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Transport and cytotoxicity of the anticancer drug 3-bromopyruvate in the yeast *Saccharomyces cerevisiae*

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Abstract We have investigated the cytotoxicity in *Saccharomyces cerevisiae* of the novel antitumor agent 3-bromopyruvate (3-BP). 3-BP enters the yeast cells through the lactate/pyruvate H^+ symporter Jen1p and inhibits its cell growth at minimal inhibitory concentration of 1.8 mM when grown on non-glucose conditions. It is not submitted to the efflux pumps conferring Pleiotropic Drug Resistance in yeast. Yeast growth is more sensitive to 3-BP than Gleevec (Imatinib methanesulfonate) which in contrast to 3-BP is submitted to the PDR network of efflux pumps. The sensitivity of yeast to 3-BP is increased considerably by mutations or chemical treatment by buthionine sulfoximine that decrease the intracellular concentration of glutathione.

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Abbreviations

3-BP	3-bromopyruvate
BSO	Buthionine sulfoximine
GSH	Glutathione
YNB	Yeast nitrogen base
HX2	Hexokinase 2
MIC	Minimal inhibitory concentration
PDR	Pleiotropic drug resistance

Introduction

The small molecule 3–bromopyruvate (3-BP) inhibits both cell motility and pyruvate release in African Trypanosomes (Barnard et al. 1993) as well as the growth of *Toxoplasma gondii* (De Lima et al. 2011). Recently it was found that 3-BP is a novel anticancer drug that reduces dramatically the level of ATP in a variety of cancer models followed by cell death (Ko et al. 2004; Mathupala et al. 2010; Ko et al. 2001; Qin et al. 2010; Porporato et al. 2010). This indicates that 3-BP inhibits both mitochondrial and glycolytic ATP production (Mathupala et al. 2010; Ko et al. 2001; Geschwind et al. 2002) in malignant tumors that are submitted to the "Warburg effect" characterized by increased glycolysis and abundant production of lactate in the presence of oxygen. The "Warburg effect" has been related to overexpression of hexokinase 2 (HX2) and its binding to mitochondria (Ko et al. 2001; Geschwind et al. 2002; Mathupala et al. 2009; Pedersen 1978; Pedersen 2007). It also involves the monocarboxylate transporters MCT1 & MCT4 and the mitochondrial phosphate carrier (PIC) (Mathupala et al.

2010; Izumi et al. 2011; Mathupala et al. 2006). However, how 3-BP selectively affects only cancer cells is not understood.

In this study we have examined the transport and the mechanism of cytotoxicity of 3-BP in the yeast *Saccharomyces cerevisiae*. This non pathogenic, unicellular eukaryotic model possesses uniquely sensitive genetic and genomic tools (Goffeau et al. 1996) that can be indicative of similar modifications occurring in the human system (Karathia et al. 2011; Mager and Winderickx 2005) including those involved in cancer chemotherapy (Suter et al. 2006; Cardenas et al. 1999; Dylag et al. 2010; Kurtz et al. 2005). We report that 3-BP is toxic to yeast growth. Its major transporter is the lactate/pyruvate permease Jen1p. 3-BP is not submitted to the efflux pumps involved in the Pleiotropic Drug Resistance (PDR) network. Its intracellular cytotoxicity is strongly modulated by the level of reduced glutathione (GSH) that can be decreased by mutations in the pathway for GSH synthesis or by treatment with buthionine sulfoximine (BSO).

Materials and methods

Strains and growth conditions

The yeast *Saccharomyces cerevisiae* haploid strains used in this work are isogenic to W303-1A, BY4741 and US50-18C parental strains, described in Table 1. Strains were grown at 28°C in standard rich (YPD) medium or in synthetic minimal medium (YNB) containing 0.67% yeast nitrogen base without aminoacids. The minimal YNB media was supplemented, when necessary, with appropriate aminoacids and/or adenine and uracil to a final concentration of 20 μg/ml and with 2% sucrose, glucose, galactose or mannose. Complete medium: 1% Bacto-peptone plus 1% yeast extract with 2% glucose or with lactic acid contained 0.5% lactate, pH=5. For plating the media were solidified with 2% of Bacto-agar. Liquid cultures were grown at 28°C and 160 rev./min.(Kaiser et al. 1994).

