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3-Bromopyruvate: A novel antifungal agent against the human pathogen *Cryptococcus neoformans*

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ABSTRACT

We have investigated the antifungal activity of the pyruvic acid analogue: 3-bromopyruvate (3-BP). Growth inhibition by 3-BP of 110 strains of yeast-like and filamentous fungi was tested by standard spot tests or microdilution method. The human pathogen *Cryptococcus neoformans* exhibited a low Minimal Inhibitory Concentration (MIC) of 0.12–0.15 mM 3-BP. The high toxicity of 3-BP toward *C. neoformans* correlated with high intracellular accumulation of 3-BP and also with low levels of intracellular ATP and glutathione. Weak cytotoxicity towards mammalian cells and lack of resistance conferred by the PDR (Pleiotropic Drug Resistance) network in the yeast *Saccharomyces cerevisiae*, are other properties of 3-BP that makes it a novel promising anticryptococcal drug.

1. Introduction

Cryptococcosis is a major life-threatening infection [1–3] that kills approximately 650 thousands HIV/AIDS patients every year [2–4]. Amphotericin B (AmB), which is a drug of choice in cryptococcosis therapy exhibits severe toxic properties [3,5]. In its less toxic liposomal formulation AmB is very expensive and is not of standard use in many hospitals [3]. Fluconazole and fluorocytosine, alone or in combination with AmB, are also used in the management of cryptococcosis [6]. However for both of these drugs many fungi including *Cryptococcus neoformans* rapidly develop resistance [7–9]. In this paper we report a novel antifungal activity of 3-BP, which is also a potent anticancer agent with no apparent side effects in animals or humans [10–14]. It was demonstrated that in *S. cerevisiae* the specific transporter of lactate/pyruvate Jen1p is involved in the cellular uptake of 3-BP [15]. Another important observation is that 3-BP is not a substrate for the efflux pumps involved in the Pleiotropic Drug Resistance (PDR) network that confers resistance to many anticancer and antifungal drugs [15–17]. We show here that 3-BP is an effective agent combined antitumor and anticryptococcal therapy. This is important as for many years the number of cancer patients who develop fungal infections is increasing dramatically and invasive fungal infections by *C. neoformans* often complicate chemotherapy [18].

2. Materials and methods

2.1. Fungal strains

A total of 52 fungal strains from 14 yeast-like species and 58 filamentous fungi strains belonging to 18 species were included in this study (Table 1). Clinical strains (57) were recovered from ambulatory patients of the Laboratory of Molecular Diagnostics "Bio-Genetik" (Wroclaw) and hospitalized patients in the Wroclaw Medical University (Poland). Moreover a total of 53 environmental strains were isolated from soil and biodeteriorated materials.

2.2. Antifungal susceptibility testing

The susceptibility tests toward 3-BP (Sigma), fluconazole (Pfizer) and amphotericin B (Sigma) were performed by standard spot tests method on SD medium: 0.67% yeast nitrogen base, 2% bacto-agar (Becton Dickinson) and 2% glucose, pH 5.5 [19]. To determine whether glucose has any impact on susceptibility to 3-BP, as it was described earlier for *S. cerevisiae* [15], the growth inhibition assays were also performed on modified SD medium where glucose was replaced by other appropriate carbon source (purchased from Sigma). Differences, if any, of growth susceptibility, were indicated in Table 1. The susceptibility tests toward fluconazole and amphotericin B were performed only for the tested *C. neoformans* strains. The fresh stock solutions 0.6 M 3-BP, 0.011 M amphotericin B and 0.16 M fluconazole were prepared in sterile

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Table 1
Susceptibility of 110 fungal strains towards 3-bromopyruvate (3-BP) evaluated by spot tests method[b] and microdilution assay[c].

