Killing multiple myeloma cells with the small molecule 3-bromopyruvate: implications for therapy

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The small molecule 3-bromopyruvate (3-BP), which has emerged recently as the first member of a new class of potent anticancer agents, was tested for its capacity to kill multiple myeloma (MM) cancer cells. Human MM cells (RPMI 8226) begin to lose viability significantly within 8 h of incubation in the presence of 3-BP. The K_m (0.3 mmol/l) for intracellular accumulation of 3-BP in MM cells is 24 times lower than that in control cells (7.2 mmol/l). Therefore, the uptake of 3-BP by MM cells is significantly higher than that by peripheral blood mononuclear cells. Further, the IC_{50} values for human MM cells and control peripheral blood mononuclear cells are 24 and 58 μmol/l, respectively. Therefore, specificity and selectivity of 3-BP toward MM cancer cells are evident on the basis of the above. In MM cells the transcription levels of the gene encoding the monocarboxylate transporter MCT1 is significantly amplified compared with control cells. The level of intracellular ATP in MM cells decreases by over 90% within 1 h after addition of 100 μmol/l 3-BP. The cytotoxicity of 3-BP, exemplified by a marked decrease in viability of MM cells, is potentiated by the inhibitor of glutathione synthesis buthionine sulfoximine. In addition, the lack of mutagenicity and its superior capacity relative to Glivec to kill MM cancer cells are presented in this study. *Anti-Cancer Drugs* 25:673–682 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

3-Bromopyruvate (3-BP) belongs to a new class of potent anticancer agents first described by Pedersen *et al.* [1,2]. 3-BP is quite different from the currently available chemo-drugs that target one or more of the following: DNA replication, cell cycle/growth, cellular signal transduction pathways, angiogenesis, and receptors. Significantly, 3-BP targets the energy metabolism of cancer cells, thus inhibiting their energy production and depleting their energy sources (reserves). 3-BP is a potent 'energy blocker' of cancer cells and is very rapid/effective in killing such cells. In addition, 3-BP at the effective concentrations that kill cancer cells has little or no effect on normal cells.

Multiple-drug resistance of eukaryotic cells to cytotoxic drugs is a major medical problem in terms of both cancer chemotherapy and infectious diseases. 3-BP, which interacts with thiol and thiomethyl groups of cysteine and methionine residues [1], is a strong alkylating agent that inhibits both cellular ATP production sources of cancer cells, that is, glycolysis and mitochondrial pathways, while leaving normal cells unharmed [2–4]. Therefore, 3-BP is a potent anticancer drug with high specificity for cancers and has been used successfully in animals and humans with no apparent side effects [5–11]. 3-BP also inhibits the energy consuming process of angiogenesis, which is critical for cancer growth and metastasis [12]. Multiple myeloma (MM) is an incurable hematologic cancer associated with cytogenetic abnormalities and genomic instability. These include primary translocations of chromosome 14q32, affecting the immunoglobulin heavy chain IgH locus, and 11q13, 16q23, and 20q11, involving the oncogenes *c-maf*, cyclin D1, D3, and FGFR3/MMSET [13,14]. MM patients may express multiple genetic aberrations that affect several oncogenic pathways and cause the accumulation of malignant plasma cells within the bone marrow [15,16]. This disease has a poor prognosis in mammals and is characterized clinically by a broad range of symptoms, including renal insufficiency, hypercalcemia, lytic bone lesions, bone pain, anemia, and finally death [17,18].

Nakano *et al.* [19] have shown in the established MM line RPMI 8226, as well as in primary MM cells from bone marrow samples of MM patients, that concentrations of 3-BP in the range of 30–90 μmol/l induce marked death

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of cancer cells. They have also shown that a combination of doxorubicin and 3-BP suppresses tumor growth in severe combined immunodeficiency mice implanted subcutaneously with RPMI 8226 cells, in which case neither drug alone had a significant effect. In this context, the reversible proteasome inhibitor bortezomib (velcade) may be much more potent than 3-BP in killing MM cells [17]. However, clinically, it is not necessary for a drug to have a highly potent cancer killing effect. Rather, for clinical treatment it is more important that the drug does not promote drug resistance and is not mutagenic. The detailed reason for the specificity of 3-BP to cancer cells is still unknown. The specificity and selectivity of 3-BP to cancer cells is mainly because of its simple and small structure, which mimics the chemical structure of lactate/pyruvate with one carboxylate moiety. This monocarboxylate moiety of 3-BP allows its specific entry through a monocarboxylate transporter (MCT), which is overexpressed in cancer cells exhibiting the Warburg effect. Therefore, 3-BP disguised as lactic acid can 'trick' the cancer cells and enter like a Trojan horse. It has little effect on normal cells as these contain very few lactic acid transporters under normal physiological conditions. In this paper, we report the novel findings that in the MM cell line RPMI 8226, 3-BP is specifically taken up and this uptake correlates with the significant overexpression of the *MCT1* gene encoding the plasma membrane lactate transporter.

Methods

Reagents and media

The following reagents were from the sources indicated in parentheses: antibiotic–antimycotic solution (Gibco BRL, Carlsbad, California, USA), 3-BP (Sigma-Aldrich, St Louis, Missouri, USA), buthionine sulfoximine (BSO; Sigma), dimethyl sulfoxide (Sigma), glutamax (Gibco BRL), heat-inactivated fetal bovine serum (Gibco BRL), Lymphoflot (Biotest AG), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma], RPMI 1640 (Lonza, Basel, Switzerland), trypan blue (Sigma), MES buffer (Sigma), HEPES buffer (Carl Roth GmbH Co. KG, Karlsruhe, Germany), and Glivec/Gleevec Imatinib methanesulfonate (Haoyuan Chemexpress Co., Shanghai, China). [^{14}C]-Labeled 3-BP was a gift from Dr Young H.K.

