Mass Spectrometry-based Label-free Quantitative Proteomics To Study the Effect of 3PO Drug at Cellular Level

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Supporting Information

ABSTRACT: Human endothelial cells (ECs) have been employed to monitor the protein changes induced by [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one] (3PO), a compound able to inhibit the glycolytic flux partially and transiently and to reduce pathological angiogenesis in a variety of disease models. Normal and TNF α induced inflamed ECs were incubated with and without 3PO at a concentration (20 μ M) able to inhibit cell proliferation without cell death. At the end of the incubation period, samples were submitted to the following steps: (a) whole protein extraction, reduction, alkylation, and digestion by trypsin; (b) peptide separation by nano-LC–MS/MS analysis using a high-resolution mass spectrometer; (c) data analysis including protein identification, quantification, and statistical analysis. An altered protein expression profiling in combination with protein network analysis was employed by using a mass spectrometry-based label-free quantification approach to explore the underlying mechanisms of 3PO at cellular level.

KEYWORDS: Quantitative proteomics, PFKFB3 inhibitor, mass spectrometry, inflammation, cell pathway modulation, 3PO

To get an improved understanding of the mechanism of action of small biologically active molecules, it is mandatory to categorize their cellular protein targets. Undoubtedly, this is a complex problem due to the lack of a unique experimental solution, which can give complete information. However, mass spectrometry-based proteomic approaches, in combination with bioinformatics, deliver a progressively developing set of tools allowing a deeper insight into such information. Liquid chromatography–mass spectrometry (LC–MS) based protein quantification is a high-throughput approach, frequently used to study the biological processes in cells, tissues, biological fluids, and organisms. These approaches not only provide a list of identified proteins but also a lot of information that allows us to understand physiological changes occurring between two or more states. For instance, disease vs control; treated vs nontreated. Over the past decade, many MS based approaches have been successfully developed to address the questions related to protein–protein interaction, post-translational modifications, and protein expressions or abundances. In shotgun proteomics, a variety of labeling approaches have been developed including isotope dilution, radiolabeled amino acid incorporation, stable isotope labeling by amino acids in cell culture (SILAC), isotope-coded affinity tags (ICAT), chemically synthesized peptide standards, isobaric tags (iTRAQ) and tandem mass tags (TMT) for relative quantification, and AQUA peptide for absolute quantitation. Nevertheless, many labeling methods have potential limitations such as long workflow or complex sample preparation, incomplete labeling, a high concentration of sample requirement, very expensive experimental proce-

dures, and a limited number of sample analyses. Therefore, to overcome these complications, in the present study we focused on the development and application of a MS-based label-free quantitative method for the comprehensive *in vitro* characterization of a chemical compound in cell lines. This approach is cost-efficient and easy to manage, it allows relative quantitation by analyzing spectral count or ion intensities.^{1,2} The development of highly reproducible nano-HPLC separation, high-resolution mass spectrometers, and dedicated computational tools greatly improved the reliability and accuracy of label-free comparative LC–MS/MS analysis.³

The present study describes an application of label-free quantitative proteomics in human ECs (EA. hy926 cell lines) to analyze the effects of 3PO [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one] (Figure 1a), a compound that, being able to partially and transiently inhibit glycolysis *in vivo*, reduces pathological angiogenesis,⁴ very likely due to its ability to inhibit the PFKFB3 enzyme,⁵ an activator of a key glycolytic enzyme, 6-phosphofructo-1-kinase (PFK-1).⁴ However, high dose of 3PO (>30 μ M) may cause tumor vessel disintegration and enhance cell dissemination.⁶ Hence, identification and characterization of EC proteins whose expression is modulated by the effect of 3PO will expand our understanding of its mode of action. ECs rely on increased glycolysis activity rather than

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Figure 1. (a) Structure of 3PO and (b) effect of 3PO on EA.hy926 cell proliferation. ECs were treated with 3PO at various concentrations (10, 20, 30, and 40 μM) for 24 h, and percentage of cell proliferation was assessed by using MTT assay. All data represented as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.001$ compared with control ($N = 3$).