Tested strains	MIC (mM)	Tested strains	MIC (mM)
C. neoformans (4C)[a]	0.12–0.15[b]	*T. mentagrophytes* (2C)	1.8–2.4
	0.15–0.23[c]	*T. rubrum* (4C)	1.8–2.4
C. uniguttulatus (2E)	2.4–3.6	*T. tonsurans* (3C)	1.8–2.4
C. lusitaniae (4C)	2.4–3.6	*M. canis* (2C)	3.0
C. guilliermondii (5C)	2.4–3.6	*M. gypseum* (2C)	3.0–3.6
C. famata (4C)	2.4–3.6	*C. herbarum* (6E)	7.2–8.4
R. glutinis (2C)	2.4–3.0	*U. chartarum* (3E)	6.0–7.2
R. rubra (3C)	3.0–3.6	*A. tenuissima* (2E)	7.2–8.4
C. lipolytica (2C)	1.8–2.4	*F. graminearum* (2E)	6.0–7.2
C. krusei (5C)	1.2–1.8	*T. viride* (4E)	7.2–8.4
C. albicans (5C)	2.4–3.6	*A. glauca* (5E)	1.8–2.4
	(1.4–1.8; GAL)[d]	*R. nigricans* (4E)	1.8–2.4
C. glabrata (5C)	2.4–3.6	*C. globosum* (3E)	1.8–3.0
	(1.2–1.8; TRE)[d]	*A. clavatus* (2E)	7.2–8.4
C. tropicalis (3C)	2.4–3.6	*A. fumigatus* (2C + 2E)	6.0–7.2
	(1.0–1.4; TRE)[d]	*F. oxysporum* (4E)	8.4–9.6
A. pullulans (4E)	7.2–8.4	*A. niger* (3E)	22.8–24.0
E. dermatitidis (4E)	8.4–9.6	*P. chrysogenum* (3E)	22.8–24.0

[a] Number of tested strains: C – clinical; E – environmental.
[b] MIC (Minimal Inhibitory Concentration) values obtained by standard spot tests method [19].
[c] MIC values determined according to microdilution assay [20], defined as the lowest compound concentration for which the lack of growth was observed.
[d] MIC values from tests performed on modified SD medium with: GAL – galactose; TRE – trehalose.

water or dimethyl sulfoxide (Sigma) and added in adequate volume to the culture medium.

Especially for 3-BP and *C. neoformans* the *in vitro* susceptibility tests were carried out according to Clinical Laboratory Standards Institute (CLSI) reference method M27-A2 [20].

The inocula of the yeast-like fungi were prepared from cultures in exponential phase of growth and were adjusted to optical density OD_{600} = 0.125. Aliquots of 3 μl were spotted in 10-fold serial dilutions onto the plates containing various concentrations of the tested compound. The 3 μl inocula of all filamentous fungi were prepared as described elsewhere [21] and spotted centrally onto the plates. The MIC values were determined based on colony diameter. The plates were incubated at 28 °C for 72 h for the yeast-like or 7 days for the black yeast-like species and 7–14 days for the filamentous fungi.

To check the role of glutathione in the natural resistance to 3-BP, the spot tests were also performed on medium supplemented with pure, reduced GSH (glutathione) or with the glutathione-depleting agent BSO (buthionine sulfoximine) at final concentrations of 5 and 10 mM, respectively. GSH (Sigma) and BSO (Sigma) solutions were prepared in water and at the used concentrations did not by itself affect the growth of strains.

All the tests were repeated at least three times.

2.3. Intracellular ATP level determination

ATP level was examined for cells cultivated in complete YPS medium (2% sucrose, 2% peptone and 1% yeast extract), pH 5.5. The 24 h cultures were diluted to OD_{600} = 0.25 ($8.5–8.7 \times 10^6$ - cells/ml). The cultures were incubated in YPS medium with 0.15 and 0.30 mM 3-BP (1/16 and 1/8 MIC value, respectively), and also in medium free of 3-BP. The ATP level was determined after 0, 15, 30, 45 min, 1 and 2 h using the ATPlite™ Luminescence Assay System (PerkinElmer) and PerkinElmer EnSpire® Multimode Plate Reader. ATP levels were compared to that of the control (free of 3-BP) and were recalculated per living cells. Viability of the cells at each time-point was determined by plating a 0.1 ml sample of culture from appropriate culture dilutions on YPD medium (2% glucose, 2% peptone and 1% yeast extract), pH 5.5. Colonies were counted after 72 h of incubation at 28 °C. Viability of the cells evaluated by this method was also determined in SD liquid medium for concentrations equal to two- and fourfold MIC of 3-BP (data not shown).

2.4. Glutathione determination

The GSH level of chosen *C. neoformans*, *Cryptococcus uniguttulatus*, *Aureobasidium pullulans* and *Exophiala dermatitidis* strains grown in the minimal SD medium were determined using boiling buffered ethanol procedure for quantitative metabolite extraction and Ellman's test as described previously [15].

