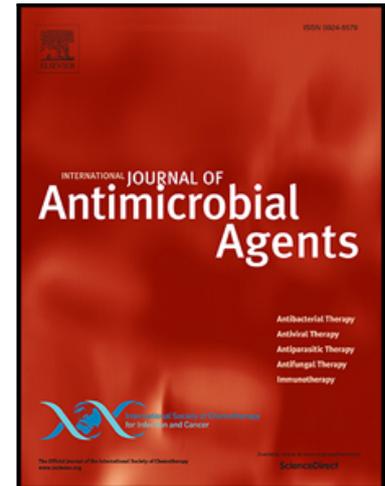# Accepted Manuscript

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Highlights

- Among ESKAPE bacteria, 3-bromopyruvate selectively inhibits *Staphylococcus aureus*
- 3-bromopyruvate kills both metabolically-active and -inactive cells
- 3-bromopyruvate has potent biofilm-disrupting activity

The antimetabolite 3-bromopyruvate selectively inhibits *Staphylococcus aureus*

Paolo Visca[a], Federica Pisa[a,b], Francesco Imperi[b] #

[a] Department of Sciences, University "Roma Tre", Rome, Italy;

[b] Department of Biology and Biotechnology Charles Darwin, Sapienza University of Rome, Laboratory

affiliated to Istituto Pasteur Italia − Fondazione Cenci Bolognetti, Rome, Italy

Address correspondence to Francesco Imperi, francesco.imperi@uniroma1.it

Abstract

The rise in antibacterial resistance jeopardizes current therapeutic strategies to control infections, soliciting the development of novel antibacterial drugs with new mechanisms of action. In this work we report the discovery of a potent and selective anti-staphylococcal activity of 3-bromopyruvate (3BP), an antimetabolite in preclinical development phase as an anticancer drug. 3BP showed bactericidal activity against *Staphylococcus aureus*, with active concentrations comparable to those reported to be effective against cancer cells. In contrast, no relevant activity was observed against other ESKAPE bacteria (*Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.). The anti-staphylococcal activity of 3BP was confirmed using a panel of human and veterinary strains, including multidrug-resistant isolates. 3BP showed highest antibacterial activity under conditions which increase 3BP stability (acidic pH) or promote *S. aureus* fermentative metabolism (anaerobiosis), although 3BP was also able to kill metabolically-inactive cells. 3BP showed synergism with gentamicin, and also disrupted preformed *S. aureus* biofilms, at concentrations only slightly higher than those inhibiting planktonic cells. This study unravels novel antibacterial and anti-biofilm activities for the anticancer drug 3BP, thus paving the way for further pre-clinical studies.

1 Introduction

Antibiotic resistance is a serious public health problem at the global level. Available antibiotics have saved millions of lives, but are progressively losing their efficacy against many bacterial pathogens. While very few new antibiotics are being developed by the pharmaceutical industry, mainly due to the inherent low reward and high risk of antibiotic research, the rapid spread of antibiotic-resistant pathogens both in hospitals and in the community calls for new investments in antibacterial drug discovery. To be effective, these investments should however support experimental approaches able to make the process of antibacterial drug discovery more rapid and economically sustainable.

Searching for side activities in drugs already approved for use in humans or in advanced phase of preclinical development represents a potential shortcut to obtain new antibacterials [1]. As compared to *de novo* drug discovery, the drug repurposing approach has a higher probability of yielding bioavailable and safe hit compounds, which can move straightforward into clinical trials or serve as leads for drug optimization programs. Moreover, drug repurposing is expected to reduce the time and costs of the conventional drug discovery process, since pharmacological/toxicological information is available for repurposing candidates. Notably, UK and US funders have recently launched programs to re-evaluate deprioritized drugs for new therapeutic uses [1].

In the last years, an increasing number of studies identified some antibacterial activity in several drugs approved for different purposes, including anticancer, antifungal, and cardiovascular therapies [2,3], strongly supporting the research on drug repurposing as a strategy to identify novel antibacterials that can be rapidly delivered to the clinical phase.