Sucrose, galactose, mannose, daunorubicin, rhodamine 6 G, glutathione and buthionine sulfoximine were purchased from Sigma-Aldrich (USA); yeast extract, Bacto-peptone, yeast nitrogen base, Bacto-agar were from Difco (USA); Imatinib methanesulfonate was from Haoyuan Chemexpress Co., China. The [^{14}C]-labeled 3-BP was kindly donated as a gift from Dr. Young H. Ko.

Spot tests

To determine MIC (minimal inhibitory concentration) of a tested compound toward the selected yeast strain and to compare the relative susceptibility of several strains, the cells were grown to mid-log phase, diluted to OD_{600}=0.25 and spotted (3 μl) in 10-fold serial dilutions onto the agar plates containing various concentrations of a tested compound. Plates were incubated at 28°C for 72–120 h and photographed. The sensitivity assays were repeated a minimum of three times. Differences in growth show variability of the tested strain in their susceptibility to the tested inhibitor (Kaiser et al. 1994).

Radioactive substrate transport assay

The uptake of the [^{14}C]-labeled 3-BP was carried out by the method reported for L-lactate transport by Casal et al. (Casal et al. 1999). Cells were grown to exponential phase at 28°C in rich (YPD) medium with 2% glucose. The cells were harvested, washed twice with ice-cold de-ionized water, resuspended in rich medium with lactic acid and incubated for 4 h. After incubation cells were harvested, washed twice and resuspended in ice-cold de-ionized water to a final concentration of 200 mg/ml (wet weight). Aliquots of 10 μl of cells were incubated for 30 s with different concentrations of the radioactive [^{14}C]-labeled 3-BP in phosphate buffer at 30°C. The reaction was stopped with ice-cold water and the suspension was filtrated on nitrocellulose Whatman filters using a vacuum filtration box (Hoefer, USA). Radioactivity of each sample was measured using a scintillation fluid (Perkin-Elmer) and a scintillation counter Beckman LS100. All charts and calculations were made using GraphPad Prism 5 program.

Determination of reduced glutathione

The yeast metabolite extracts were prepared according to Gonzales et al. (Gonzalez et al. 1997). The 5 ml overnight yeast cultures grown on minimal medium with sucrose up to OD_{600}=1.5–1.8 were centrifuged and added to 5 ml of the buffered, boiling ethanol (75% ethanol, 70 mM HEPES, pH 7.5). After incubation for 3 min at 80°C, the samples were cooled on ice for 3 min and the volume of the extraction mixture was reduced by evaporation for 3 h at 45°C. The residue was re-suspended in 250 μl of double distilled water and centrifuged for 5 min, at 14,000 rpm to remove the insoluble material. The whole solubilized sample was added to 2.5 ml of 0.1 M sodium phosphate buffer (pH 8.0) and mixed and 50 μl of 4 mg/ml DTNB (5,5′-dithio-bis-[2-nitrobenzoic acid]) was added (Ellman 1959). The reaction was developed for 15 min and the absorbance at 412 nm was measured with a Varian Cary 300 Spectrophotometer in the the double beam mode using 2.75 ml of the described sodium phosphate buffer with 50 μl of 4 mg/ml DTNB as the reference. The final concentration of the glutathione in the sample was determined on the basis of a standard curve using purchased glutathione as standard.