Cell cultures

The human myeloma cell line RPMI 8226 (ATCC CCL-155, plasmacytoma) was obtained from the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers. The MM cell line and PBMCs were propagated in tissue culture flasks (Nalgene NUNC, Penfield, New York, USA) in complete growth medium containing RPMI 8226 supplemented with 2 mmol/l glutamax, 1% antibiotic–antimycotic solution, and 10% heat-inactivated fetal bovine serum. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. RPMI 8226 cells propagated between 6 and 15 passages were used for tests.

Isolation of human peripheral blood mononuclear cells

PBMCs from healthy adult volunteers were isolated from venous blood samples, collected in the presence of Li-heparin (7.5 ml) as an anticoagulating agent, using density gradient centrifugation (2000 rpm/30 min) and Lymphophlot (1.077 g/ml). After two washes (1400 rpm/10 min) in PBS, PBMCs were resuspended to a final concentration of 10^7 cells/ml in complete growth medium. Cell viability was determined to be greater than 97% by trypan blue exclusion.

The study reported here was approved by the Research Ethics Committee of the Medical University of Wroclaw, Poland, and consent was obtained from all volunteers.

Evaluation of 3-bromopyruvate cytotoxicity

The cytotoxic effect of various concentrations of 3-BP was evaluated using the myeloma cell line RPMI 8226. Control experiments were performed on isolated human PBMCs. Before (24 h) addition of 3-BP, the exponentially growing cells were seeded in flat-bottomed 96-well microplates (NUNC) at a density of 2.5×10^4 cells/well for RPMI 8226 and 2.5×10^5 cells/well for PBMCs in a final volume of 100 µl of complete growth medium. In some experiments cells were pretreated with BSO for 24 h before the addition of 3-BP. Before application, 3-BP was diluted in RPMI 1640 supplemented with antibiotics. To test the sensitivity of the RPMI 8226 myeloma cell line to 3-BP in the presence of BSO, concentrations of 12.5–50 µmol/l 3-BP were used, and kinetic studies were performed after 4, 8, 12, 24, and 48 h of growth. Cell viability was determined by the MTT colorimetric assay as described previously [20]. Briefly, at the end of the incubation, 20 µl MTT (5 mg/ml) was added to each well and the plate was incubated for a further 4 h. The formazan produced was solubilized with 180 µl of dimethyl sulfoxide for 15 min with shaking. After addition of 12.5 µl of Sørensen's phosphate buffer [(0.133 mol/l phosphate, pH 7.2): 0.133 mol/l Na_2HPO_4 and 0.133 mol/l KH_2PO_4, prepared by mixing 71.5 ml of Na_2HPO_4 and 28.5 ml of KH_2PO_4 to obtain a pH of 7.2], the concentration of formazan was determined by optical density measurement at 570 nm using the ASYS UVM 340 spectrophotometer (Biochrom, Cambridge, UK). The cytotoxic activity of the studied compounds is represented by their IC_{50} values, the dose that inhibits the proliferation rate of treated cells by 50% as compared with control untreated cells.

Assay of L-lactate

Assay of L-lactate in the extracellular medium and cell-free extracts of myeloma culture was carried out enzymatically using lactate dehydrogenase from rabbit muscle (Sigma;

148 U/mg). To shift the equilibrium to pyruvate, alkaline buffer and hydrazine as a pyruvate-binding reagent were used [21] (*http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/llacticacidmw.Par.0001.File.tmp/llacticacidmw.pdf*). The assay was conducted in a semikinetic mode using a reagent mixture with the following composition: 0.5 mol/l glycine-KOH buffer with 0.4 mol/l hydrazine sulfate, pH 9.0; 2.5 mmol/l NAD^+; 4.0 U/ml lactate dehydrogenase. Calibration was performed by adding L-lactate at final concentrations of 0.25, 0.75, 1.25, and 2.0 mmol/l. After incubation for 30 min, the difference in absorbance at 340 nm was measured for samples compared with blank at the start point (near 0 min) and after 30 min of incubation. The samples of extracellular medium, treated with trichloroacetic acid, were neutralized with KOH. Extraction from the cells was performed using hot 75% ethanol plus 70 mmol/l HEPES (pH = 7.5): incubation at 80°C for 5 min, followed by drying and solubilization in water up to a final volume equal to the aliquot of the culture (7 ml).

Influence of 3-bromopyruvate on the intracellular ATP level

RPMI 8226 cells (2.5×10^4 or 5×10^4) were plated in 96-well microplates and incubated in 100 µl of complete growth medium. After 24 h of incubation, aliquots of 100 µl of various concentrations of 3-BP diluted in RPMI 1640 were added, and the cells were further incubated up to 120 min. The cell lysates were prepared at different time points (0, 15, 30, 60, 90, 120 min). Intracellular ATP levels were determined using the ATPlite Luminescence Assay System (PerkinElmer Inc., Waltham, Massachusetts, USA) and the PerkinElmer EnSpire Multimode Plate Reader.

Radioactive substrate transport assay

Assay of the uptake of [^{14}C]-labeled 3-BP was carried out by the method reported for L-lactate transport [22]. The harvested RPMI 8226 cells and PBMCs were washed twice in HEPES buffer (pH 7.4) and resuspended to a final concentration of 5×10^7 cells/ml in the transport buffer (25 mmol/l D-glucose, 137 mmol/l NaCl, 5.37 mmol/l KCl, 0.3 mmol/l Na_2HPO_4, 0.44 mmol/l KH_2PO_4, 4.17 mmol/l $NaHCO_3$, 1.26 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$), with 10 mmol/l MES for pH 6.0 or 10 mmol/l HEPES for pH 7.4. Aliquots of 10 µl were incubated at 37°C with various concentrations of the radioactive [^{14}C]-labeled 3-BP solution in HEPES/MES buffer. The reaction was stopped rapidly by washing with ice-cold HEPES/MES buffer, and filtration on a nitrocellulose filter was carried out thereafter. Radioactivity of each sample in scintillation fluid was measured using the Beckman LS100 scintillation counter (PerkinElmer).