3-Bromopyruvate (3BP) is a chemically synthesized halogenated derivative of pyruvate that has been used by biochemists for several decades. Indeed, alkylating properties have made 3BP suitable for use in enzymatic studies in vitro, with preferential targets focused on metabolic enzymes. About 15 years ago, 3BP was proven to also act as a powerful anticancer agent [4]. 3BP treatment abolishes ATP

production in cancer cells mainly by glycolysis inhibition [5], inducing rapid cancer cell death both in vitro and in several animal tumour models, including hepatocarcinomas, colon, breast and lung cancers [6]. Irrespective of the strong alkylating activity of 3BP in vitro, this compound showed an outstanding selectivity towards cancer cells in animal models, which appears to be related to i) the much higher expression of the main 3BP transporter (monocarboxylate transporter 1, MCT-1) in tumour cells as compared to normal cells, and ii) the metabolic differences between cancer cells and healthy ones. Indeed, while glycolytic metabolism is exacerbated in cancer cells, and indeed these cells display high rates of aerobic glycolysis even in the presence of oxygen (i.e. the "Warburg effect"), normal cells mainly rely on mitochondrial oxidative phosphorylation for energy production, thus resulting barely sensitive to the anti-glycolytic effect of 3BP [6,7]. The tumour-eradicating activity of 3BP has been documented in different animal models. While the majority of *in vivo* studies did not report relevant adverse effects of 3BP treatment on healthy cells or tissues at therapeutically-active doses, some liver toxicity was observed when very high dosing regimens and systemic delivery were used (reviewed in ref. [6]). Few studies have already described the use of 3BP in volunteer cancer patients [8,9], and 3BP appears to be currently exploited as a last-resort treatment in some cancer clinics worldwide [10, https://www.cancertreatmentsresearch.com/3-bromopyruvate/]. However, while the U.S. company PreScience Labs stated that the FDA approval for a Phase I clinical evaluation of 3BP in liver cancer patients was gained in 2013, 3BP has not yet undergone formal clinical trials, possibly because of lack of funding and/or the existence of competing patent applications [10]. A recent case of 3BP misuse by an unlicensed practitioner, who was accused of causing three deaths in a German alternative medicine clinic in 2016, may have somehow complicated the road of this candidate drug to the clinic [10].

Regarding the possible activity of 3BP on microorganisms, a couple of studies highlighted the capability of 3BP to inhibit the growth of eukaryotic pathogens, such as the fungus *Cryptococcus neoformans* and the microalgae *Prototheca* spp. [11,12]. Although a patent application for use of anti-

glycolytic compounds, including 3BP, to prevent or treat bacterial infections was submitted in 2011 [13], the antibacterial activity of 3BP was never investigated experimentally, with the only exception of a recent study showing mild growth inhibition of *Mycobacterium tuberculosis* exposed to millimolar concentrations of 3BP [14].

This work was intended to assess the possibility of 3BP repurposing as an antibacterial agent against pathogens of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) for which new drugs are urgently needed. Our working hypothesis originated from the following: (i) bacterial pathogens possess analogues of eukaryotic glycolytic enzymes, (ii) pyruvate is an intermediate of the energy metabolism in many pathogenic bacteria, and (iii) 3BP inhibits some bacterial glycolytic enzymes *in vitro* [12]. Our results revealed that 3BP has bactericidal and anti-biofilm activity against *S. aureus*, including multidrug-resistant isolates, while it does not inhibit all the other ESKAPE bacteria, and suggest that the specificity of 3BP towards *S. aureus* is likely due to unique metabolic features of this species.

2   Materials and methods

2.1   Bacterial strains and growth conditions. Bacterial strains were routinely maintained in Luria-Bertani (LB) medium supplemented with 1.5% agar at 37°C. Muller-Hinton (MH) broth was used for antibacterial assays. When indicated, MH was adjusted at different pH values by the addition of HCl and sterilized by filtration, or supplemented with glucose or pyruvate at 5 or 50 mM concentration. Anaerobic conditions were obtained by incubating plates in a GENBox Jar 2.5L in the presence of a GENbox anaerobic generator (BioMérieux). To support anaerobic growth of *S. aureus*, MH medium

was supplemented with 5 g/l glucose. Tryptic Soy Broth (TSB) supplemented with 2% glucose and 2% NaCl (TSB-GN) was used for biofilm assays [15]. 3BP was purchased from Sigma-Aldrich, and the stock solutions was prepared in water and stored at -20°C before use.

2.2   Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. The MIC of 3BP and antibiotics was determined using the broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines [16], after 24-h incubation at 37° C under static conditions. The MBC of 3BP for *S. aureus* isolates was determined by plating dilutions from each well showing no bacterial growth on LB agar plates. Colonies were counted after 24 h-incubation at 37°C. The MBC was defined as the lowest concentration of 3BP required to kill 99.9% of the initial inoculum [17].