2.5. Transport assay

3-BP uptake assays were performed using [^{14}C]-labeled 3-bromopyruvate (kindly donated by Young H. Ko, Baltimore), based on the method previously described for the radioactive L-lactate uptake assay [22]. Cells from exponential phase of growth in YPS medium, at 28 °C were washed twice and resuspended in ice-cold, sterile, de-ionized water to obtain the concentration of approximately 200 mg/ml. Aliquots of 200 μl of cells suspension were mixed with 100 μl of 20% of sucrose, 100 μl of 1 M phosphate buffer (pH 5.0) and 400 μl of de-ionized water and incubated for 10 min at 30 °C. 200 μl of [^{14}C]-labeled 3-BP solution in appropriate concentrations prepared in 0.1 M phosphate buffer (pH 5.0) were added. Samples were incubated for 15, 30, 60, 120 s, 5, 10 and 20 min at 30 °C. At each time-point the reaction was stopped using ice-cold water and the sample was filtrated on nitrocellulose filters (Whatman), using a vacuum filtration box (Hoefer, USA). Radioactivity of each sample was measured using a Beckman LS100 scintillation counter. All charts and calculations were made using the GraphPad Prism 5 program.

3. Results

3.1. Antifungal activity of 3-BP

The MIC values for most of the 110 tested fungal strains were in the range of 2.4–3.6 mM 3-BP (Table 1). The highest antifungal activity of 3-BP was observed for the basidiomycetous pathogen *C. neoformans*. The MIC values determined by spot tests and microdilution methods were in the range of 0.12–0.15 mM [20–25 μg/ml] and 0.15–0.23 mM [25–38 μg/ml] 3-BP, respectively. The MIC values for fluconazole and amphotericin B of the tested *C. neoformans* strains were in the range of 0.02–0.03 mM [6.25–8.5 μg/ml] and 0.43–0.54 mM^{-3} [0.4–0.5 μg/ml], respectively (data not shown).

Strains belonging to Glomeromycetes, as well as Ascomycetes strains from the Trichophyton genus and also *Candida krusei*, *Candida lipolytica*, and *Chaetomium globosum* exhibited average susceptibility toward 3-BP (MIC of 1.8–2.4 mM). For fungal species such as *Cladosporium herbarum*, *Ulocladium chartarum*, *Alternaria tenuissima*, *Trichoderma viride*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus clavatus and Aspergillus fumigatus* the MIC values were higher (7.2–8.4 mM 3-BP). The black yeast-like fungi *E. dermatitidis* and *A. pullulans* exhibited low susceptibility with MIC values of 8.4–9.6 mM 3-BP and 7.2–8.4 mM 3-BP, respectively. *Penicillium chrysogenum* and *Aspergillus niger* showed the highest resistance with MIC values between 22.8 and 24 mM 3-BP.

3.2. Influence of 3-BP on intracellular ATP levels in C. neoformans

We have determined cellular ATP level in *C. neoformans* var. *neoformans* after administration of 0.15 and 0.30 mM 3-BP (1/16

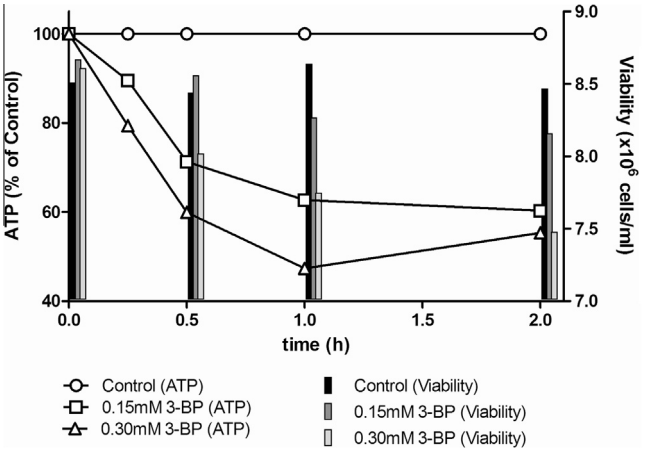

Fig. 1. Influence of 3-bromopyruvate on intracellular ATP (adenosine 5′-triphosphate) levels and viability of *Cryptococcus neoformans* varietas *neoformans*.

and 1/8 of the MIC, respectively). For both 3-BP concentrations, the intracellular ATP level decreased by 10% or 20% respectively after 15 min and 35% or 55% after 1 h of treatment (Fig. 1). It was also observed that these concentrations of 3-BP caused about 8–10% decrease of viability after 2 h. However, approximately 100% decrease of cell viability was observed after 24 h of cultivating in the presence of 3-PB concentration corresponding to fourfold MIC value (data not shown).