Table 1 Yeast strains used

Strain	Background	Description and usage	References
W303-1A	W303-1A; (*ade2-1, leu2-112, his3-11,15, trp1-1, ura3-1*)	Wild-type strain, isogenic to BLC203 except for the presence of *JEN1*.	Thomas et al.(Thomas and Rothstein 1989).
BLC203 (ΔJEN1)	W303-1A; (*ade2-1, leu2-112, his3-11,15, trp1-1, ura3-1, ΔJEN1*)	Strain in which the lactate transporter gene *JEN1* is deleted. Used to test 3-BP transport as a negative control.	Casal et al. (Casal et al. 1999).
EM01	US50-18 C; (*ura3, his1*)	Wild-type strain, isogenic to US50-18 C and AD strains.	From Maciaszczyk-Dzubińska E. [not published]
US50-18 C	US50-18 C; (*ura3, his1, pdr1-3*)	Strain with the activating mutation *pdr1-3 in the* gene encoding the transcription factor Pdr1p. This strain is hyper-resistant to all drugs which are Pdrp substrates.	Balzi et al. (Balzi et al. 1987).
AD1-3	US50-18 C; (*ura3, his1, pdr1-3, Δyor1, Δsnq2, Δpdr5*)	Strain with deletion of 3 genes encoding the major Pdr transporters Yor1p, Snq2p and Pdr5p. The *pdr1-3* mutation results in overexpression of the non-deleted genes controlled by *PDR1*	Decottignies et al. (Decottignies et al. 1988).
AD1-7	US50-18 C; (*ura3, his1, pdr1-3, Δyor1, Δsnq2, Δpdr5, Δpdr10, Δpdr11, Δycf1, Δpdr3*)	Strain with deletion of 6 genes encoding the transporters Yor1p, Snq2p, Pdr5p, Pdr10p, Pdr11p, Ycf1p and the transcription factor Pdr3p.The *pdr1-3* mutation results in overexpression of the non-deleted genes controlled by *PDR1*	Decottignies et al. (Decottignies et al. 1988).
AD1-8	US50-18 C; (*ura3, his1, pdr1-3, Δyor1, Δsnq2, Δpdr5, Δpdr10, Δpdr11, Δpdr15, Δycf1, Δpdr3*)	Strain with deletion of 7 genes encoding the transporters Yor1p, Snq2p, Pdr5p, Pdr10p, Pdr11p, Pdr15p, Ycf1p and the transcription factor Pdr3p. The *pdr1-3* mutation results in overexpression of the non-deleted genes controlled by *PDR1*.	Decottignies et al. (Decottignies et al. 1988).
AD1-9	US50-18 C; (*ura3, his1, pdr1-3, Δyor1, Δsnq2, Δpdr5, Δpdr10, Δpdr11, Δpdr15, Δycf1, Δpdr3, Δpdr1*)	Strain with deletion of 7 genes encoding the transporters Yor1p, Snq2p, Pdr5p, Pdr10p, Pdr11p, Pdr15p, Ycf1p and both transcription factors *Pdr1p* and *Pdr3p* .	Decottignies et al. (Decottignies et al. 1988).
BY4741	BY4741; (*his3Δ; leu2Δ; met15Δ; ura3Δ*)	Parental strain, isogenic to ΔGSH1, ΔGSH2 and ΔGLR1 strains, encoding enzymes involved in glutathione biosynthesis.	From EUROSCARF
ΔGSH1	BY4741; (*his3Δ; leu2Δ; met15Δ; ura3Δ; GSH1Δ*)	Strain with deletion of the gene encoding γ-glutamylcysteine synthetase, which catalyzes the first step in glutathione biosynthesis (Ohtake et al. 1990).	From EUROSCARF
ΔGSH2	BY4741; (*his3Δ; leu2Δ; met15Δ; ura3Δ; GSH2Δ*)	Strain with deletion of the gene encoding glutathione synthetase, which catalyzes the synthesis of glutathione from gamma-glutamylcysteine and glycine (Ohtake et al. 1990).	From EUROSCARF
ΔGLR1	BY4741; (*his3Δ; leu2Δ; met15Δ; ura3Δ; GSH2Δ*)	Strain with deletion of the gene encoding glutathione reductase, which converts oxidized glutathione to the reduced form GSH (Outten et al. 2005).	From EUROSCARF

Results and discussion

3-bromopyruvic acid is taken up in yeast cells by the glucose-repressible lactate /pyruvate transporter Jen1p

At external pH=5.0 of minimal (YNB) medium the minimal inhibitory concentration (MIC) of 1.95 mM 3-BP inhibits the growth of the parental strain W303-1A (Fig. 1a).