2.3   Time-kill assays. Time-kill assay were performed using the CLSI M26-A standard method [17] with some modifications. Briefly, *S. aureus* ATCC25923 was cultured in MH for 6-8 h and then diluted in MH at a final OD_{600}=0.01 (corresponding to *ca.* 10^6 CFU/ml) in the absence or in the presence of 3BP and/or gentamicin. Bacterial cultures were incubated at 37°C under vigorous shaking (200 rpm). At different time points, serial dilutions were prepared in saline (0.9% NaCl) and aliquots of each dilution were plated on LB agar plates to determine the number of viable cells (CFU).

2.4   Checkerboard assay. The checkerboard broth microdilution method [18] was performed to investigate the interaction of 3BP with ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol or ofloxacin. Two-fold serial dilutions of 3BP and each antibiotic in MH were perpendicularly dispensed in 96-well microtiter plates, and each well was inoculated with *S. aureus* ATCC25923 at *ca*. 5×10^5 CFU/ml. Microtiter plates were incubated at 37° C under static conditions for

24 h, and bacterial growth was visually assessed. Results were expressed as Fractional Inhibitory Concentration index (FICI), which corresponds to the sum of the FICs of the two antibacterial compounds, where the FIC for each drug is defined as the MIC of the drug in combination divided by the MIC of the drug used alone. The FICI was interpreted as follows: ≤0.5, synergy; >0.5–4, indifference; >4, antagonism [18].

2.5   Biofilm assays. Biofilm formation was evaluated in TSB-GN as previously described, with few modifications [15]. *S. aureus* strains were cultured in TSB at 37°C for 6-8 h, refreshed in TSB-GN at $OD_{600} = 0.01$, and 100-μl aliquots were dispensed in the wells of tissue-culture treated 96-well microtiter plates. After 24 h at 37°C under static conditions, planktonic cells were removed and microtiter plates were washed three times with distilled water to remove non-adherent cells. Microtiter plates were air-dried, and adherent biofilms were fixed with 100 μl of 95% ethanol for 10 min. Ethanol was discarded, microtiter plates were air-dried and fixed biofilm were stained with 100 μl of 1% crystal violet for 10 minutes. Unbound crystal violet was removed and microtiter plates were washed three times with distilled water and air-dried. The biofilm-bound dye was solubilised with 100 μl of 33% glacial acetic acid and, after 10 minutes, quantified by measuring the OD_{600} in a Wallac 1420 Victor3V plate reader. To assess the anti-biofilm activity of 3BP, 24-h old biofilms were incubated under static conditions at 37°C for further 24 h in TSB-GN supplemented or not with 3BP at 1×, 2×, 4× or 8×MIC. Then, biofilms were fixed, stained and quantified as described above.

2.6   Construction of the deletion mutant *P. aeruginosa* PAO1 Δ*gshA*

Deletion mutagenesis in *P. aeruginosa* was performed as previously described [19], using the suicide vector pDM4Δ*gshA*, which was generated by cloning ca. 450-bp long DNA fragments corresponding to

the upstream and downstream genomic regions of *gshA* (PA5203) into the plasmid pDM4. Plasmids and primers used for cloning are listed in Table S1.

2.7   Statistical analysis. Statistical analysis was performed with the software GraphPad Instat, using one-way analysis of variance (ANOVA).

3. Results

3.1   Antibacterial activity of 3BP against ESKAPE pathogens

Preliminarily, the MIC of 3BP was determined on a small selection of bacterial strains belonging to the ESKAPE group. 3BP showed antimicrobial activity against the two *S. aureus* strains, ATCC25923 and the methicillin-resistant ATCC43300, with MIC values of 20 and 40 μg/ml, respectively. In contrast, all the other ESKAPE species were insensitive to 3BP up to 320 μg/ml (Table 1). The anti-*S. aureus* activity of 3BP was further evaluated on 20 clinical and veterinary isolates, including some cystic fibrosis isolates and several antibiotic resistant strains (Table S2). 3BP was found to inhibit the growth of all *S. aureus* isolates tested, with MIC values ranging between 20 and 80 μg/ml (Table S2).