oxidative phosphorylation to generate energy for maintaining the cellular functions in angiogenesis. The glycolytic activity of ECs is critical for angiogenesis, and it has been demonstrated that reduction of glycolysis by silencing or blocking PFKFB3, results in impairment of EC proliferation, migration, and vascular sprouting *in vitro*.^{7,8} 3PO reduces vessel sprouting also *in vivo* (IP injection) in EC spheroids, zebrafish embryos, and the mouse retina by inhibiting EC proliferation and migration.^{4,9,10} Moreover, the compound has been shown to be selectively cytostatic to transformed cells, to suppress the tumorigenic growth of breast adenocarcinoma, leukemia, and lung adenocarcinoma cells *in vivo*.^{4,5} More recently, it has been demonstrated that 3PO significantly represses intraplaque angiogenesis and hemorrhages in mice, demonstrating its potential to prevent plaque rupture.¹¹ Therefore, PFKFBs play a crucial role in maintaining glucose homeostasis and control the rate of glycolysis in other tissues. Among four existing PFKFB3 isoforms, PFKFB3 is upregulated by inflammatory stimuli. Inflammation, a complex biological response that has a fundamental role in various diseases (autoimmune, neurodegenerative, cardiovascular, cancer, and microbial infectious diseases) is now recognized as a key factor involved in all the stages of disease progression of atherosclerosis, and novel findings provide an important link among EC dysfunction, angiogenesis, inflammation, and EC metabolism in several diseases.^{12–15} Moreover, an inflammatory activation of monocytes/macrophages, via Toll-like receptor ligands or pro-inflammatory cytokines, switches their metabolism from oxidative phosphorylation to aerobic glycolysis, further potentiating the inflammatory process.¹⁶ Notwithstanding the plethora of evidence indicating the key role of 3PO in reducing angiogenesis, the molecular mechanism of action of 3PO at EC level under physiological or inflamed conditions remains to be established. Recent findings indicate in fact that 3PO might act through mechanisms that are unrelated to PFKFB3 inhibition,¹⁷ and recent studies carried out with recombinant

PFKFB3 and microscale thermophoresis (to evaluate its interaction with 3PO) did not confirm any binding of the molecule to the enzyme (personal communication). Hence, the present study is focused on understanding the quantitative modifications of protein profiles in 3PO-treated ECs, in inflamed ECs and in inflamed 3PO-treated ECs.

To understand the 3PO molecular mechanism of action in the endothelium, in the presence or in absence of an inflammatory agent, EA.hy926 cells, a permanent human endothelial cell line¹⁸ were used as model and tumor necrosis factor alpha (TNF- α), as an inflammatory agent. In order to check the level at which concentration of 3PO inhibited EC proliferation, EA.hy926 cells were treated with different 3PO concentrations (10–40 μM) for 24 h, and cell proliferation percentages were determined using MMT assay (Experimental Procedure in the Supporting Information). As shown in Figure 1b, we observed a significant cell proliferation inhibitory effect at 20 μM concentration of 3PO. Therefore, we selected this concentration for further analysis. To induce the inflammation of endothelium, we treated the cells with 10 ng/mL concentration of TNF α for 24 h (Experimental Procedures mentioned in the Supporting Information).¹⁹

Figure 2 shows the workflow of proteomics approach. In the first step, we incubated EA.hy926 cells as described above. After all the treatments, cells were collected and subjected to the following steps: (a) protein extraction, reduction, alkylation, and in-solution digestion by trypsin; (b) peptide separation by nano-LC–MS/MS analysis by using an Orbitrap

Figure 2. Work-flow employed for the MS-based label-free quantitative proteomic analysis.

Fusion high-resolution MS. The resulting raw files from MS analysis were subjected to quantitative analysis. The quality and reproducibility of biological replicates were determined by using multiscatter plot analysis; the coefficient of correlation was measured based on the LFQ intensities generated from the latest version of MaxQuant software (v 1.6.2.3). The average Pearson coefficient was higher than 0.98 among all samples, indicating the high degree of reproducibility as shown in Figure S1.

