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Letters

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Bioorthogonal Profiling of a Cancer Cell Proteome Identifies a Large Set of 3-Bromopyruvate Targets beyond Glycolysis

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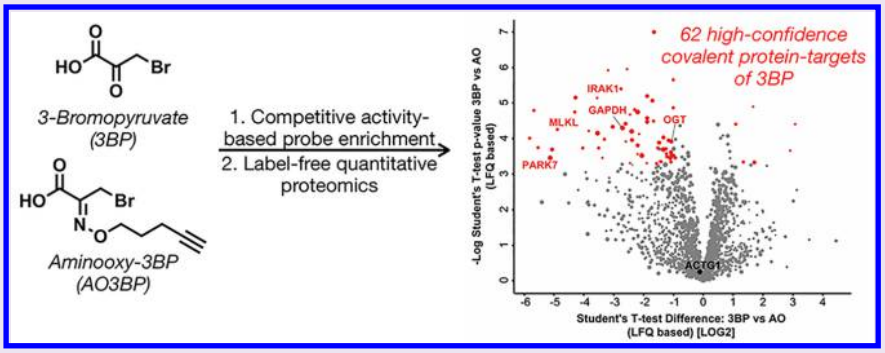
Supporting Information



ABSTRACT: 3-Bromopyruvate (3BP) is a potential anticancer agent viewed as a glycolytic inhibitor that preferentially kills cancer cells through inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), resulting in severe energy depletion. We previously identified four cysteine residues in GAPDH that are alkylated by 3BP, resulting in its inactivation. However, we also showed that addition of excess pyruvate, the final product of glycolysis, was unable to rescue cells from 3BP treatment. This result indicates that GAPDH may not be the only relevant target and is consistent with the chemical reactivity of 3BP that should result in the modification of cysteine residues in many different proteins. To directly test this hypothesis, we first synthesized a probe of 3BP activity bearing an alkyne functionality, termed AO3BP, and then demonstrated that this probe could modify a variety of proteins in living cells. Subsequent competition of AO3BP labeling with pretreatment by 3BP identified 62 statistically significant proteins of various functions as targets of 3BP, confirming that 3BP labeling is indeed widespread. We conclude that 3BP's cytotoxic impact on cancer cells does not only result from selective inhibition of glycolysis but rather from a more widespread effect on cellular proteins that could be driven by the pharmacokinetics of the 3BP. These pleiotropic consequences should be considered when thinking about the potential toxicity of this highly reactive compound.

3-Bromopyruvate (3BP or 3-bromopyruvic acid, Figure 1) is a reactive, electrophilic derivative of pyruvate that has generated interest as a potential anticancer therapeutic agent.[1] It has demonstrated efficacy in a number of animal tumor models,[2−5] although liver toxicity was noted.[6,7] Two case studies have also been reported on 3BP administration to patients, where one patient with hepatocellular carcinoma (HCC) showed a favorable response to treatment.[8,9] This result was encouraging enough to catalyze the scheduling of a phase I study of 3BP (as PS-101) in HCC patients in 2015, but it has not started.

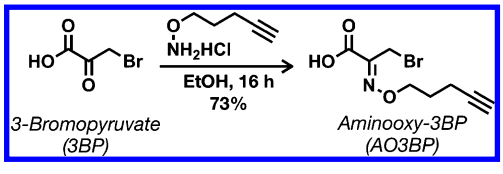


Figure 1. 3-Bromopyruvate and our bioorthogonal probe aminooxy-3BP (AO3BP).

3BP is hypothesized to target cancer cells through inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to a shut down of glycolysis, and selective killing of glycolytically active tumor cells over normal cells (the Warburg effect).[10−12] However, given the inherently reactive structure of 3BP, it is not surprising that it has been found to impact a number of other enzymatic and biological processes. More specifically, 3BP has been found to inhibit methylisocitrate lyase, 3-phosphoglycerate kinase, succinate dehydrogenase

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(SDH; complex II), and hexokinase II,[13−21] although the impact on the last was not observed consistently.[10,15,22] Moreover, 3-BP was reported to cause oxidative stress via depletion of intracellular glutathione and its impact on mitochondria, leading to increased levels of reactive oxygen species (ROS).[22−26] More recently, additional pleiotropic effects of 3-BP, including stimulation of autophagy,[27] induction of endoplasmic reticulum stress,[28] altered expression of cell death-regulatory proteins,[29] and dysregulation of two key intracellular signal transduction pathways, the Akt/mTOR and the MAP kinase pathways,[30] were reported.