Relative quantification by real-time polymerase chain reaction

Total RNA was isolated from fresh human myeloma cells and from PBMCs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were determined using the Nanophotometer Pearl, IMPLEN by measuring the absorbance at 260 and 280 nm. The total RNA samples were treated with DNase for 30 min at 37°C (Thermo Scientific, Waltham, Massachusetts, USA). The isolated RNA from each sample was reverse-transcribed to single-stranded cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California, USA). Relative quantifications were performed on a 7500 real-time PCR System (Applied Biosystems) using the DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific Finnzymes). The thermal cycling program was set as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. The primers specific to human genes were designed on the basis of their sequences in the GenBank database (accession no.: NM_003051.3 – *MCT1*; NM_004731.4 – *MCT2*; NM_013356.2 – *MCT3*; NM_004207.3 – *MCT4*). The sequences of the specific forward and reverse primers were as follows: 5′-GTGGCTCAGCTCCGTATTGT-3′ and 5′-GAGCCGACCTAAAAGTGGTG-3′ for *MCT1*; 5′-CATTATATTCTTGGCTCCATATGCTAAA-3′ and 5′-TACTGAATTCGAGGTCGAATATATTTG-3′ for *MCT2*; 5′-GGCCGCACGTCCCGTATCTG-3′ and 5′-GGGCGG TCCGATGAGCACAG-3′ for *MCT3*; 5′-AAGGTGCGG CCCTACTCCGT-3′ and 5′-GCCGTAGAACCCGCCAGG TC-3′ for *MCT4*; 5′GGCATGGCCTTCCGTGTCCC-3′ and 5′-TGCCAGCCCCAGCGTCAAAG-3′ for *GAPDH* as a housekeeping gene. Standard curves were prepared for each target and housekeeping gene. The software provided by the real-time PCR system producer (Applied Biosystems) calculates the relative amount of the target gene and the housekeeping gene on the basis of the C_q values. The final results were processed according to that followed in the study by Bustin *et al.* [23]. Statistical analysis was carried out using Student's *t*-test in the program Statistica 7 (StatSoft, Tulsa, Oklahoma, USA).

Ames test

The mutagenicity test was performed using a classic preincubation assay [24,25] with 3-BP or Glivec/Gleevec. Four *Salmonella typhimurium* strains sensitive to frame-shift mutations: TA97, TA98, TA1537, TA1538, were used, as well as two strains sensitive to substitution mutations: TA100, TA1535. *S. typhimurium* strains were obtained from the Polish Collection of Microorganisms of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. All tests were repeated at least three times. All charts and calculations were made using the GraphPad Prism 5 program (GraphPad Software, La Jolla, California, USA).

Results

In-vitro 3-bromopyruvate is more potent against myeloma RPMI 8226 cells than normal peripheral blood

mononuclear cells because the uptake of 3-bromopyruvate by RPMI 8226 cells is significantly higher than that by peripheral blood mononuclear cells

The human myeloma cells RPMI 8226 actively divided *in vitro*, and after 24 h of growth the content of L-lactate in the extracellular liquid of these cancer cells was 3 mmol/l compared with 1 mmol/l at time zero of incubation (data not shown). However, in the case of PBMCs used as a controls *in vitro*, no secretion of L-lactate was observed in the complete growth medium. This assures that the cancer cells, RPMI 8226, have high glycolytic metabolism compared with control cells. To determine whether 3-BP has a stronger cytotoxic effect against myeloma RPMI 8226 cells compared with normal PMBCs, we performed a cytotoxic MTT assay. During the 24-h treatment of cancer cells (RPMI 8226) with various concentrations of 3-BP, the viability of the cells was significantly decreased. As shown in Fig. 1a, after 24 h of growth in the presence of the studied agent, the myeloma cells were more sensitive to 3-BP than normal cells. The IC_{50} for 3-BP in the case of RPMI 8226 myeloma cancer cells and normal PMBCs was 23.7 ± 1.0 and 57.9 ± 1.1 μmol/l, respectively.

To assess whether the toxicity of 3-BP toward RPMI 8226 cells is a consequence of its increased uptake, we performed a transport assay comparing uptake velocities of radiolabeled 3-BP in PBMCs and RPMI 8226 cells (Fig. 1b). The data show a significantly higher level of uptake of 3-BP in RPMI 8226 cells (with a K_m of 0.3 mmol/l) compared with control PBMCs (with a K_m 7.2 mmol/l). Thus the K_m value of 3-BP uptake for RPMI 8226 cells was 24-fold lower than that for PBMCs. These data implicate the uptake differences as one of the crucial factors in the selective toxicity of 3-BP toward cancer cells. We also tested whether the uptake of 3-BP in RPMI 8226 cells is pH-dependent. An accumulation assay using two concentrations of 3-BP (0.05 and 0.5 mmol/l) at pH 6 and pH 7.4 was performed in 20 min (Fig. 1c). At a concentration of 0.05 mmol/l 3-BP, the accumulation level was low and did not exceed 1 nmol/10^6 cells throughout the assay. The pH change had no effect on accumulation at this concentration of 3-BP. However, at the end of the assay (after 20 min) with the higher concentration of 3-BP (0.5 mmol/l), the accumulation of 3-BP at pH 6 was almost 4 nmol/10^6 cells compared with almost 2.5 nmol/10^6 cells at a pH of 7.4. This clearly shows the influence of the proton motive force on the uptake efficiency of 3-BP. This result suggests that a proton symporter such as MCT may be the pathway for the entry of 3-BP into the cells and confirms the data on increased MCT expression in RPMI 8226 cells.

The higher uptake of 3-bromopyruvate probably resulted from the higher expression of the *MCT1* gene in the RPMI 8226 cell line

To test for the expression of genes encoding MCTs, real-time PCR was performed. In the experiment, the relative expressions of the *MCT1*, *MCT2*, *MCT3*, and *MCT4* genes in myeloma RPMI 8226 cells in comparison with PBMCs were determined (Fig. 2). *MCT1* expression in MM cells was significantly higher than that in control cells and almost 12 times higher than that of *MCT4*. The differences in the expressions of that gene was statistically significant at P less than 0.01. The expression levels of *MCT2*, *MCT3*, and *MCT4* in MM cells were at the same level as or only slightly higher than that in PBMCs, and the difference was not statistically significant.