In total, 2214 proteins and 18869 unique peptides were identified and quantified by the Andromeda search engine in MaxQuant using Uniprot *Homo sapiens* database; search parameters were 10 ppm tolerance on peptides, 0.8 Da on fragments, and less than 1% false discovery rate (Table S1). A two-sample *t* test was employed using the latest version of Perseus software (v 1.6.1.3) to define the proteins that were differentially regulated in the treated and control groups of samples. The following criteria were applied: S0 value was set to 2 on both sides, permutation-based FDR value set at 0.05. Proteins with a *P*-value less than 0.05 were considered statistically significant. We quantified 130 and 161 up- and down-regulated proteins in the 3PO treated ECs compared to the control group (Table S2). The distribution of differentially regulated proteins in ECs upon 3PO treatment is shown in Figure 3a. In this study, we did not identify many changes in up-regulated proteins. Therefore, we used down-regulated proteins for further analysis.

To describe the protein network, influenced by the drugs (3PO and 3PO/TNF α), we used the Clue-GO plug-in in the latest version of Cytoscape software (v 3.6.0) and Ingenuity Pathway Analyses (IPA, Qiagen). Both software interrogate the Gene Ontology term database related to protein accession numbers, such as KEGG terms, Reactome terms, and cellular component; molecular and biological processes terms. They assign a *P*-value analyzing the data by statistical tests. Figure 4a shows examples of differentially regulated pathways. Clearly 3PO targets mitochondria and down-regulated substrates such as cytochrome complex, mitochondrial respiratory chain complexes I, III, and IV, oxidoreductase complex, mitochondrial respiratory chain, mitochondrial membrane, and protein complex present in the mitochondrial inner membrane (Table S2). Most of the down-regulated proteins identified in the 3PO treatment originated from the mitochondrion inner membrane: CYC1 and ATP5L, respectively. CYC1 is a heme-containing component of the Cytochrome *b*-c1 complex that shuttles electrons between complex III and complex IV in the respiratory chain. In normal conditions, ATP5L or ATP synthase subunit g is a part of complex V that produces ATP from ADP in the presence of the proton gradient generated across the membrane by electron transport complexes of the mitochondrial respiratory chain. However, based on our results we can say that 3PO might be blocking the proton gradient across the membrane via inhibition of the proteins associated with the multiplex complexes of the electron transport chain, which may lead to the reduction of ATP synthesis. Therefore, the energy required for cellular metabolic activities and cell proliferation decreases.

Gene ontology enrichment analysis of molecular functions revealed that nucleoside-triphosphatase activity, electron transfer activity, GTPase activity, and coenzyme binding categories were significantly over-represented. In addition, Reactome pathway analysis (Figure 4a) revealed also the inhibition of the citric acid (TCA) cycle, ATP synthesis by

Figure 3. Distribution of differentially regulated proteins in ECs (a) after 24 h exposure with 3PO, (b) after the induction of inflammation with TNF α in the absence of 3PO, and (c) after induction of inflammation with TNF α in the presence of 3PO. Scatter plots of log2 fold change on x-axis against $-\log P$ -value on y-axis of significantly quantified proteins. Green color indicates down-regulation, and red color represents up-regulation.

chemiosmotic coupling, and pyruvate metabolism pathways. Under physiological conditions, pyruvate generated from glycolysis is converted into acetyl coA that enters into the TCA cycle, and the resulting NADH enters into the electron transport chain to produce ATP. This is essential for cell proliferation and cell metabolic activities. Studies have shown that increased levels of TCA intermediates increase anaerobic

Figure 4. Main pathways and respective genes found in different treatments of ECs. ClueGo plugin for Cytoscape software was used for the Reactome network pathway analysis. (a,b) Key pathways of down-regulated proteins in ECs upon 3PO exposure for 24 h. (c) Major pathways associated with down-regulated genes in inflammatory condition stimulated by using $\text{TNF}\alpha$, in the presence of 3PO. Significance of the clustering is shown by color code and the size of nodes. Panel b: Green color indicates down-regulation, and red color represents upregulation of proteins.

pathways and aerobic fatty acid oxidation for ATP production in macrophage-rich atherosclerotic arteries that further potentiate the cell proliferation.²⁰ Moreover, in the angiogenesis process, pathological cells generate more amount of ATP for their cellular activities. Additionally, as shown in Figure 4b, we also observed an inhibition of vasculogenesis

pathway by 3PO. The tetraspanin family related cell surface glycoprotein such as CD9, which is a crucial protein in the suppression of cancer cell motility, metastasis, differentiation, signal transduction, and cell adhesion²¹ showed down-regulation. It also involves the platelet activation and aggregation.