3.3. Uptake of 3-BP

To check whether the differences in sensitivity to 3-BP between clinical *C. neoformans* and environmental *Cryptococcus uniguttulatus* strains are caused by different permeability properties, we performed uptake assays using [^{14}C]-labeled 3-BP. Fig. 2A and B shows that the level of accumulated 3-BP and the velocity of its uptake into the cells are both significantly higher in *C. neoformans* var. *neoformans* than in *C. uniguttulatus* strain. Although at the first four time-points (15, 30, 60 and 120 s) the level of 3-BP accumulation was about twofold higher in *C. neoformans* var. *neoformans* than in *C. uniguttulatus*, it was over fivefold higher after 20 min of incubation. The highest uptake velocity of 3-BP was observed at the beginning of the experiment (approximately 1.5 nmol/min $\times$ 10^6 cells after 15 s) and rapidly decreased later, but not to zero level. Similarly for *C. uniguttulatus* and *E. dermatitidis* the highest values of uptake velocity (0.3 and 0.15 nmol/min $\times$ 10^6 cells, respectively) were also observed within the first 15 s. In *E. dermatitidis* we

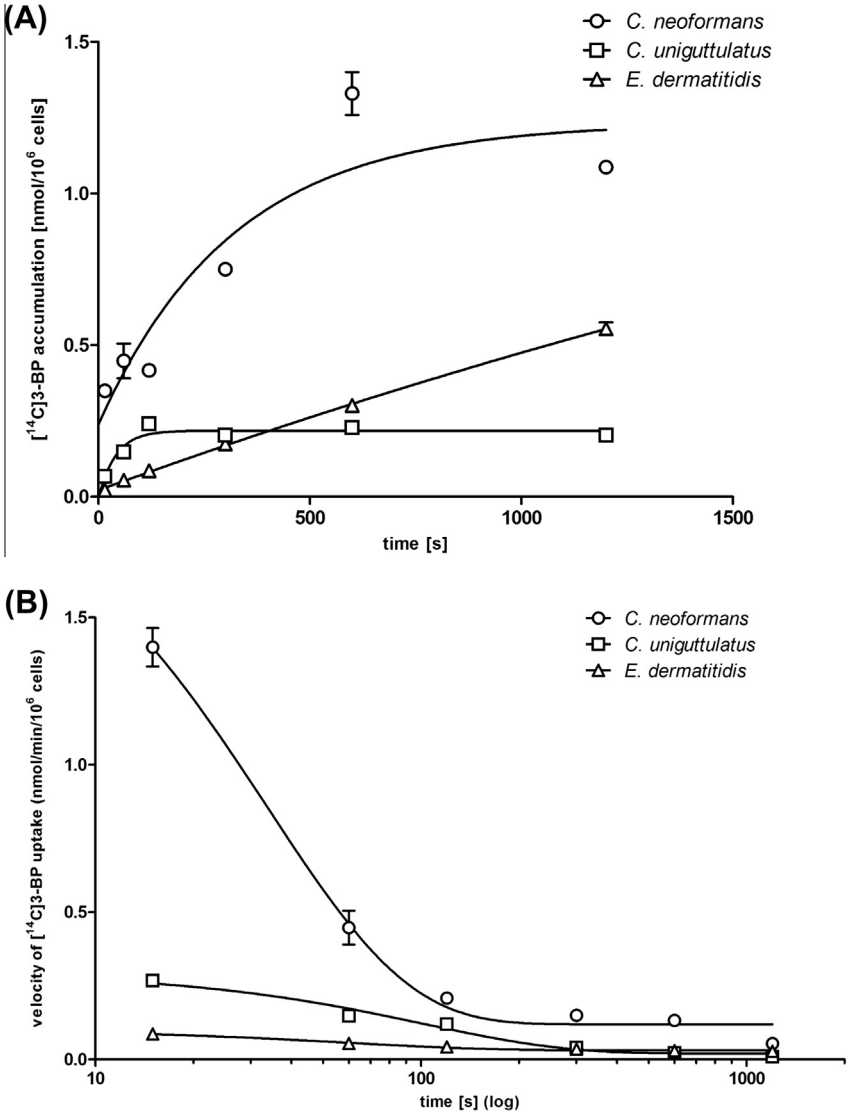

Fig. 2. Time-dependent accumulation (A) and uptake velocity (B) of [^{14}C]-labeled 3-bromopyruvate in chosen fungal strains.