A rapid decrease in intracellular ATP levels caused by 3-bromopyruvate is the main reason for cell death

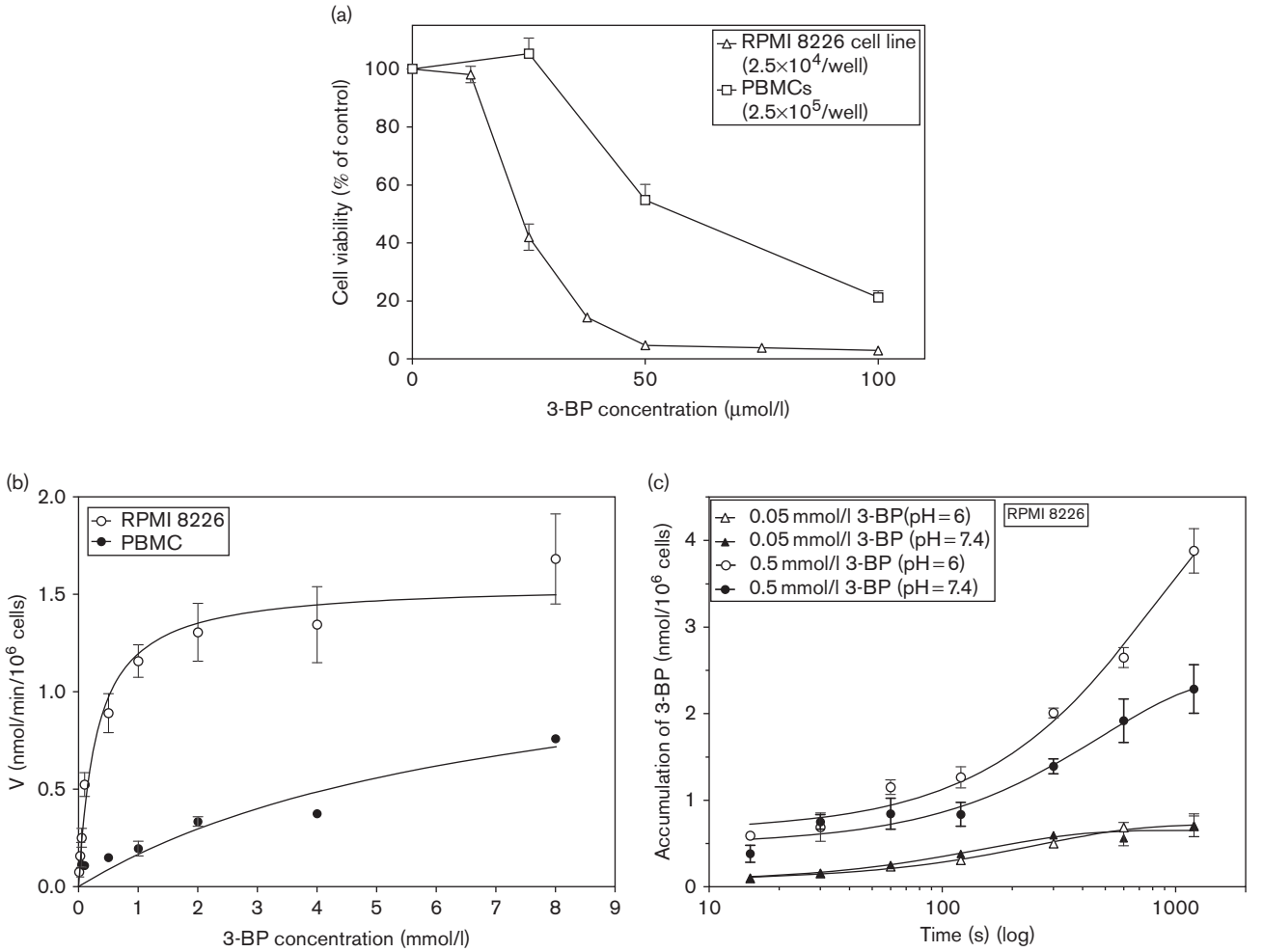
To assess the influence of 3-BP on the intracellular levels of ATP in the RPMI 8226 cells, we performed an ATP assay with the ATP levels being measured six times during an incubation of 120 min. Figure 3 shows the change in the intracellular ATP levels in the RPMI 8226 cell line during incubation with 10, 20, 50, and 100 μmol/l 3-BP. The studied agent at the concentrations of 10 and 20 μmol/l did not cause any decrease in the ATP level, and one can even observe a weak stimulation. However, when 50 and 100 μmol/l of 3-BP were used, the intracellular ATP levels decreased by over 70 and 90%, respectively, within 120 min.

Buthionine sulfoximine increases sensitivity of RPMI 8226 cancer cells to 3-bromopyruvate by increasing ATP depletion at the beginning of treatment

The intracellular level of glutathione influences the sensitivity of different cancers to 3-BP such as MM [26] and solid tumors of the liver, that is, hepatocarcinomas [3]. Therefore, in our study we used BSO, a known glutathione-depleting agent that is not toxic to mammalian cells [27]. We determined whether the potentiating effect of BSO on the cytotoxic activity of 3-BP against cancer cells is dependent on its concentration. The purpose of this study was to determine the IC_{50} value of 3-BP for killing RPMI 8226 cancer cells in the absence and also in the presence of BSO. Figure 4 shows that pretreatment of RPMI 8226 cells with 100, 200, and 500 μmol/l BSO decreases the IC_{50} for 3-BP from $\sim$24 to $\sim$13 μmol/l and thus decreases the effective concentration of 3-BP by two-fold independently of the amount of BSO used.