Our own work confirms 3BP inhibition of GAPDH, as we identified four cysteine residues that were alkylated by 3BP, resulting in inhibition of enzymatic activity and consequential shutdown of glycolysis.[31] However, we also found that extraneous addition of excess pyruvate (the end product of the glycolysis) was unable to prevent the ensuing cell death.[31] These results indicate that inhibition of glycolysis is not solely responsible for cellular toxicity. At best, this implies that the current model of 3BP action is incomplete and calls into question whether 3BP can be used to selectively target the Warburg effect in cancer. In fact, 3BP treatment is suspected to have caused the deaths of three patients treated at an alternative medicine clinic in Germany,[32] further underscoring the urgency to characterize this compound's biological impact in greater detail.

As 3BP appears to be largely a covalent inhibitor, the most direct next step in understanding its effects is the direct identification of 3BP protein-targets. To accomplish this goal, we first synthesized a probe of similar structure to 3BP, 3-bromo-2-((hex-5-yn-1-yloxy)imino)propanoic acid (AO3BP, Figure 1 and Scheme S1), which bears an alkyne for copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC). By combining AO3BP with CuAAC and a fluorescent tag, we first characterized its protein-labeling in living cancer cells. We then used an enrichment tag and competition with unmodified 3BP to identify 62 high-probability target proteins with a variety of cellular functions. These results further support a "pleiotropic" effect of 3BP treatment that should be considered in any further clinical development of this compound.

To generate a bioorthogonal probe for 3BP modifications, we wanted to minimize the structural alterations to the molecule while considering the ease of the synthetic route. We therefore settled on a strategy where the inherent reactivity of the 3BP ketone was exploited in an oxime-formation reaction with 1-(aminooxy)pent-4-yl to give AO3BP (Figure 1). GAPDH was then treated with various concentrations of either 3BP or AO3BP *in vitro* for 6 min before the enzymatic activity was measured. Both compounds resulted in enzyme inhibition, while we did observe a small decrease in AO3BP potency compared to 3BP (Figure S1). As an additional control, we also synthesize a nonclickable version of AO3BP though an oxime-formation reaction with commercially available *O*-ethylhydroxylamine to give Et3BP (Scheme S1). Notably, Et3BP was also able to inhibit GAPDH, with a potency essentially identical to AO3BP (Figure S1). These results show that AO3BP and at least one other oxime-containing compound (Et3BP) are able to block GAPDH activity, an established activity of 3BP.

We next tested AO3BP as a probe in living cells by treating cancer cells with various concentrations (25−200 μM) or DMSO vehicle for 5 h. The corresponding cell lysates were then subjected to CuAAC with an azido-TAMRA dye and analyzed by in-gel fluorescence (Figure 2A). As is clear from the "low fluorescence" image, AO3BP strongly labeled a protein of approximately 35 kDa in size that quite likely represents GAPDH. However, when the fluorescence intensity is increased (high fluorescence) a wide range of labeled proteins becomes clearly visible, particularly at higher AO3BP