The yeast displays at least two distinct transporters involved in the uptake of monocarboxylates across the plasma membrane: the Lactate/Pyruvate: H^+ Symporter Jen1p and the Acetate: H^+ Transporter (Ady2p). Additionally, the yeast genome encodes five members of the Monocarboxylate Porter (MCP) Family (De Hertogh et al. 2002), but their role in importing monocarboxylic acids has not been proven (Makuc et al. 2001; Reihl and Stolz 2005).

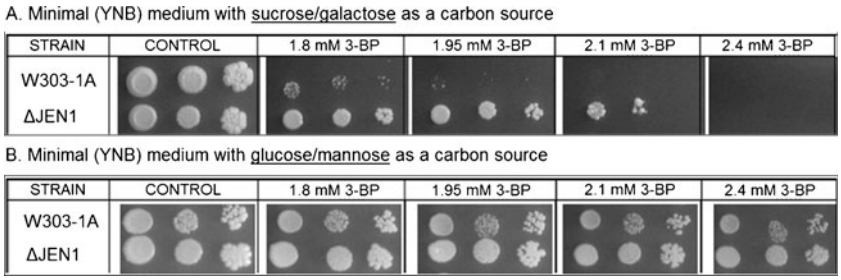

Fig. 1 Differences in growth sensitivity of the parental strain (W303-1A) and Δ*JEN1* mutant to 3-bromopyruvate on media (YNB) with a non-repressing (sucrose/galactose) and repressing (glucose/mannose) carbon source

The Jen1p transporter was an obvious candidate for the 3-BP transporter as it transports pyruvate as well as lactate (Casal et al. 1999; Cassio et al. 1987). Figure 1a shows that the growth of the Δ*JEN1* strain is more resistant to 3-BP (MIC=2.4 mM) than the parental strain W303-1A (MIC= 1.95 mM). This effect is observed under culture conditions (sucrose/galactose) that derepress Jen1p but is not visible in medium containing glucose/mannose. Indeed, Fig. 1b shows that in this medium a MIC higher than 2.4 mM is observed both for parental W303-1A and Δ*JEN1* strains probably due to the well known glucose repression of Jen1p (Casal et al. 1999).

Figure 2 shows that the uptake of [^{14}C] 3-BP is markedly decreased in the Δ*JEN1* strain compared to the parental strain W303-1A. Transport parameters in the parental strain were consistent with Michaelis-Menten kinetics with a Km=2 mM 3-BP and a V_{max}=0.57 nmol 3-BP taken up /min x 10^6 cells. Uptake rate in the Δ*JEN1* strain is over 2-fold lower with a V_{max}=0.22 nmol/min x10^6 cells. A small amount of 3-BP is transported in the ΔJEN1 strain. This may be due to the simple diffusion combined to weak activities of other transporters such as the acetate transporter Ady2p and the monocarboxylate tansporters Mch1p to Mch5p, unrelated to Jen1p (data not shown). It has to be noted however that there is no lactate/pyruvate transporter similar to Jen1p in mammalian cells. It remains thus to identify the monocarboxylate transporters which take up specifically 3-BP in cancer cells.

