to an approximately 45% decrease in cellular ATP (Figure 1A). The response to BP at 50 and 100 μM was more dose-dependent and led to greater loss of ATP, or 65% and 90% loss, respectively (Figure 1A). Higher concentrations of DG were not used here, since the K_i of glycolysis inhibition is 15 mM, and for BP 2.4 mM (Ko et al., 2001). The doses used here thus represent 1–2 × K_i for DG, and for BP 0.02–0.04 × K_i, suggesting that the effects of BP were not due to glycolysis inhibition.

Inhibition of glycolytic activity leads to decreased levels of reducing equivalents (NADH and NADPH). By reflecting levels of NAD(P)H mainly derived from glycolysis and the citric acid cycle, the MTT assay is often used for assessing viability (Berridge et al., 2005). Here, we used it for an indirect assessment of rapid effects of DG and BP on energy metabolism. HCT116 cells were treated with 10 mM DG for 3 h, at which time there is no apoptosis and no loss of cells. There was, however, a 40% decrease in MTT signal (Figure 1B), indicating an effect on glycolysis/citric acid cycle. As shown above, BP at 50 μM leads to decreased ATP but was nevertheless found to have no effect in the MTT assay, and indeed had to be increased to 100 μM to have any effect (Figure 1B). Increasing BP to 2.4 mM, i.e., to its K_i value for glycolysis, did decrease the MTT signal considerably, but virtually all the cells were heavily blebbed and clearly dying within this time frame. No MTT effects with 50 and 100 μM BP were seen also at 5 h of treatment (not shown) and in MCF-7 breast carcinoma cells (not shown).

These data suggested that the effect of up to 50 μM BP on ATP levels at 5 h involves mitochondria.

2.2. Effects of DG and BP on mitochondrial integrity

Formation of ATP by oxidative phosphorylation in the mitochondria depends on the integrity of the respiratory chain and the mitochondrial inner membrane potential (∆ψ). The fluorescent probe tetramethylrhodamine ethyl ester perchlorate (TMRE) is taken up only by mitochondria with intact ∆ψ. In control cells, about 90% of the population showed TMRE staining (Figure 1C), and the remaining 10% to the left of the peak thus represent de-energized and dying cells. After 3–6 h treatment with DG at 40 mM, the TMRE stain remained intact (Figure 1C). BP at 50 μM caused a small but discernible loss of TMRE, and 100 μM led to depolarization in 50% of the cells within 3 h (Figure 1C). The experiment was performed three times, and the difference between the effect of 40 mM DG and 50 μM BP was supported by a p value of 0.04 (Student’s t-test).

2.3. Effects of 2-deoxyglucose and 3-bromopyruvate on glucose consumption and growth

To directly examine effects on glycolysis, we assessed glucose consumption over 24 h, as well as cell survival based on total protein in each sample. The results (Figure 2A) show that DG
at 10 and 20 mM, and BP at 25 mM have a similar anti-proliferative effect over 24 h. However, glucose consumption is markedly lower with 20 mM DG (1.3 × $K_i$) than with 25 mM BP (0.1 × $K_i$). Furthermore, 50 µM BP was so cytotoxic that only few cells remained after 24 h and almost no glucose had been consumed, in accordance with a very rapid effect.

We conclude that treatment for 3–5 h with 10–20 mM DG leads to loss of glycolytic activity, and that it reduces ATP levels without affecting mitochondrial integrity. By contrast, and over the same time, BP at 50 µM does not reduce glycolytic activity but does inhibit ATP production, and it has a small but discernible effect on mitochondrial integrity. The observed anti-glycolytic effect of BP over 24 h thus appears to be slower than that of DG, and, importantly, slower than its effect on mitochondria. We conclude that despite their similar use as glycolysis/hexokinase inhibitors DG and BP have different effects on glycolysis and mitochondria. Although we have not identified the additional molecular target(s) of BP, we speculate that at least one is mitochondrial and that it is alkylated by BP. Our results are furthermore in accordance with the view that BP is able to target both a mitochondrial and a glycolysis-related protein (Pedersen, 2007), and with the recent report that BP may disrupt intracellular proton gradients (Dell’antone, 2006). The difference we report between DG and BP may speak in favour of BP as a clinical drug, since its double inhibitory effect should allow the use of low doses and should reduce side effects on non-tumour, glucose-related processes, e.g., in the liver and the brain.