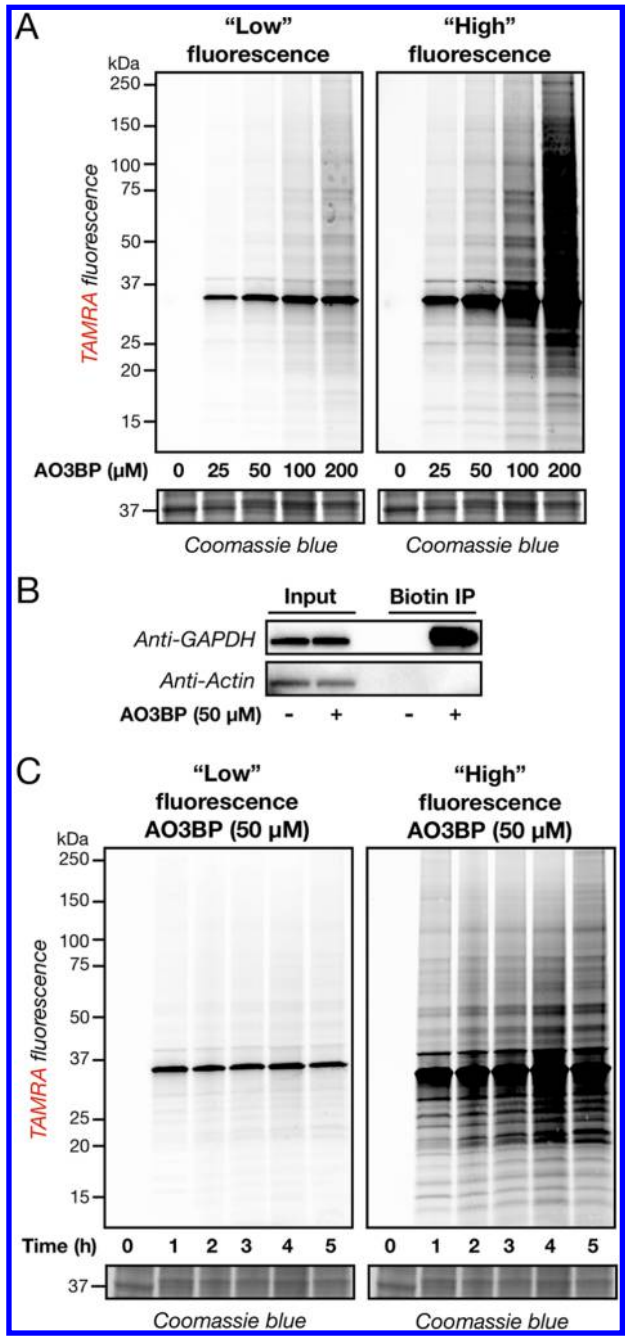

Figure 2. AO3BP is a covalent modifier for proteins in living cells. (A) H1299 cells were treated with the indicated concentrations of AO3BP for 5 h before CuAAC with azido-TAMRA and in-gel fluorescent scanning. (B) H1299 cells were treated with AO3BP (50 μM) for 5 h, followed by CuAAC with azido-azo-biotin and enrichment with neutravidin-coated beads. After washing and elution, the indicated proteins were visualized by Western blotting. (C) H1299 cells were treated with AO3BP (50 μM) for the indicated lengths of time before CuAAC with azido-TAMRA and in-gel fluorescent scanning.

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concentrations. Notably, the pattern of modified proteins remained consistent at all of the tested concentrations.

To confirm that GAPDH was indeed a direct target of AO3BP, cultured cells were treated with AO3BP (50 μM) for 5 h. Then, the lysates were subjected to CuAAC with a cleavable biotin tag (azido-azo-biotin). The labeled proteins were then captured with NeutrAvidin beads, followed by stringent washing. The enriched proteins were eluted by treatment with sodium dithionite and subjected to SDS-PAGE and Western blotting (Figure 2B). GAPDH was indeed selectively enriched from the AO3BP-treated cells, while actin, another abundant cysteine-containing protein was not, indicating some selectivity for AO3BP labeling.

As a final characterization experiment, we tested the kinetics of labeling. Accordingly, H1299 cells were treated with AO3BP (50 μM) for different lengths of time (1−5 h) before lysis, CuAAC with azido-TAMRA, and in-gel fluorescence (Figure 2C). Interestingly, we found that protein labeling was already maximal at 1 h with no major changes at later time points, supporting the high reactivity of 3BP in cells.

Next, we further validated AO3BP as a reasonable probe to identify 3BP reactivity by performing a competitive-labeling experiment. More specifically, H1299 cells were first treated with either 3BP (50 μM) or Et3BP (50 μM) for 1 h, after which time the medium was exchanged for fresh medium containing AO3BP (50 μM) for an additional hour. The cells were then lysed, subjected to CuAAC, and analyzed by in-gel fluorescence (Figure 3). In both competition experiments, we