To further investigate the anti-staphylococcal activity of 3BP, the MBC was also determined for each isolate. Notably, 3BP MBC was identical to the MIC for 77% of the strains (17 out of 22), and only 2×MIC for the remaining strains (Tables 1 and S1), suggesting that 3BP is primarily endowed with bactericidal activity against *S. aureus*. To confirm this, a time kill assay was performed using *S. aureus* ATCC25923 as the reference strain and 3BP at concentrations corresponding to 1×, 2× or 4×MIC. 3BP was able to kill *S. aureus* cells in a dose-dependent manner, with a very fast bactericidal activity, as 97.3, 98.7 and 99.7% of cells died within 1 h of 3BP treatment at 1×, 2× and 4×MIC, respectively.

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(Figure 1). Under these experimental conditions, the 3 $\log_{10}$ reduction in the number of CFU, which is required to define an antibiotic as bactericidal [17], was obtained with 80 μg/ml of 3BP after ≥ 2 h of treatment (Figure 1).

3.2   Effect of culture conditions on the anti-staphylococcal activity of 3BP

It has been reported that the stability of 3BP in aqueous solutions is significantly higher at acidic pH as compared with neutral (physiological) or basic pH [23]. To investigate any pH dependence of antibacterial activity, the 3BP MIC was determined for the reference strain *S. aureus* ATCC25923 in MH adjusted at different pH values (range 5-8; Table 2). While the different pHs did not relevantly affect the ability of *S. aureus* ATCC25923 to grow under the tested conditions, the growth rate was slightly reduced at pH 5 as compared to other pHs (Figure S1). Notably, a significant decrease in 3BP MIC was observed from basic to acidic pH. Indeed, 3BP MIC was 4-fold reduced at pH 5 and 8-fold increased at pH 8 with respect to the MIC at pH 7 (Table 2), indicating that the acidic pH significantly enhances the antibacterial activity of 3BP, probably as a result of increased stability of the compound.

Notably, exogenous pyruvate protected *S. aureus* from the antibacterial activity of 3BP in a dose-dependent manner, while equivalent concentrations of glucose did not (Table 2), suggesting that pyruvate and 3BP compete for transporter(s) and/or specific intracellular target(s).

3BP activity against *S. aureus* was also assessed during anaerobic growth, which was reported to increase the rate of glycolysis in this bacterium [24,25]. The MIC of 3BP for ATCC25293 was four-fold lower than that obtained under aerobiosis (Table 2), implying that *S. aureus* cells are more sensitive to 3BP under anaerobic conditions.

To verify whether active metabolism is essential for the antibacterial activity of 3BP, we tested the bactericidal activity of 4×MIC 3BP (80 μg/ml) in saline against *S. aureus* ATCC25923 pre-incubated or not at 4°C for 16 h in saline to reduce metabolic activities. No increase in the resistance to 3BP-

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mediated killing was observed in metabolically-inactive (or poorly active) cells (Figure 2), suggesting that 3BP treatment could also be effective against dormant *S. aureus* cells.

3.3   Interaction between 3BP and antibiotics

To assess 3BP interaction(s) with currently-available antibacterial drugs, checkerboard assays with 3BP and several antibiotics characterized by different modes of action were conducted using *S. aureus* ATCC25923 as test strain. 3BP had a synergistic effect only with gentamicin (Table 3). Although indifference was observed with all other antibiotics, ampicillin-3BP and tetracycline-3BP combinations showed a FICI close to 0.5 (Table 3), suggestive of some additive effect. Notably, no drugs pair showed antagonistic effect (FICI>4) (Table 3).

The synergy between 3BP and gentamicin was confirmed by time-kill assays; *S. aureus* ATCC25923 was treated with 0.5×MIC of 3BP (10 µg/ml), gentamicin (0.156 µg/ml) or both compounds, and the number of CFU/ml was counted over time (Figure 3). Notably, the number of CFU/ml decreased in the first 4 h of treatment with both compounds, either alone or in combination. However, while *S. aureus* ATCC25923 treated with sub-MIC concentrations of 3BP or gentamicin alone attained growth levels almost comparable to the untreated control (20-h incubation), the combination of 3BP with gentamicin at 0.5×MIC completely abrogated re-growth. Since synergism is defined as a ≥ 2 log10 decrease in CFU/ml for the drug combination as compared to each drug alone at 20 h [26], this experiment confirms that 3BP and gentamicin exert a synergistic effect on *S. aureus*.