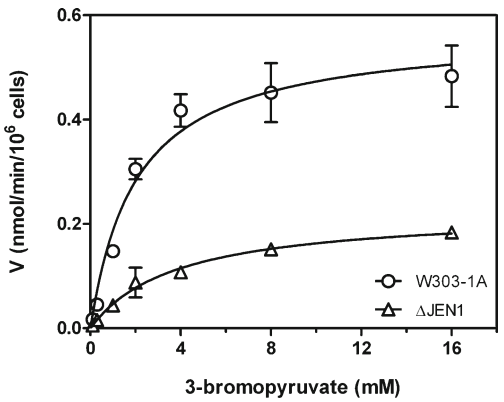

Fig. 2 Transport of [^{14}C] 3-BP in W303-1A and ΔJEN1 strains; (Km=2 mM 3-BP, V_{max}=0.57 nmol 3-BP/min×10^6 cells

The Yeast PDR network is not involved in conferring resistance to 3-bromopyruvate

In *Saccharomyces cerevisiae* the Pleiotropic Drug Resistance network of genes confers resistance to a variety of small cytotoxic molecules by activating their cellular efflux (Cannon et al. 2009; Prasad and Goffeau 2012). Single and multiple deletions in ABC efflux pumps and of their transcription regulators renders the cells very sensitive to inhibitiors. We have compared the growth of the various PDR-hypersensitive deleted mutants AD1-3, AD1-7, AD1-8, AD1-9 and of the upregulated multidrug resistant mutant US50-18 C (Balzi et al. 1987; Decottignies et al. 1988) compared to the EM01 parental isogenic strain on media containing various concentrations of 3-BP. Figure 3a shows that neither 3-BP–resistance nor sensitive phenotype were observed. 3-BP is thus not a substrate of any of the tested drug-efflux pumps belonging to the ABC family of transporters. The phenotype is totally different when comparing growth (Fig. 3c and d) in the presence of the well known multidrug substrates daunorubicin and rhodamine 6 G (Kolaczkowski et al. 1998; Decottignies et al. 2001). Figure 3b shows that the well know anticancer drug Gleevec (Imatinib methanesulfonate) (Hoepfl et al. 2002; Cohen et al. 2002) inhibits growth at concentrations similar to those which are toxic for 3-BP. However, the parental strain EM01 is more resistant (MIC=2.2 mM) to Imatinib methanesulfonate than the Pdrp-activated strain US50-18 C (MIC=1.8 mM) while the supersensitive AD deleted strains are more sensitive to Imatinib methanesulfonate (MIC 1.6 mM) than the parental EM01. Moreover, an aqua-Molar content of 3-BP is almost 4 times lower than Gleevec (M.W. 166.96 versus 589.71, respectively). From a practical point of view it is worth to mention that the first drug is much cheaper. This important new finding suggests that 3-BP is less likely to develop multidrug resistance in the course of chemiotherapy (Endicott and Ling 1989; Gottesman and Pastan 1993) than Imatinib methanesulfonate and many other drugs (such as daunorubicin, vinblastine and mitomycine).

Intracellular concentration of reduced glutathione correlates with 3-bromopyruvate sensitivity

In Fig. 4 we have tested the susceptibility to 3-BP of three strains defective in glutathione metabolism (Ohtake et al.