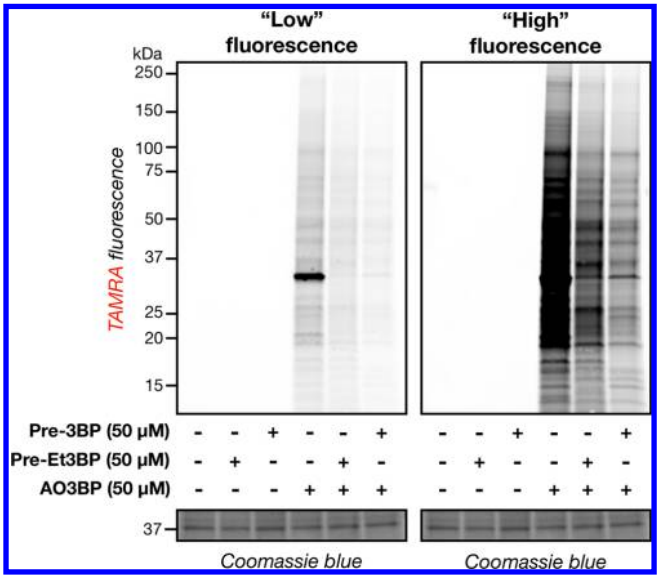

Figure 3. AO3BP labeling can be completed by preincubation with 3BP. H1299 cells were treated with either 3BP (50 μM), Et3BP (50 μM), or vehicle for 1 h before addition of AO3BP (50 μM) for an additional hour. CuAAC with azido-TAMRA followed by in-gel fluorescent scanning shows that 3BP and AO3BP have overlapping targets.

found a noticeable loss of AO3BP-labeling throughout the proteome with a greater reduction in lower molecular-weight targets between 15 and 50 kDa, including the major band at 35 kDa. These results indicate that at least a fraction of the AO3BP protein targets are indeed labeled at the same cysteine residues by 3BP. Additionally, we see slightly different patterns of competition between Et3BP and 3BP, highlighting the power of competitive activity-based probe experiments for identification of targets. To support this result, we repeated this competition experiment (in duplicate) and then reacted the cell lysates with the cleavable biotin tag. After enrichment, washing, and elution, the captured proteins were visualized by Colloidal Blue staining (Figure S2). Gratifyingly, these results were very similar to the fluorescence analysis in Figure 3, with 3BP pretreatment resulting in less enrichment of many different proteins, thus validating AO3BP as an effective 3BP mimetic.

Combined, our results support the application of AO3BP, in combination with 3BP competition, to identify 3BP targets by proteomics. Accordingly, cultured cells were subjected to competitive labeling in triplicate as above. The corresponding lysates were subjected to CuAAC with azido-biotin, followed by neutravidin enrichment and on-bead trypsinolysis. We then performed label free quantitative (LFQ) mass spectrometry[33] (n = 3 biological replicates; Table S1), resulting in the identification of 62 proteins (Figure 4A and Table S2) that gratifyingly contained GAPDH. To confirm these results, we repeated this competitive enrichment using azido-azo-biotin, released the labeled proteins and performed Western blotting against GAPDH and four other potential targets (Figure 4B). All five proteins were indeed enriched by AO3BP, which could be competed by pretreatment with 3BP, providing confirmation of our label-free proteomics and supporting the rather broad reactivity of 3BP across the proteome.

The development of 3BP as an anticancer agent has been based on a model where this agent is a GAPDH inhibitor suited to target aerobic glycolysis in tumor cells.[10−12,34] In support of this model, biochemical analysis from ourselves and others has identified GAPDH as a direct target of 3BP, where alkylation of several cysteines of this protein by 3-BP results in potent inhibition of GAPDH enzymatic activity.[10,31] Furthermore, depletion of cellular ATP pools in response to cellular treatment with 3BP has been thoroughly established.[11,22,31,34,35] However, a number of observations have revealed detrimental cellular effects of 3-BP that are not directly related to the inhibition of glycolysis,[22−30] providing accumulating evidence that 3BP perturbs a variety of cellular functions as a minimally selective alkylation reagent, rather than acting specifically on the Warburg effect of cancer cells.