Figure 1 – Early effects of 2-deoxyglucose and 3-bromopyruvate on ATP and reducing equivalents. (A) HCT116 cells were treated with the indicated concentrations of 2-deoxyglucose (DG) and 3-bromopyruvate (BP) for 5 h. Cellular ATP levels were then determined using the Aposensor ATP Assay kit (Alexis Biochemicals) and are expressed as fold signal in untreated control samples. Data represent the averages of three independent experiments. Bars indicate standard error of the mean (SEM). (B) HCT116 cells were treated with the indicated concentrations of DG and BP for 3 h, and were then subjected to the MTT reduction assay (Promega) to assess levels of reducing equivalents (NAD(P)H) to reflect glycolytic activity. Results are shown as fold signal in untreated control samples. Data represent the averages of three independent experiments, except with 2.4 mM BP, which was done twice. Bars indicate standard error of the mean (SEM). (C) HCT116 cells were treated with the indicated concentrations of DG and BP for 3 and 6 h, and the mitochondrial inner membrane potential was then examined using the fluorescent TMRE dye which is taken up only by mitochondria with intact membrane potential. The experiment was performed three times and assessment of the M1 populations yielded a statistically significant difference ($p = 0.04$; Student’s $t$-test) between 40 mM DG and 50 µM BP treatments at 3 h.
Mitochondria are a major source of intracellular reactive oxygen species (ROS) via electron leakage in the electron transport chain. Disruption of the mitochondrial membrane gradient leads to increased electron leakage, and thereby to potentially toxic ROS levels (Orrenius, 2007). ROS might therefore be involved in the observed anti-proliferative effect of BP, whereas they would not be expected to have an equally prominent role in the effect of DG. To test this hypothesis, the ROS scavenger N-acetylcysteine (NAC, 5 mM) was added to cells concomitantly with the drugs, and resulting levels of cell survival were assessed after 24 h using the SRB total protein assay. NAC was found to have no or little effect on the anti-proliferative effect of DG, while it protected almost completely against the cytotoxicity of 50 µM BP (Figure 2B).

Of potential importance for the general use of metabolism-based viability assays was a similar observation supporting ROS-mediated cytotoxicity of BP. Using the WST-1 viability assay which is based on reactive oxygen production through metabolite production (Tan and Berridge, 2000; Berridge et al., 2005), we say which is based on reactive oxygen production through metabolism of BP. Using the WST-1 viability assay was a similar observation supporting the fact that DG leads to some accumulation of cells in G1, at the expense of S phase, whereas BP increased the S and G2/M phases at the expense of the G1 fraction (Figure 3A). At 24 h of treatment, apoptosis induced by 20 mM DG and 50 µM BP was quantified using the Apoptosense ELISA-type assay based on caspase cleavage of cytokeratin-18 to a stable and apoptosis-specific fragment and which thus quantitates accumulated apoptosis levels (Hägg et al., 2002; Kramer et al., 2004). Neither drug induced any significant apoptosis over 24 h, either in the HCT116 cells or in three other tumour cell lines (Figure 3B). After 48 h, the effect of DG was still mainly low-apoptotic, as seen by annexin-V/propidium iodide staining and flow cytometry. By contrast, among the remaining cells in the BP-treated sample, very few were healthy, i.e., double-negative (Figure 3C). Significant release of LDH into the surrounding medium without signs of apoptosis suggested some form of lysis or necrosis in BP-treated cells (see below).

The total anti-proliferative effects of 10–20 mM DG and 25–50 µM BP over 24 h are shown in Figure 2A. We also assessed the effects over 48 h by counting the numbers of surviving, attached cells, and similarly also in a HCT116 subline which is deficient in p53 (HCT116 p53−/−). BP treatment resulted in a sharper decline in viability than did DG (Figure 3D, left). The dose–response patterns of the HCT116 p53−/− are similar to those of the wild-type cells, especially for DG (Figure 3D, right). The slight resistance to BP in these cells is consistent with the report that HCT116 p53−/− cells are adapted to a reduced mitochondrial membrane potential (Δψm) compared...
Figure 3 – Anti-proliferative effects of 2-deoxyglucose and 3-bromopyruvate. (A) HCT116 cells were treated with 20 mM DG or 50 µM BP for 18 h, after which time samples were prepared for cell cycle analysis. Data represent the averages of two separate experiments. (B) Four different human tumour cell lines were treated for 24 h with 20 mM DG or 50 µM BP. Accumulated apoptosis was then quantitated using the Apoptosense assay (PEVIVA, Sweden). Data represent averages ± SEM of at least three experiments per cell line. (C) HCT116 cells were treated with 20 mM DG for 48 h, and low-level cell death (upper right quadrant) was confirmed using the propidium iodide/annexin-V assay by flow cytometry, while 50 µM BP for 48 h led to near complete loss of viable cells (lower left quadrant). (D) HCT116 wild-type-p53 and HCT116 p53−/− cells were treated with the indicated concentrations of DG and BP for 48 h, after which time survival was assessed by cell counting. Data represent averages ± SEM of three separate experiments.
2.6. Potentiation of platinum drugs by BP