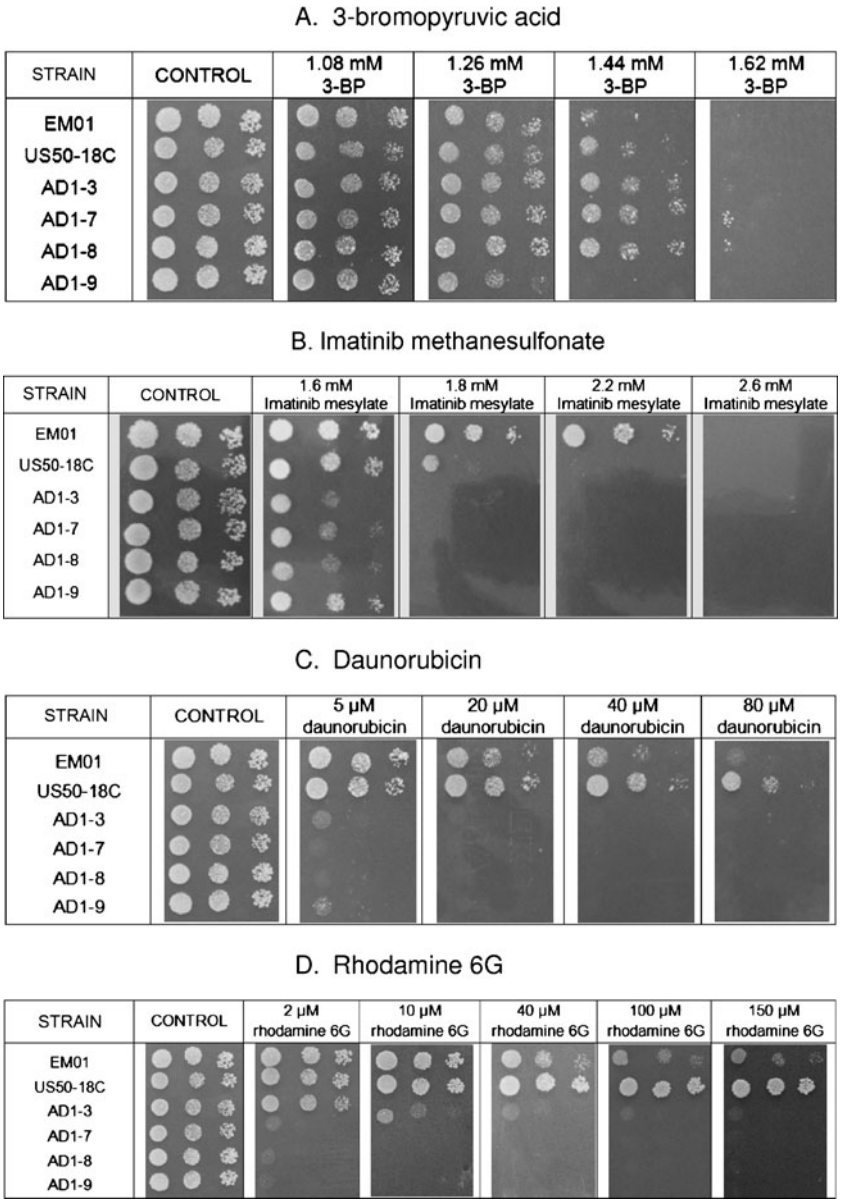

Fig. 3 Differences in sensitivity of PDR-mutants to 3-BP, Imatinib methanesulfonate, daunorubicin and rhodamine 6 G. Minimal medium (YNB) with sucrose

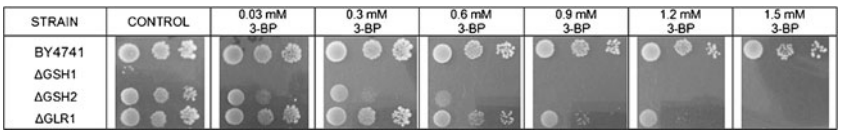

Fig. 4 Sensitivity of three strains defective in glutathione metabolism (ΔGSH1, ΔGSH2, ΔGLR1) to 3-BP, compared to the parental strain (BY4741)

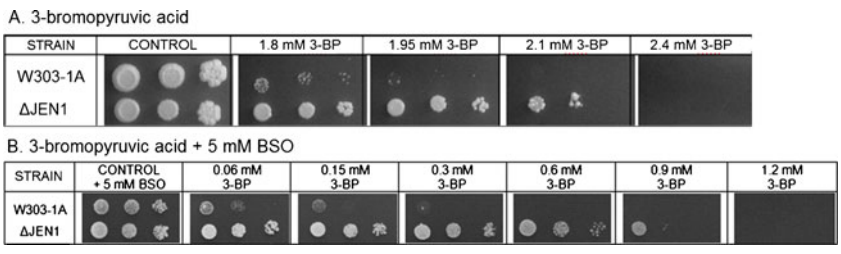

Fig. 5 Influence of 5 mM BSO (buthionine sulfoximine) to sensitivity to 3-bromopyruvate. Minimal (YNB) medium with sucrose