To gain more insight into the pool of cellular proteins that can be covalently modified by 3BP, we employed a bioorthogonal probe strategy. First, we synthesized and characterized an alkyne-bearing probe of 3BP activity, AO3BP, demonstrating that this probe can indeed label a large number of proteins in living cells. We then showed that a notable fraction of this labeling can be reduced by pretreatment with 3BP, enabling us to combine this competition with label-free quantitative proteomics to identify 62 statistically significant 3BP targets. These targets represent proteins involved in a variety of cellular processes. Notably, several of these proteins have been identified to have highly reactive cysteines, including IRAK1, MLKL, OGT, and PARK7, and the target list is consistent with the general reactivity of other α-halo-ketones toward reactive cysteine residues.[36−39] The quality of these targets relies upon the overlap in the targets between AO3BP and 3BP. Our competition data show that this is true for many proteins, but the subtle differences between Et3BP and 3BP competition indicates that additional 3BP targets could likely be identified by using multiple clickable probes that have different overlapping target profiles

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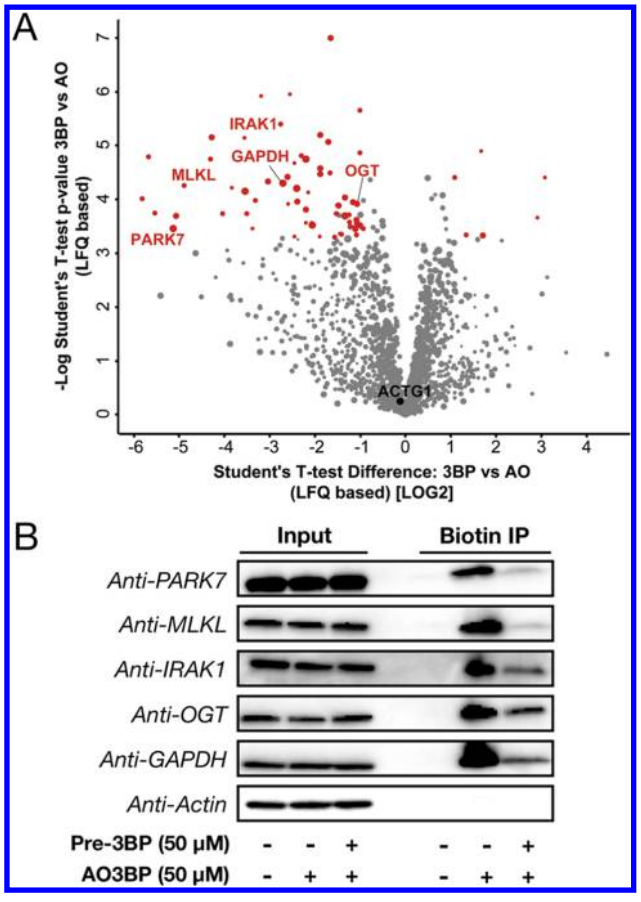

Figure 4. Unbiased identification of 3BP targets by competitive labeling and label-free quantitative proteomics. (A) The results of the *t* test based on label free quantitation is shown as a volanco plot (*x*-axis: log 2 transformed fold differences, *y*-axis; −log 10 *p*-value). Significant changing proteins (permutation based FDR of 0.01) that differ 2-linear-fold or more are marked in red. Selected proteins are labeled by the gene name. The size of the filled circle indicates the abundance of a given protein. (B) Confirmation of 3BP labeling of identified proteins. H1299 cells were treated with either 3BP (50 μM) or vehicle for 1 h before addition of AO3BP (50 μM) for an additional hour. This labeling was followed by CuAAC with azido-azo-biotin, enrichment, and selective elution. The indicated proteins were visualized by Western blotting and showed reduced AO3BP labeling with 3BP pretreatment.

with 3BP. The large number of potential targets for 3BP raises some important questions. Is the general alkylation of this diversity of proteins responsible for toxicity or are there specific targets critically involved in mediating drug-induced cell death? Likewise, what mechanism would explain any selective toxicity to tumors over healthy tissue? In summary, 3BP appears to be a general alkylating agent in living cells, consistent with its chemical structure. We believe that our results suggest that the mode or modes of action of this potential drug need to be further elucidated before its application in the clinic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00743.

Experimental methods and characterization of synthetic compounds, figures of AO3BP GAPDH inhibition and 3BP pre-treatment results, and NMR spectra (PDF)

proteomics data (XLSX)

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Notes

The authors declare no competing financial interest.

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