observed a slow increase of 3-BP accumulation and low, but constant, velocity of its uptake. After 20 min of incubation the level of accumulated 3-BP was equal to 1.25 nmol 3-BP/10^6 cells for *C. neoformans* var. *neoformans*, 0.5 nmol 3-BP/10^6 cells for *E. dermatitidis* and 0.25 nmol 3-BP/10^6 cells for *C. uniguttulatus*.

3.4. Resistance to 3-BP related to intracellular GSH level

Exploring the other factors that could be responsible for observed differences in susceptibility to 3-BP we determined the intracellular GSH content in cells of chosen fungi (Fig. 3). The highest levels of intracellular GSH was observed for the black yeast-like fungi *A. pullulans* and *E. dermatitidis* grown in minimal SD medium (0.15 and 0.18 nmol GSH/10^6 cells, respectively). These high levels of GSH appears to be related with high MIC values (7.2 and 8.4 mM 3-BP, respectively). This correlation was also observed when *C. neoformans* var. *neoformans* and *C. uniguttulatus* were compared. The MIC value for *C. neoformans* on SD medium was equal to 0.15 mM 3-BP and the corresponding GSH level was equal to 0.06 nmol GSH/10^6 cells. In the case of *C. uniguttulatus* these values were equal to 2.4 mM 3-BP and 0.139 nmol GSH/10^6 cells, respectively. Thus *C. uniguttulatus* showed about 16-fold lower susceptibility of growth to 3-BP and about twofold higher intracellular level of GSH than *C. neoformans*.

The influence of the glutathione depleting agent BSO [23] on fungal susceptibility to 3-BP was also evaluated. In the case of *E. dermatitidis*, the presence of 10 mM BSO in the SD medium decreased considerably the MIC values to 0.66–0.72 mM 3-BP compared to the control MIC values which amounted to 8.4–9.6 mM 3-BP without BSO. For the *A. pullulans* strains the MIC values in the presence of BSO were in the range of 0.54–0.6 mM 3-BP compared to 7.2–8.4 mM 3-BP in control. It was concluded that BSO acts synergistically with 3-BP to increase the growth susceptibility in the case of *E. dermatitidis* and *A. pullulans*. The synergistic effect between these two compounds was confirmed for all *Cryptococcus* spp. strains tested. While in the control the MIC values for *C. neoformans* were in the range of 0.12–0.15 mM 3-BP, the addition of 10 mM BSO had more limited synergistic effect as MIC values of 0.08–0.1 mM 3-BP were observed. Similarly in *C. uniguttulatus* the MIC values of 2.4–3.6 mM 3-BP observed in the absence of BSO were decreased to 1.4–1.8 mM 3-BP by 10 mM BSO.

Additional evidences for the important role of GSH in 3-BP susceptibility were obtained when growth was estimated by spot tests. Growth susceptibility to 3-BP of all *E. dermatitidis* and *A. pullulans* tested strains was about twofold lower when 5 mM synthetic GSH was added to SD medium. This effect was even more visible in the case of all tested *C. neoformans* and *C. uniguttulatus* strains where growth susceptibility to 3-BP was decreased eight and twofold by 5 mM synthetic GSH, with MIC values of 3-BP in the range of 1.0–1.2 and 4.8–6.8 mM 3BP, respectively.