3.4   Anti-biofilm activity of 3BP

Biofilm formation during infection is a primary cause of antibiotic treatment failure, as bacterial cells encased into biofilms are generally more resistant to antibiotics than their planktonic counterparts [27]. To assess whether 3BP retains its antibacterial activity on biofilm cells, pre-formed biofilms of *S.*

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aureus ATCC25923 were treated for 24 h with increasing concentrations of 3BP, corresponding to 1×, 2×, 4× and 8×MIC. Notably, a complete disruption of ATCC25923 biofilm was observed in the presence of 3BP concentrations ≥ 2×MIC (Figure 4). No significant differences in biomass were observed between 24 or 48-h old untreated biofilms and biofilms treated with 3BP at 1×MIC (Figure 4).

To verify whether the anti-biofilm properties of 3BP are shared by different *S. aureus* strains, the same assay was performed on the strains ATCC43300, BG2 and BG7, endowed with different levels of sensitivity to 3BP in the MIC and MBC assays (Table S1). Overall, 3BP showed biofilm-disrupting activity against all strains, although some differences were observed. For instance, ATCC43300 biofilms were almost completely disrupted at ≥ 2×MIC (similarly to ATCC25923), while 3BP at 1×MIC only inhibited biofilm growth without causing biofilm disruption (Figure 4). Biofilm disruption at ≥ 2×MIC was also observed for BG7, although the effect was weaker than that observed with ATCC25923 and ATCC43300 (Figure 4). Finally, BG2 was a bit more resistant to the anti-biofilm activity of 3BP, as significant biofilm disruption was only observed at ≥ 4×MIC (Figure 4). In the whole, this experiment demonstrates that 3BP disrupts *S. aureus* biofilms, though at concentrations slightly higher than those active against planktonic cells.

4. Discussion

The rise of resistance among bacterial pathogens leads to a growing need for novel antibacterials. Here, we assessed the antibacterial activity of a well-known antimetabolite, 3BP, which is in preclinical phase of development as an antitumor drug. We found that 3BP has bactericidal activity against *S. aureus*, with MIC and MBC values for different strains overall comparable to the concentrations active

against cancer cell lines [6,28] or fungal cells [11] (Table 1 and S1). In contrast, 3BP does not inhibit the growth of any other ESKAPE species (Table 1), either Gram-negative or Gram-positive, suggesting that the antibacterial activity of 3BP is specific to *S. aureus* (and maybe closely-related species). The biological and molecular basis of such specificity is unknown. Considering that 3BP susceptibility in eukaryotic cells (i) positively correlates with 3BP uptake rates and (ii) negatively correlates with intracellular levels of glutathione [11], it is tempting to discuss whether these two factors could somehow justify the observed selectivity of 3BP towards *S. aureus*. Regarding 3BP uptake, at least two of the species found to be highly resistant to 3BP (i.e., *A. baumannii* and *P. aeruginosa*) are able to grow and/or obtain energy in minimal media containing lactate or pyruvate as the sole carbon source [29-32], indicating that these species possess transporter(s) for lactate/pyruvate uptake, which could also mediate internalization of the 3BP analogue. However, considering that in bacteria enzymes and transporters involved in catabolism are often tightly regulated [33], it cannot be excluded that the expression of these systems in these two species is somehow repressed during growth in the nutritionally rich MH medium. Unfortunately, the knowledge on lactate and pyruvate transport systems in bacteria is still scarce [34], and thus the presence and/or expression of these transporters cannot be simply predicted by genomics and/or transcriptomic analyses. 3BP uptake was measured in fungal cells by using [14C]-labeled 3BP [11]; however, this radioactive compound is not commercially available, hampering the direct assessment of 3BP internalization by bacteria in the present work. Thus, it cannot be ruled out that some specific feature(s) of the cell envelope can somehow influence the uptake of 3BP and, thus, its antibacterial activity in different bacteria.

Interestingly, a putative correlation can instead be predicted between glutathione and 3BP antibacterial activity. Indeed, among ESKAPE bacteria, *S. aureus* is the only one which does not produce glutathione [35,36]. In both cancer and fungal cells glutathione has been proposed to protect from 3BP toxicity by preventing oxidative stress [11,37] and/or by directly interacting with and titrating 3BP,

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ultimately reducing its toxic levels in the cytoplasm [38]. It can be hypothesized that the higher sensitivity of *S. aureus* to 3BP could, at least partially, be related to the lack of glutathione-based detoxification mechanism(s). To test whether endogenous glutathione production can *per se* account for 3BP resistance, we deleted in *P. aeruginosa* PAO1 the *gshA* gene, which is essential for glutathione biosynthesis [39]. Notably, we found that the MIC of 3BP for the glutathione-deficient *P. aeruginosa* Δ*gshA* mutant was 320 µg/ml, i.e. just 2-fold lower than that of the wild type (640 µg/ml), and still 4-16 fold higher than that for *S. aureus* strains (Table S1). This strongly suggests that the inability to produce glutathione does not represent the only reason for the specificity of 3BP towards *S. aureus*.