Pharmacological potentiation of anticancer agents is an important strategy for overcoming drug resistance as well as for avoiding high dosage and the concomitant undesirable side effects of chemotherapy. Because tumour cells have unique energy requirements, we reason that even partial deprivation of energy/ATP may help break resistance, e.g., by reducing macromolecule synthesis and DNA repair. Accordingly, we have earlier established that in HCT116 cells, both DG and the fatty acid β-oxidation inhibitor etomoxir, which like DG was found to reduce cellular ATP levels, potentiate the anti-proliferative effects of the cornerstone chemotherapeutic agent cisplatin (manuscript under revision). Since the present data showed that BP also leads to reduced ATP levels, albeit via a third, different mechanism, we have here examined BP for potentiation of cisplatin and of oxaliplatin.

BP did not significantly potentiate apoptosis over 24 h when combined with either cisplatin or oxaliplatin at doses up to 20 μM (not shown). However, other responses are possible, notably necrosis, since extreme loss of cellular ATP and/or high levels of reactive oxygen species may per se lead to necrosis (Proskuryakov et al., 2003; Orrenius, 2007). Necrosis induction was therefore examined after 48 h using the LDH release assay. Release was monitored as the ratio of accumulated LDH in the supernatant and total LDH in supernatant plus cell lysates. By itself, BP at 50 μM induced significant LDH release without any marked increase in apoptosis as seen by the Apoptosense assay (Figures 4A and 3A). For reference, 20 μM cisplatin for 20 h will in the HCT116 cells induce an 8–12 fold increase in apoptosis, and nuclear fragmentation in >50% of the cells, while 5 μM does not induce either (Berndtsson et al., 2007, and not shown). When combined with cisplatin at 5 μM, BP induced an additive LDH release, while the effect was lesser with 5 μM oxaliplatin (Figure 4A). The figure also includes data showing the lack of apoptosis potentiation after the same treatments, confirming that necrosis is at hand and that the combination treatment completely bypasses apoptosis.

Using cell counts as read-out, we then went on to assess potentiation (regardless of anti-proliferative mode) of even lower doses of platinum drug. Cisplatin and oxaliplatin were used at 3 and 1 μM, respectively, and BP at 25 μM. The numbers of surviving attached cells were assessed after 48 h, and compared to cell numbers in untreated controls. The results show order to let 10-fold induction represent a “maximum” in the sense that this is apoptosis induction by cisplatin at 20 μM, i.e., a near-maximal clinically attainable concentration. Data represent the average of two experiments with triplicate or quadruplicate samples in each. (B, C) BP-induced potentiation of overall anti-proliferative effects of 3 μM cisplatin and 1 μM oxaliplatin, respectively, was examined after 48 h in HCT116 p53-wild-type (B) and in HCT116 p53-deficient (C) cells. The numbers of remaining cells were counted using a Burker chamber, and the results are expressed as percent of numbers in untreated controls. Data represent averages ± SEM of three separate experiments.
that BP potentiated both agents in HCT116 cells (Figure 4B) and also in HCT116 p53−/− cells (Figure 4C).

Similar to colon cancer in general, the HCT116 cells are fairly resistant to cisplatin-induced apoptosis, and the HCT116 p53−/− cells are even more resistant, requiring 30 μM for a 2–3 fold apoptosis induction (not shown). Our data confirm the overall resistance of the HCT116 cells to cisplatin, and demonstrate that resistance to cisplatin due to lack of p53 could be broken by BP (Figure 4C). The high p53 dependence of the oxaliplatin response is obvious in the lack of response in the p53−/− cells compared to the wild-type, but again, BP clearly potentiated the effect in these resistant cells (Figure 4C). It may be noted that in control samples, cells are still proliferating, and, since results are expressed as percent of control, cells treated with only platinum actually grow within 48 h, while the combination of platinum and BP leads to a net loss.

In conclusion, we find that together with favourable reports on the lack of adverse side effects of BP (Ko et al., 2004), our results indicate the potential use of BP in the clinic not only as single-drug treatment of hepatocarcinomas (Ko et al., 2004), but, in particular, we suggest that BP may find important use as a chemopotentiator and that its efficacy is due to effects not only on glycolysis, but also on mitochondria and to induction of ROS. Chemopotentiation implies the use of multiple agents at low doses. In the current literature, in investigations of BP as single agent, it has been used at 0.1–5 mM, and in the animal model i.p. injections of BP at 2 mM were used (Ko et al., 2004). Although the in vivo concentrations and pharmacokinetics of BP have to our knowledge not been investigated, the 25 μM we have used here is a substantially lower dose, and thus more likely to be attainable in vivo.