4. Discussion

In the case of all tested *C. neoformans* strains comparing the MIC values determined for 3-BP (Table 1) with those obtained for fluconazole (0.02–0.03 mM) and amphotericin B (0.43–0.54 mM^{-3}) we conclude that 3-BP is an efficient antifungal agent in comparison to fluconazole. Large differences in growth susceptibility to 3-BP were observed, even between species from the same genus such as *C. neoformans* and *C. uniguttulatus*. Unlike was reported in *S. cerevisiae* [15], no differences in susceptibility depending on carbon source (glucose or sucrose) were observed for these species. On the other hand the MIC values obtained for *Candida glabrata* and *Candida tropicalis* were slightly lower on trehalose (1.2–1.8 and 1.0–1.4 mM 3-BP, respectively) than on glucose (2.4–3.6 mM 3-BP). Similarly the MIC values for *Candida albicans* were lower on galactose (1.4–1.8 mM 3-BP) than on glucose (2.4–3.6 mM 3-BP). A similar differential sensitivity to 3-BP in galactose versus glucose grown cells was described earlier for *S. cerevisiae* [15] and is probably related to the well known glucose repression of the monocarboxylate transporter Jen1p [22]. In the case of *C. albicans* a Jen1p homologue was found [24] and a glucose-repressible lactate uptake was described [25]. Similar mechanisms may exist in *C. tropicalis* which is closely related with *C. albicans* [26].

Significant differences in the uptake and accumulation of 3-BP explain different susceptibility toward 3-BP in the case of two *Cryptococcus* species examined in this study. In the case of *C. neoformans* var. *neoformans*, it was shown that the initial uptake velocity as well as maximum accumulation level were over fivefold higher than in *C. uniguttulatus*. Moreover, preincubation with lactate did not modify the uptake of 3-BP in *C. neoformans* var. *neoformans* cells (data not shown). This suggests that lactate/pyruvate transport in *C. neoformans*, unlike in *S. cerevisiae*, is rather constitutive. It was recently established that in the case of *S. cerevisiae* the Jen1p transporter is involved in 3-BP uptake and that its repression by glucose increases the MIC of 3-BP [15].

S. cerevisiae Jen1p homologues were found in genomes of pathogenic fungi as such as *C. albicans*, *Candida utilis* and *A. fumigatus* as

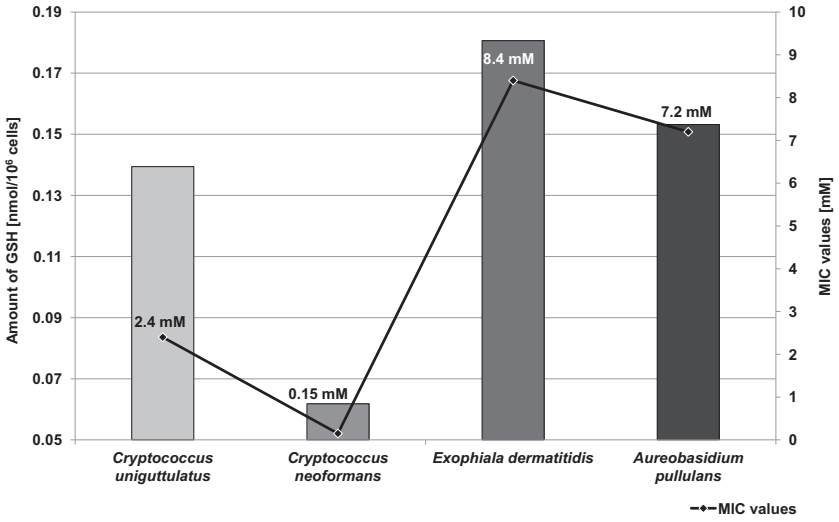

Fig. 3. Comparison of minimal inhibitory concentration MIC values of 3-bromopyruvate and the amounts of intracellular glutathione (reduced) in the case of four fungal strains.

well as in Ascomycetes species closely related to *S. cerevisiae* [24]. Based on currently available sequenced genome of *C. neoformans* var. *neoformans* JEC21 [27] we performed BLAST searches with the Jen1p sequence from *S. cerevisiae* as query and confirmed that a homolog of Jen1p does not exist in the *C. neoformans* proteome. Therefore transporter of lactate/pyruvate other than Jen1p must exist. It is well known that *C. neoformans* is able to proliferate in human blood and cerebrospinal fluid, which are rich in L-lactate [28,29]. Our studies based on growth curves comparison also confirm better utilization of L-lactate by *C. neoformans* than by the nonpathogenic *C. uniguttulatus* (data not shown). This is probably connected with expression of unidentified lactate/pyruvate transporter(s) in *C. neoformans* or with the lack of such transporters in *C. uniguttulatus*. This observation also could be explain the differences in 3-BP uptake into *C. neoformans* and *C. uniguttulatus* cells (Fig. 2A and B). This could be related to the fact that during adaptation to mammalian hosts, the central carbon metabolism of *C. neoformans* is remodeled and that alternative carbon sources such as acetate, lactate or fatty acids are utilized [29]. Furthermore, the lactate–proton symport paralogues lacA and lacB recently identified in the genome sequence of *Rhizopus oryzae*, belonging to the Glomeromycetes, have no homology to the yeasts monocarboxylate transporters [30]. Thus, it can be assumed that the different susceptibility to 3-BP observed among fungi, are due in part to the presence or absence as well as phylogenetic diversity of these transporters.