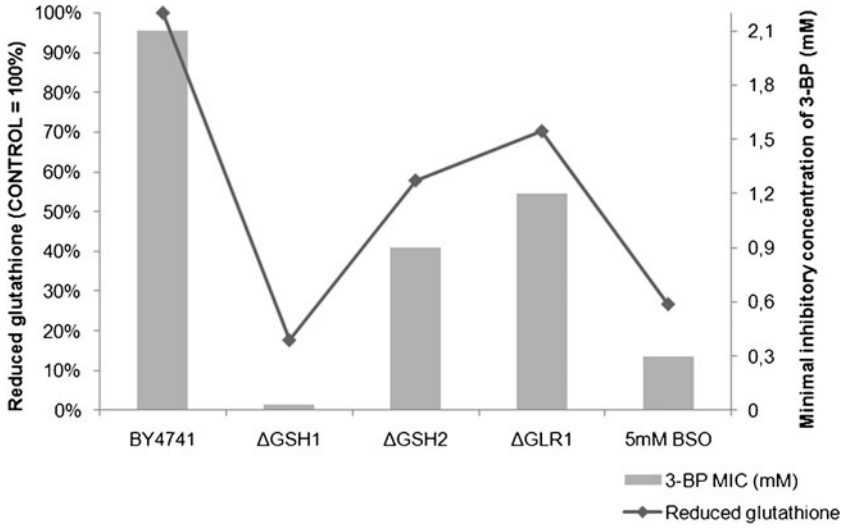

Fig. 6 Correlation of 3-BP sensitivity (MIC values) for BY4741, ΔGSH1, ΔGSH2, ΔGLR1 strains and BY4741 strain grown on medium supplemented with 5 mM BSO, and the relative intracellular levels of reduced glutathione in these strains

1990; Outten et al. 2005). Growth of a strain with deletion of the gene encoding gamma-glutamylcysteine synthetase (*GSH1*), which catalyses the first step in glutathione biosynthesis is strongly weakened even on medium without 3-BP. It is totally inhibited by the very low concentrations of 0.03 mM 3-BP. Difference in growth between the strain deleted in the *GSH2* gene (encoding glutathione syntethase, which catalyzes the final step of synthesis of glutathione from gamma-glutamylcysteine and glycine) and parental strain is also clearly visible on medium with 3-BP (MIC=0.9 mM), with no significant difference on control medium (MIC=2.1 mM). Deletion of the *GLR1* gene (encoding glutathione reductase) also increased the sensitivity to 3-BP (MIC=1.5 mM).

Figure 5 shows that 0.5 mM buthionine sulfoximine (BSO), a known inhibitor of gamma-glutamylcysteine synthetase, (Reliene and Schiestl 2006) increases about 30 times the susceptibility of the parental yeast cells to 3-BP indicating a spectacular synergistic toxic effect. As expected the deletion of the *JEN1* gene restores resistance to 3-BP. This shows that BSO acts intracellularly. Notably the cytotoxicity of doxorubicin shown to be enhanced by BSO has also been reported in multi-drug resistant cells from human breast tumor (Dusre et al. 1989). Figure 6 shows a strong correlation between the intracellular level of GSH and the sensitivity to 3-bromopyruvate. These results indicate that glutathione plays a significant role in resistance to 3-BP in yeast, either directly through interaction with 3-BP or indirectly. An amount of 2 mM BSO treatment resulted in a 45% lower GSH and 30% higher frequency of genomic DNA deletions during mouse development (Reliene and Schiestl 2006). As BSO in minimally cytotoxic doses has limited secondary effects both on yeast (this study) and mammalian cells one can conclude that 3-BP in combination with BSO might be useful in anticancer therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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