To test the sensitivity of RPMI 8226 cells to 3-BP (concentrations from 12.5 to 50 μmol/l) in the presence of BSO (100 μmol/l), kinetic studies were performed within 48 h of growth. As presented in Fig. 5, we found that BSO significantly ($0.000001 < P < 0.05$) increases the cytotoxic effect of 3-BP, showing a decrease in cell viability, usually between 13 and 48% for particular time points within 48 h of incubation. The weakest potentiating effect of BSO on the cytotoxicity of 3-BP was observed at the highest 3-BP concentration (50 μmol/l). The results indicate that within the first 12 h of incubation, 3-BP in combination with BSO affected approximately

Fig. 1



(a) Viability of cancer and normal cells after 3-BP treatment. RPMI 8226 cells (MM cells) and PBMCs (normal cells) at initial cell densities of 2.5×10^4 and 2.5×10^5, respectively, were grown in 100 μl of complete growth medium for 24 h and then 100 μl of serially diluted anti-cancer agent was added for an additional 24 h. The cell viability was determined using the MTT assay. The results of two experiments with six replicates each were averaged. The results are presented as mean±SEM. (b) The 3-BP uptake assay in the cancer RPMI 8226 cells and control PBMCs. The uptake of radiolabeled 3-BP in cancer RPMI 8226 cells is much more efficient than in control cells. Moreover, the K_m is about 24-fold lower in cancer cells than in control cells. (c) Characteristics of 3-BP accumulation in the RPMI 8226 cells during 20 min of incubation under the indicated conditions. 3-BP, 3-bromopyruvate; MM, multiple myeloma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMCs, peripheral blood mononuclear cells.

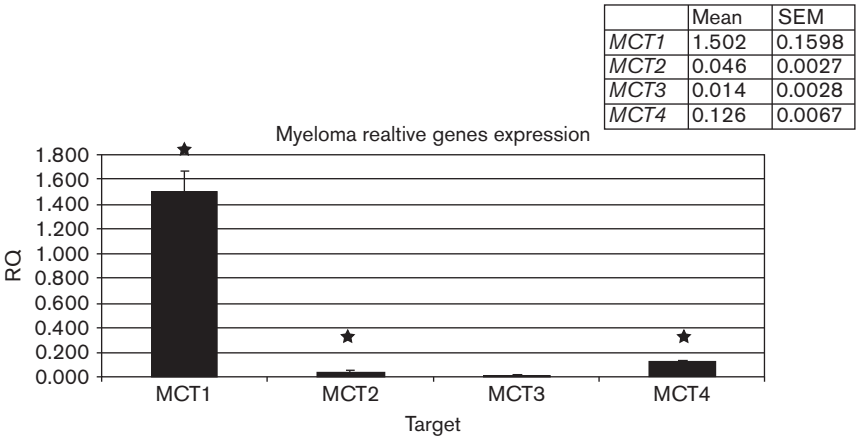
the same number of cells at least 4 h faster than 3-BP alone. The results clearly show the synergistic cytotoxic action of 3-BP and BSO against myeloma cancer cells.

Consequently, in our study we have also assessed the influence of BSO on the levels of the intracellular ATP deprivation caused by 3-BP in RPMI 8266 cells (Fig. 6). Cotreatment of 3-BP with BSO had no significant short-term effect on the ATP levels in the RPMI 8226 cell line. However, the effect is visible in the first time point after 15 min of incubation with 50 μmol/l 3-BP. Thus, there is a 20% decrease in ATP concentration compared with a decrease of 60% when using 50 μmol/l 3-BP together with 100 μmol/l BSO. These data are coherent with the hypothesis that the obvious synergistic effect of BSO and 3-BP is the result of long-term glutathione deprivation, which leads to increased mortality of the cancer cells.

A test that excludes mutagenicity supports the use of 3-bromopyruvate as a therapeutic drug

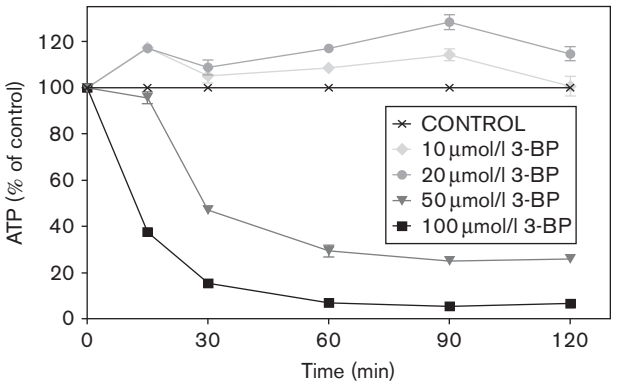
Being a potential therapeutic, 3-BP should exhibit minimal mutagenicity. As this information was not found in the literature, we performed the Ames' test [24] to determine possible mutagenic properties of 3-BP compared with the known anticancer drug Glivec/Gleevec (Fig. 7). The Ames' test allows the screening for specific induced point mutations, that is, insertions, deletions, and substitutions. Six *S. typhimurium* strains were used – four sensitive to frame-shift mutations: TA97, TA98,

Fig. 2



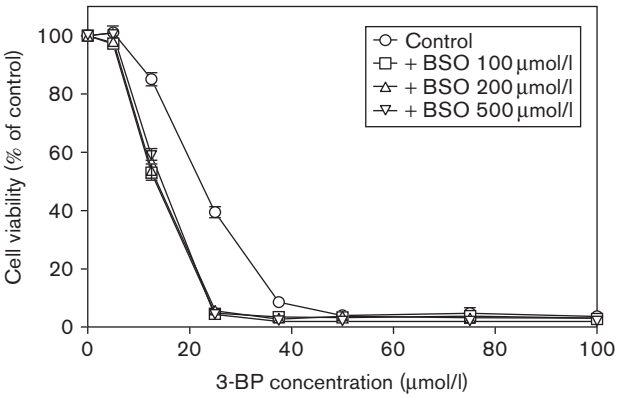
The relative quantification of *MCT1*, *MCT2*, *MCT3*, and *MCT4* genes using real-time PCR. The diagram shows the relative quantification of *MCT* genes in cancer cells relative to their expression in PBMCs. Means and SEM were calculated on the basis of independent values obtained in three experiments performed in triplicate. The values of gene expressions are shown as *x*-fold of the gene expressions in controls. *Statistically significant results at $P<0.01$, as determined by Student's *t*-test with Bonferroni correction. The final results were processed according to Bustin *et al.* [23]. MCT, monocarboxylate transporter; RQ, relative quantification.