Studies with *C. neoformans* also show that 3-BP affects the management of cellular energy. As the intracellular ATP level decreased significantly even in the presence of low sub-MIC values of 3-BP, it can be concluded that 3-BP acts primary by depletion of intracellular ATP, which leads to catabolic perturbation and finally to cell death through induction of apoptosis [31]. A similar decrease of ATP was described earlier for mammalian cancer cells [11]. In viable cells of *C. neoformans*, the incubation with 3-BP at a concentration equivalent to only 1/8 of MIC value caused decrease of intracellular ATP of about 20% after 15 min, 40% after 30 min and 55% after 1 h, (Fig. 1). However, a clear decrease in cell viability was observed after 6–8 h of cultivation in the presence of 3-BP concentration corresponding to fourfold MIC value (data not shown). We assume that rapid decrease in ATP level during the first hour of experiment is probably due to primary inhibition of mitochondrial respiration [13]. Subsequent slight increase in ATP level may be due to temporal upregulation of glycolytic flux.

We have observed a strong correlation between intracellular levels of reduced glutathione and resistance to 3-BP in all the tested fungal strains. It is well known, that GSH plays an important role in cell defense against oxidative stress, as well as in detoxification of xenobiotics and heavy metals [32]. This process is due to the formation of glutathione-S-conjugates (GS-X) with electronophilic xenobiotics. They are sequestrated in vacuole and/or are excreted from fungal cell, especially by multidrug transporter protein (MRP1), which exports glutathione-S-conjugates [33,34]. Our susceptibility assays performed both in the presence of synthetic 5 mM GSH, as well as with 10 mM BSO confirmed the role of glutathione in resistance to 3-BP. It is well known that BSO irreversibly inhibits γ-glutamylcysteine synthetase (γ-GCS) and contributes to intracellular GSH depletion and oxidative stress induction [23]. Our experiences with BSO and synthetic GSH additionally conclude that the susceptibility of fungi towards 3-BP is strongly related to natural level of intracellular glutathione.

In conclusion, our studies demonstrate that 3-bromopyruvate exhibits various antifungal properties. It is fungistatic to most of the 110 tested fungal strains. Moreover it shows strong fungicidal effects on sensitive *C. neoformans* strains where 3-BP caused rapid intracellular ATP depletion before *C. neoformans* cell death even at concentrations as low as 1/8 or 1/16 of the MIC value. Different susceptibility towards 3-BP observed among the studied fungi is probably connected with different lactate/pyruvate metabolism and different levels of intracellular GSH. Uptake assays of [^{14}C]-labeled 3-BP shows that natural resistance to 3-BP is probably related to different substrate specificity or absence of the pyruvate/lactate transporters in these fungi. As shown earlier in *S. cerevisiae*, different activity of these transporters may be responsible for different level of 3-BP accumulation and its toxicity. This may also be the case for *C. neoformans*, *C. uniguttulatus* and *E. dermatitidis*. Synergistic effects of 3-BP and BSO were also observed on SD medium supplemented by 10 mM BSO, as reported earlier for *S. cerevisiae*. The MIC of 3-BP was reduced from 0.15 to 0.08 mM for *C. neoformans* and from 2.4 to 1.4 mM in case of *C. uniguttulatus*.

Taking into account the growing population of fungal strains resistant to azoles [9] and the fact that amphotericin B shows severe toxic effects toward mammalian cells [3,5] is not difficult to see that the current treatment options for cryptococcosis are becoming more limited. Considering the weak cytotoxicity of 3-BP towards healthy mammalian cells [10,12,14] and unlike fluconazole [8,9] its invulnerability to the PDR network driving multidrug resistance phenotype in fungi, 3-BP may be a promising novel antifungal drug, especially against the human and animal pathogen *C. neoformans* which is very sensitive to 3-BP.

The results of our work are the subject of patent notification number, P.399978 (Polish Patent Office).

Conflict of interest

None.

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