Finally, the anti-*S. aureus* activity of 3BP could be related to specific metabolic features of this bacterium. The ESKAPE group contains a strictly aerobic bacterium (*A. baumannii*) and five facultative anaerobes, which can use either anaerobic respiration and fermentation (*K. pneumoniae*, *Enterobacter* spp., *P. aeruginosa*) or only fermentation (*S. aureus*, *Enterococcus* spp.) for energy production during anaerobic growth. Thus, the susceptibility of *S. aureus* to 3BP does not appear to be related to a distinctive catabolic potential of this bacterium. However, it cannot be excluded that the presence of unique metabolic pathway(s), and/or possible differences in the sequence or level of expression/activity of specific metabolic enzymes could account for the higher sensitivity of *S. aureus* to 3BP, as compared to other bacteria. Experiments are in progress to tentatively identify the transport mechanism(s) and intracellular target(s) of 3BP in *S. aureus* and to define its mechanism of action as antibacterial agent.

In addition to its bactericidal activity on planktonic cells, 3BP has also shown potent biofilm-disrupting properties, causing almost complete detachment of *S. aureus* biofilms at relatively low concentrations (only 2-4 fold higher than the MIC) (Figure 4). This result indicates that, differently from the majority of antibiotics which are much less effective against biofilms relative to planktonic cells, 3BP retains its anti-*S. aureus* activity also against biofilm-forming cells. The high tolerance to antibiotics of biofilm-

forming cells is generally ascribed to many factors, such as expression of specific resistance genes, low growth rates, poor diffusion of antibiotics across the biofilm matrix, and lower antibiotics activity under low-pH and/or low-oxygen conditions, which are often present in bacterial biofilms [40-42]. In this view, the high anti-biofilm activity of 3BP could be justified by (i) its low molecular weight and high water solubility, that likely promote diffusion across the exopolysaccharide matrix and/or water channels of the biofilm, (ii) its high stability at low pH [23], and (iii) its activity also against cells that adopt a fermentative metabolism and/or metabolically-inactive cells (Table 2 and Figure 2), such as those present in the deepest biofilm layers.

In conclusion, this study describes the promising *in vitro* activity of the antimetabolite 3BP against both planktonic and biofilm *S. aureus* cells. This compound is currently in preclinical development for anticancer therapy. Although many studies have reported its efficacy and safety in several animal tumour models involving different administration routes, such as intravenous, intraperitoneal, inhalation or intratumoral delivery (reviewed in [6]), clinical trials on 3BP have not been conducted yet and detailed pharmacokinetics studies are not available, either in animals or in humans. The concentrations of 3BP active against *S. aureus* are quite high with respect to conventional antibiotics (Table 1 and S1), but are similar to those active against cancer cell lines [6,28], suggesting that 3BP administration could attain *in vivo* 3BP concentrations that are effective also against *S. aureus* cells, at least in animal models. Moreover, considering the high anti-biofilm activity of this compound and its increased activity under anaerobic and acidic conditions, which are common in bacterial biofilms [40-42], 3BP could have potential for the topical treatment of surface-exposed biofilm-related infections, such as chronic wound and ocular *S. aureus* infections [43], which would allow to administer relatively high 3BP doses with a lower risk of systemic toxicity. In this view, our *in vitro* results could thus pave the way for the evaluation of 3BP as a narrow-spectrum antibacterial agent for the treatment of *S. aureus* in acute and/or chronic infections models. More relevantly, the characterization of the

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mechanism(s) of action of this antimetabolite in *S. aureus* could highlight novel molecular target(s) and, therefore, drive the rational design of more active compounds against this important human pathogen.