Fig. 3



Influence of 3-BP on the intracellular levels of ATP in RPMI 8266 cells in the first 120 min after application. Data are represented as a percentage of the ATP levels in the control cells (not treated). The chart illustrates results from four experiments (mean±SEM) using a starting cell density of 2.5×10^4 cells/ml. Similar results were obtained with cell densities of 2.5×10^5 cells/ml. 3-BP, 3-bromopyruvate.

Fig. 4



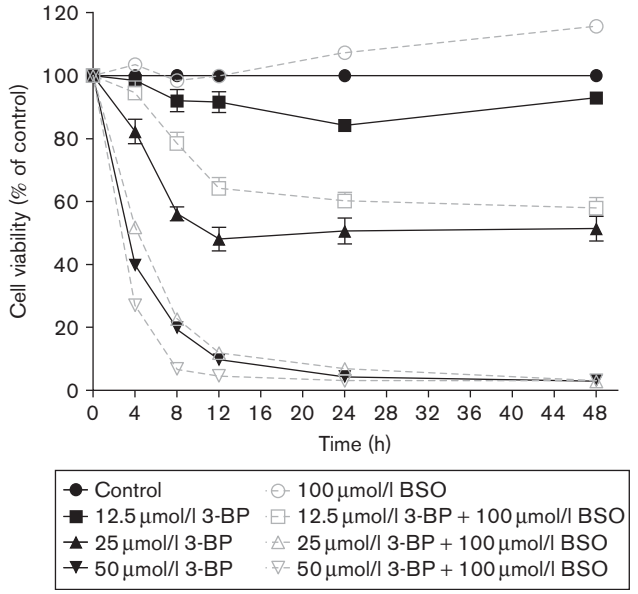
Cytotoxicity of 3-BP on MM cells pretreated with different concentrations of BSO. The cytotoxic effect of exposure to 3-BP over 24 h on the RPMI 8226 cancer cell line at an initial density of 2.5×10^4 was determined in the absence or presence of 100–500 µmol/l BSO. For each point the cell viability was determined using the MTT assay. Each result was expressed as the mean±SEM of six replicates. 3-BP, 3-bromopyruvate; BSO, buthionine sulfoximine; MM, multiple myeloma.

TA1537, TA1538, and two sensitive to substitution mutations: TA100, TA1535. Sodium azide and acriflavin were used as positive controls for TA100 and TA1535, and TA97, TA98, TA1537, and TA1538, respectively. The results are shown as a percentage of the number of revertants in the positive control. The negative control represents the number of spontaneous reversions. Compared with positive and negative controls, none of the tested 3-BP concentrations induced a significant amount of revertants in *S. typhimurium* TA strains. As in the negative control, the maximum number of revertants induced by 3-BP did not exceed 3% of those induced by positive controls. Glivec/Gleevec was shown to induce less than 4% (0.2 and 0.4 mmol/l) and less than 6% (1 mmol/l) revertants compared with the positive control.

Compared with Glivec/Gleevec, 3-bromopyruvate showed superior anticancer activity

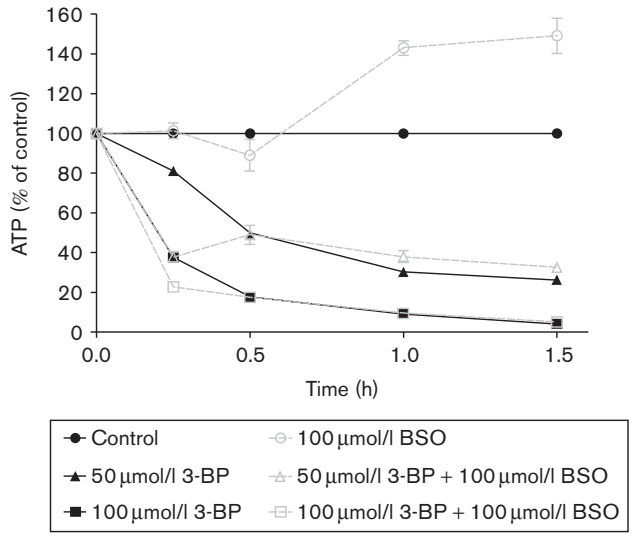
To determine whether 3-BP had a stronger cytotoxic effect than Glivec/Gleevec, a well-known antimyeloma agent, we performed a cytotoxic MTT assay. During a

Fig. 5



Kinetics of the cytotoxicity of 3-BP on RPMI 8226 cells in the presence of BSO during a 48-h incubation. Cells at a density 2.5×10^4 cells/well were pretreated with 100 µmol/l BSO for 24 h before the addition of 3-BP. BSO remained present throughout the experiment. For each time point, the cell viability was determined using the MTT assay. Each result is expressed as the mean±SEM of six replicates. 3-BP, 3-bromopyruvate; BSO, buthionine sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Fig. 6



Influence of 3-BP and BSO cotreatment on the intracellular levels of ATP in RPMI 8266 cells in the first 90 min after application. Data are represented as a percentage of the ATP levels in the control cells (not treated). The chart illustrates results from four experiments (mean±SEM) using a starting cell density of 2.5×10^4 cells/ml. Similar results were obtained with cell densities of 2.5×10^5 cells/ml. ATP levels were determined as described in the Materials and methods section. 3-BP, 3-bromopyruvate; BSO, buthionine sulfoximine.