Together with our results with DG and the fatty acid β-oxidation inhibitor etomoxir, the present data on BP furthermore indicate that ATP deprivation could be a general strategy for potentiation of the anticancer effects of the clinically important platinum drugs. One possible mechanism may involve decreased DNA repair, although it remains unclear whether this would be due to energy depletion or some type of glucose- and GRP-related stress (Yamada et al., 1999). Furthermore, if sufficiently great, loss of ATP can contribute to drug potentiation by switching the death signal from apoptosis to necrosis in an already stressed cell (Proskuryakov et al., 2003). We believe that this is not the whole explanation for potentiation in the fairly resistant HCT116 cells, since we saw significant BP potentiation also of cisplatin doses far below the apoptotic threshold of circa 10 μM cisplatin. Thus, since BP has the capability to induce ATP deprivation via two different processes (as pointed out also in (Pedersen, 2007)), and is cytotoxic via induction of ROS, it may well potentiate platinum drugs in different tumour cell types with varying glycolytic dependence and varying growth rates.

3. Experimental procedures

3.1. Cell culture and drug treatment

HCT116 colon carcinoma cell lines (p53 wt and a p53−/− subclone) were maintained in McCoy’s 5A modified medium supplemented with 10% foetal calf serum, 1-glutamine, and penicillin/streptomycin. Cells were kept under standard conditions (37 °C and 5% CO₂). For experiments, they were seeded at densities adjusted to the size of the plastic culture dishes and to reach an approximately 75% confluence the next day when cells were treated with drugs. Drugs were then present throughout the experimental incubation. 2-Deoxyglucose, 3-bromopyruvate and oxaliplatin were from Sigma–Aldrich. Cisplatin (Platinol®) was from Bristol–Myers–Squibb. Oxaliplatin was prepared in pure distilled water at the Karolinska Hospital Pharmacy and kept in small single-use aliquots at ~70 °C.

3.2. Metabolite assays

ATP levels were determined using the luciferase-based Apo-sensor ATP Depletion Assay Kit (Alexis Biochemicals) according to the manufacturer’s instructions. The MTT assay and the lactate dehydrogenase (LDH) release assay (both kits from Promega) were performed at the indicated time points and according to the manufacturer’s instructions. LDH levels were assessed both in supernatants and in the remaining cells, in order to assess percent released LDH. Glucose concentrations in culture supernatants and fresh medium were assessed with the QuantiChrome Glucose Assay (BioAssay Systems) according to the manufacturer’s instructions.

3.3. Flow cytometry

To detect changes in mitochondrial inner membrane potential (Δψm), cells were stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes Inc.) which is taken up only by mitochondria with intact membrane potential. Briefly, 10⁶ cells/sample were collected, and TMRE was added to a final concentration of 25 nM, a concentration that remained throughout the experiment. After 30 min of incubation, cells were pelleted, washed once in PBS and TMRE, and then incubated for 10 min in the dark in 100 μl of incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂, 25 mM TMRE). Prior to flow cytometric analysis, another 400 μl of incubation buffer was added. The samples were analysed on a FACS Calibur flow cytometer (Becton Dickinson) using CellQuest Pro software.

3.4. Cell cycle analysis

Cells were fixed in 4% formaldehyde for 18 h, resuspended in 95% ethanol, followed by 1 h of rehydration in distilled water. After treatment with subtilisin–Carlsberg solution (0.1% Sigma protease XXIV, 0.1 M Tris and 0.07 M NaCl (pH 7.5)) and staining with DAPI–sulphorhodamine solution (8 mM DAPI, 50 mM sulphorhodamine 101, 0.1 M Tris and 0.07 M NaCl (pH 7.5)), samples were analysed using a PAS II flow cytometer (Partec, Münster, Germany). DAPI fluorescence was measured above 435 nm. The multicycle program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA) was used for histogram analysis and at least 40,000 nuclei per histogram were analysed.
3.5. Assessment of caspase-mediated apoptosis

Caspase-specific cleavage of cytokeratin-18 (CK18) results in a stable fragment with a neoepitope (CK18-Asp396) that is recognized by the M30 antibody and quantified using the Apopto-sense® ELISA-type assay (Hägg et al., 2002; Kramer et al., 2004).

The assay was performed essentially according to the manufacturer’s instructions (Peviva AB, Sweden). Briefly, 8000–10,000 cells/well were seeded in 96-well plates and treated with drugs as indicated. At the end of treatment, NP-40 was added to the tissue culture medium to 0.5% to lyse floating as well as attached cells. Twenty-five microlitres from each well was removed for the assay.

3.6. Viability assays

Using a Burkger chamber for cell counting, viability was assessed as the number of attached cells remaining after drug treatment for 48 h and expressed as percent of the number of cells in untreated controls. Alternatively, total protein was quantified after precipitation with trichloroacetic acid and staining with sulphorhodamine-B (Sigma–Aldrich), according to the manufacturer’s instructions.

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