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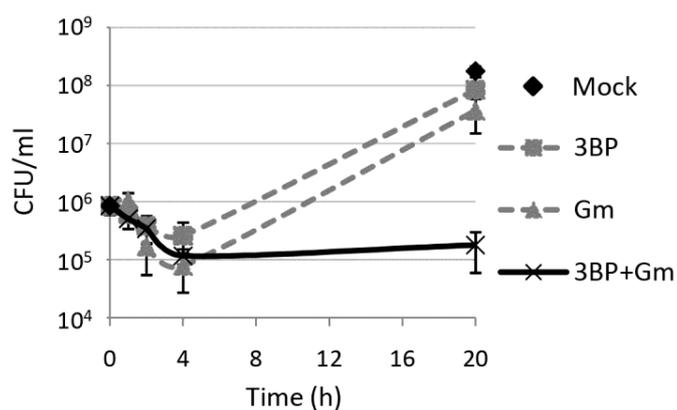
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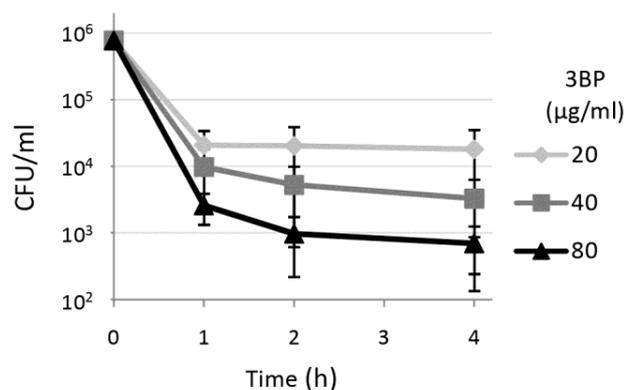
Figure 1. Time-kill curves of *S. aureus* ATCC25923 exposed to 3BP. *S. aureus* ATCC25923 was cultured in MH, adjusted at *ca.* 10^6 CFU/ml in MH containing 3BP at 20, 40 or 80 μg/ml and incubated at 37°C with vigorous agitation. At the indicated time points (0, 1, 2 and 4 h), cell viability was assessed by CFU counting on agar plates. The results are the mean (±SD) of at least four independent assays.
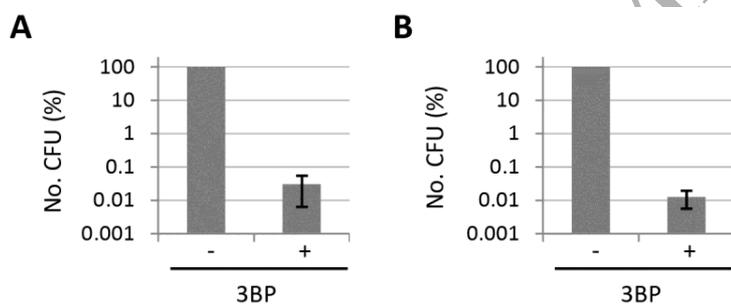
Figure 2. Killing activity of 3BP on metabolically-inactive cells. *S. aureus* ATCC25923 was cultured in MH, adjusted at ca. 10^6 CFU/ml in saline and (A) immediately treated with 3BP at 4×MIC (80 μg/ml) in saline for 2 h at 37°C or (B) incubated at 4°C for 16 h and then treated with 3BP (80 μg/ml) in saline for 2 h at 37°C. Results are reported as percentage of CFU with respect to the untreated controls (100%), and represent the mean (±SD) of three independent assays.

Figure 3. Time-kill curves of *S. aureus* ATCC25923 exposed to 3BP and gentamicin alone or in combination. *S. aureus* ATCC25923 was cultured in MH, adjusted at ca. 10^6 CFU/ml in MH containing either 3BP or gentamicin or both at 0.5×MIC (10 µg/ml and 0.156 µg/ml, respectively), and incubated at 37°C with vigorous agitation. At the indicated time points (0, 1, 2, 4 and 20 h), cell viability was assessed by CFU counting on agar plates. The results are the mean (±SD) of three independent assays.