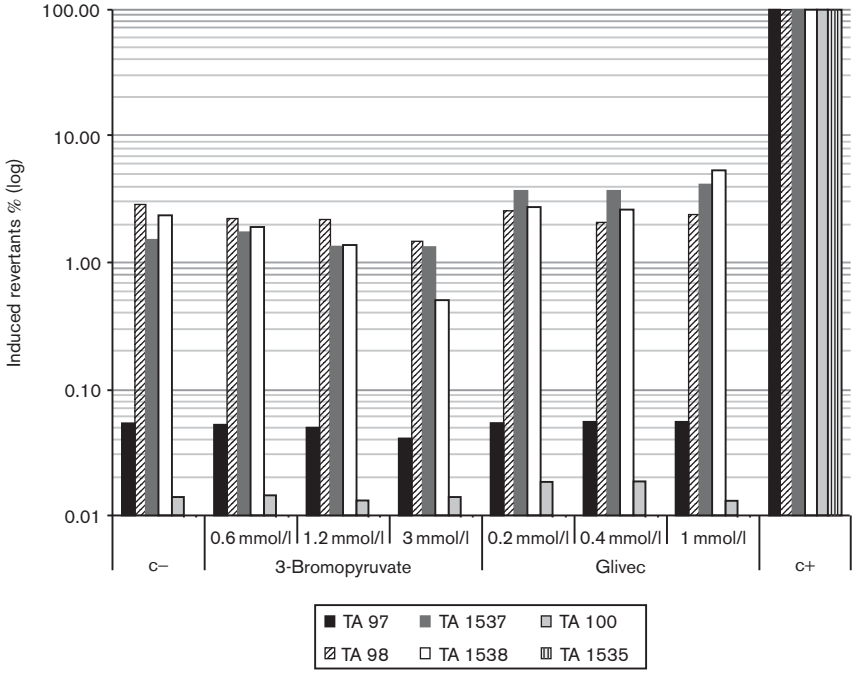
24-h treatment of cancer cells (RPMI 8226) with various concentrations of Glivec/Gleevec, the viability of the cells significantly decreased. Both Glivec/Gleevec (Fig. 8a) and 3-BP (Fig. 1) acted in a dose-dependent manner. However, as shown in Fig. 8a, after 24 h of growth in the presence of the studied agent, the myeloma cells were less sensitive to Glivec/Gleevec compared with 3-BP (Fig. 1). The IC_{50} for Glivec/Gleevec was 33.2±1.0 µmol/l, whereas that for 3-BP was 23.7±1.0 µmol/l. The sensitivity of cancer cells to both agents was higher than that of control PBMCs, showing an ~1.5-fold increased anticancer potency for Glivec/Gleevec and ~2.5-fold increased anticancer potency for 3-BP. The IC_{50} doses of Glivec/Gleevec and 3-BP for PBMCs were 45.9±1.1 and 57.9±1.1 µmol/l, respectively.

To assess the influence of Glivec/Gleevec on the intracellular levels of ATP in the RPMI 8226 cells, we performed an ATP assay with the ATP levels being measured six times during the incubation period of 120 min. Figure 8b shows the change in the intracellular ATP levels in the RPMI 8226 cell line during incubation with 10, 20, 50, and 100 µmol/l Glivec/Gleevec. 3-BP at concentrations of 10 and 20 µmol/l did not cause any decrease in the ATP level (Fig. 3). In contrast, 20 µmol/l Glivec/Gleevec caused a 30% decrease in ATP after the first 15 min. Nevertheless, this did not lead to a further fall in the ATP level. However, a 120-min incubation with 50 µmol/l Glivec/Gleevec results in a greater than 70% decrease in the intracellular ATP level, and an almost 100% decrease at a concentration of 100 µmol/l. These data show that in the RPMI 8226 cell line, Glivec/Gleevec is a more efficient ATP-depleting agent when compared with 3-BP when considering short-term effects.

Discussion

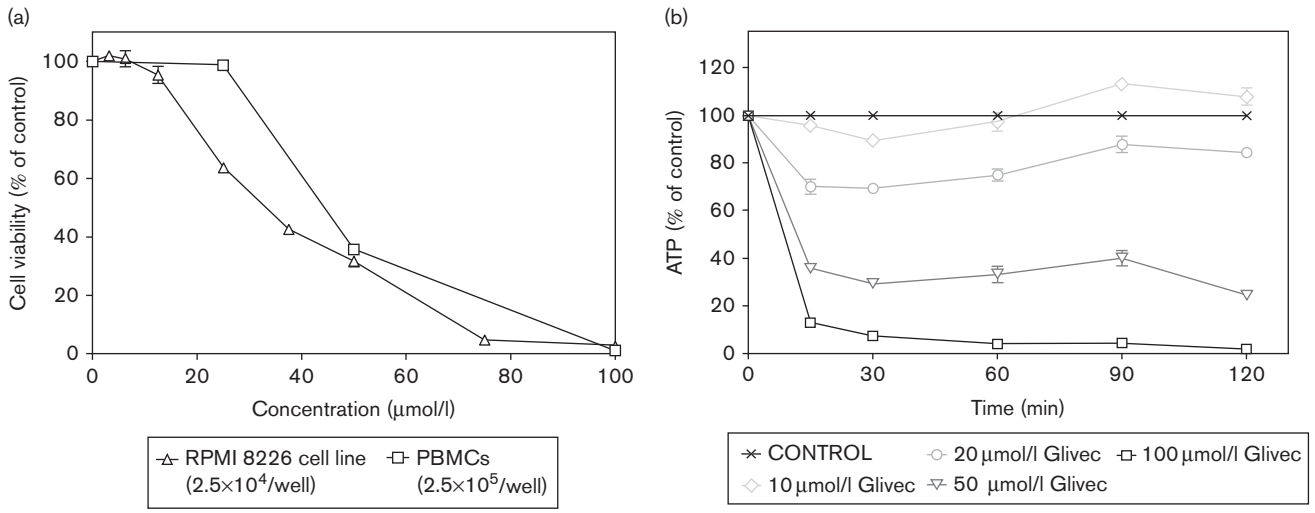
Our studies demonstrate that 3-BP exhibits antimyeloma activity. 3-BP causes rapid intracellular ATP depletion, that is, before MM cell death. This occurs even at concentrations as low as the IC_{50} value. Preferential accumulation of 3-BP in cancer cells explains the differences in susceptibility of MM cells and PMBCs toward 3-BP. Compared with control cells, hematologic MM cancer cells highly express the gene encoding the MCT1 transporter, which is likely responsible for 3-BP uptake. Moreover, as shown earlier, different levels of Jen1 transporter activity [28] may be responsible for different levels of accumulation of 3-BP and toxicity in the yeast *Saccharomyces cerevisiae* [29]. Different susceptibilities toward 3-BP may be connected with different levels of lactate/pyruvate metabolism and thus different levels of intracellular lactate and glutathione. Natural resistance to 3-BP is probably related to different levels of activity of the pyruvate/lactate transporters in these cancer cells. Different cancer cell types, for example, MM, melanoma, liver, and other cancers, overexpress different MCT transporters to different levels [30–33]. Our results indicate that MM cells highly express the