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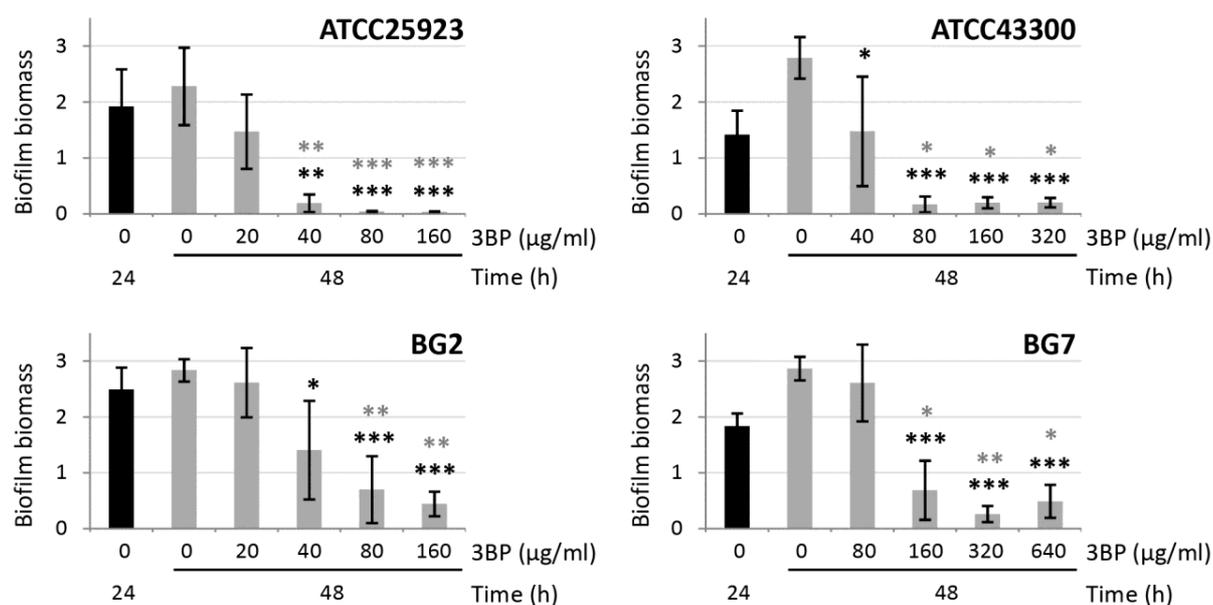
Figure 4. 3BP-mediated disruption of *S. aureus* biofilms. Biofilms of *S. aureus* strains ATCC25923, ATCC43300, BG2 and BG7 were developed in microtiter plates containing TSB-GN medium. After 24 h, supernatants were discarded, and fresh TSB-GN containing increasing 3BP concentrations was added (1×, 2×, 4× or 8×MIC). Then plates were incubated for further 24 h. Biofilm biomass was quantified as described in Materials and methods. The results are the mean (±SD) of four independent assays. Black histograms represent the biomass of 24-h old biofilms before treatment. Grey and black asterisks denote statistically significant differences with respect to 24-h old biofilms or untreated 48-h old biofilms, respectively. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (ANOVA).

Table 1. **3BP activity against ESKAPE bacteria.[1]**

Species	Strain	Reference/source	MIC (µg/ml)	MBC (µg/ml)
E. faecium	3137	Rossolini's Lab collection	>320	NA
E. faecalis	ATCC29212	ATCC	>320	NA
S. aureus	ATCC25923	ATCC	20	40
	ATCC43300 (MRSA)	ATCC	40	80
K. pneumoniae	ATCC27736	ATCC	>320	NA
	17830 (MDR)	[20]	>320	NA
A. baumannii	ATCC19606	ATCC	>320	NA
	ACICU (MDR)	[21]	>320	NA
P. aeruginosa	ATCC15692	ATCC	>320	NA
	SP1 (MDR)	[22]	>320	NA
E. cloacae	ATCC13047	ATCC	>320	NA
E. aerogenes	ATCC13048	ATCC	>320	NA

[1] Abbreviations: MDR, multidrug resistant; MRSA, methicillin-resistant *S. aureus*; ATCC, American Type Culture Collection; NA, not assessed.

Table 2. Effect of pH and anaerobiosis on 3BP MIC for *S. aureus* ATCC25923

Condition	pH	Supplement	MIC (µg/ml)
Aerobiosis	5		5
	6		10
	6.5		10
	7		20
	7.5		40-80
	8		160
	7	Glucose (50 mM)	20
	7	Glucose (5 mM)	20
	7	Pyruvate (50 mM)	320
	7	Pyruvate (5 mM)	80
Anaerobiosis	7		5

[1] MIC fold change relative to the MIC at pH 7 under aerobic conditions (20 µg/ml, corresponding to 120 µM).

Table 3. Interaction between 3BP and selected antibiotics against *S. aureus* ATCC25923

Antibiotic	MIC (μg/ml)	FIC index (FICI)[1]	Interpretation
Gentamicin	0.312	0.438	Synergy
Ampicillin	0.156	0.625	Indifference
Tetracycline	0.25	0.563	Indifference
Rifampicin	0.004	2	Indifference
Chloramphenicol	8	1	Indifference
Ofloxacin	0.25	2	Indifference

[1] FICI: < 0.5, synergy; 0.5–4, indifference; >4, antagonism.