Fig. 7



Results of the Ames' test performed on six *Salmonella typhimurium* strains – TA97, TA98, TA1537, TA1538, TA100, and TA1535 – with 3-BP and Glivec/Gleevec. Charts represent the percentage of induced revertants compared with the positive control C+ (sodium azide and acriflavine) and negative control C–. 3-BP, 3-bromopyruvate.

Fig. 8



(a) Viability of cancer and normal cells after Glivec/Gleevec treatment. RPMI 8226 cells (MM cells) and PBMCs (normal cells) at initial cell densities of 2.5×10^4 and 2.5×10^5, respectively, were grown in 100 μl of complete growth medium for 24 h. Thereafter, 100 μl of serially diluted anticancer agent was added for an additional 24 h. The cell viability was determined using the MTT assay. The results of two experiments with six replicates each were averaged. The results are presented as mean±SEM. (b) Influence of Glivec/Gleevec on the intracellular levels of ATP in RPMI 8266 cells in the first 120 min after application. Data are represented as a percentage of the ATP levels in the control cells (not treated). The chart illustrates results from four experiments (mean±SEM) using a starting cell density of 2.5×10^4 cells/ml. Similar results were obtained with cell densities of 2.5×10^5 cells/ml. 3-BP, 3-bromopyruvate; MM, multiple myeloma; PBMCs, peripheral blood mononuclear cells.

MCT1 gene that encodes a transporter that is likely responsible for 3-BP uptake. Moreover, BSO reduces the IC_{50} from 24 to 13 μmol/l 3-BP. This indicates that the resistance toward 3-BP is related to a natural level of intracellular glutathione. Therefore, we demonstrated that BSO at 100 μmol/l achieved its maximal effect on the intracellular glutathione level using higher concentrations of BSO. It is well known that BSO irreversibly inhibits γ-glutamylcysteine synthetase and contributes to intracellular glutathione depletion and oxidative stress induction [34].

We propose the following mechanism, which seems valid not only for all tested solid tumors but also for blood and skin cancers cells such as MM and melanoma as well. The development of cancer requires a series of mutations, which elicit the 'Warburg' phenotype characterized by stimulation of the rate of glycolysis, as well as by a decreased but significant mitochondrial activity [35,36]. Increased glycolysis induced by the overexpression and binding of hexokinase-2 to the mitochondria assures proper channeling of mitochondrial ATP into the cytoplasm [2]. In addition, increased glycolysis leads to the accumulation of L-lactate in cancer cells. Intracellular L-lactate induces the expression of one or more of the reversible monocarboxylate/proton symporters MCT1, MCT2, MCT3, MCT4, and/or SMCT1, which allow L-lactate to exit cancer cells. Externally added 3-BP enters preferentially into cancer cells because of the low K_m (0.30 mmol/l 3-BP) of the induced MCT(s) at the plasma membrane level. As 3-BP is an alkylating agent, it pyruvylates and inactivates the SH group of accessible cysteine residues [37]. Among these, certain enzymes and/or complexes involved in ATP producing pathways (glycolysis and oxidative phosphorylation) such as hexokinase-2, glyceraldehyde 3-phosphodehydrogenase (glycolysis), and respiratory complexes II and/or III (mitochondrial oxidative phosphorylation) are particularly sensitive to 3-BP [2]. Thus, an increased permeability to 3-BP induced by the MCT1 transporter decreases the level of intracellular ATP and leads to apoptosis [38]. Fortunately, 3-BP is a polar compound and is thus not a substrate for the multiple-drug resistance network, which inactivates many anticancer drugs in mammals [39,40]. This was confirmed by our earlier study on the pleiotropic drug resistance network in the yeast *S. cerevisiae* [29,41]. Considering the antifungal activity of 3-BP [42], its strong anticancer activity in solid and blood tissues, its weak cytotoxicity and genotoxicity, as well as its invulnerability to the multiple-drug resistance/pleiotropic drug resistance network, this small molecule may prove to be an effective anticancer drug [43] for almost all human and animal cancer cell types regardless of origin.

Finally, 3-BP is compared with Gleevec, a well-known tyrosine kinase inhibitor used mostly in the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia [44]. 3-BP is more specific and effective in killing MM cancer cells than Gleevec. In addition, 3-BP is less mutagenic and non-drug resistant than Gleevec. It is worth noting that Gleevec kills the RPMI 8226 cancer cells by depleting cellular ATP. Therefore, it is speculated that Gleevec acts as a metabolic inhibitor by binding nonspecifically to several tyrosine kinases and ATP binding/hydrolyzing proteins including ATP synthasomes [45–47]. Significantly, our results are consistent with the view of Dr Thomas Seyfried that cancer is a disease related to energy metabolism (i.e. metabolic disease) [48].

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Conflicts of interest

There are no conflicts of interest.

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