The down-regulation of FGF2 by 3PO indicates the reduction of cell division, cell differentiation, cell migration, and angiogenesis.²² In addition to this, 3PO reduced the expression of PROCN protein, which is implicated in blood coagulation, venous thromboembolism, myocardial infarction, and cancer.²³ Based on these results, we can hypothesize that 3PO might play a vital role in reducing platelet aggregation, blood coagulation, angiogenesis, and tumor formation. However, we also identified the down-regulation of oxidative phosphorylation, VEGF, cytokine and focal adhesion, and cell movement of endothelial cell signaling pathways by 3PO (Figure S2a–e).

In the second step, under the TNF α induced inflamed ECs in the absence of 3PO, we identified 75 up- and 48 down-regulated proteins. The distribution of differentially regulated proteins is reported in Figure 3b, and their log₂ fold change variations are reported in Table S3. As we already expected, we identified an upregulation of inflammatory and oxidative stress-related proteins such as HLA class I histocompatibility antigen A/B, superoxide dismutase (SOD2), and kynureninase (KYNU) proteins. Additionally, in gene ontology enrichment analysis, we identified more than 80% of upregulated proteins involved in the inflammatory-related biological process including response to interferon-gamma (36.36%), type I interferon signaling pathway (36.36%), and negative regulation of type I interferon production (9.09%). However, we found more than 88% of cellular components belong to the MHC class I protein complex. Moreover, network analysis of up-regulated proteins revealed the up-regulation of pathway represented by enriched KEGG terms: interferon signaling, antigen presentation process, activation of NF-kappa B, and regulation of Hypoxia-inducible factor by oxygen (Table S4). These results significantly explain the successful induction of inflammation in ECs with the use of TNF α .

In the third step, we evaluated the anti-inflammatory property of 3PO. ECs were treated with 20 μ M of 3PO for 1 h, followed by 24 h incubation with 10 ng/mL concentration of TNF α (experimental procedures mentioned in Supporting Information). In this analysis, we identified 40 up- and 140 down-regulated proteins, respectively. The distribution of differentially regulated proteins in the presence of 3PO in TNF α induced inflamed ECs were reported in Figure 3c, and their precise log₂ fold change variations is reported in Table S5. Interestingly, the inflammatory and oxidative stress-related proteins expression completely reversed (Table S6), thanks to the 3PO action that minimizes the effect of subsequent TNF α treatment. For example, HLA-A showed a log₂ fold change of 1.56 in inflamed cells and -0.75 in inflamed cells previously treated with 3PO (Figure 3b,c) and SOD 2 showed a log₂ fold change of 2.69 in inflamed cells and -0.53 in inflamed cells previously treated with 3PO (Figure 3b,c; Tables S3 and S5). In addition, we found intercellular adhesion molecule 1 (ICAM 1), a specific marker of inflammation, that was down-regulated in the presence of 3PO (log₂ fold change of -0.47 , Table S5). Moreover, we also found an inhibition of the TCA cycle, with respiratory electron transport chain pathways exactly as in the 3PO treated cells in the absence of inflammation. The corresponding down-regulated proteins and their interactions in network pathways are reported in Figure 4c. Several studies have reported that 3PO reduces angiogenesis by inhibiting the activity of vascular endothelial growth factor (VEGF).^{4,24} Similarly, in our study 3PO showed an inhibition of VEGFA-VEGFR2 and VEGF signaling pathways

(Figure 4c). Interestingly, in the presence of 3PO in TNF α induced inflammatory ECs, we found an inhibition of these pathways that exactly resembles the anti-inflammatory property of 3PO. The network analyses highlighted other pathways, such as RAB geranyl-geranylation, neutrophil degranulation, and platelet activation, signaling, and aggregation pathways due to the down-regulation of corresponding proteins (Figure 4c).

To further validate the anti-inflammatory effect of 3PO, we performed RT-PCR analysis at the transcriptome level. As shown in Figure 5, the inflammatory marker proteins were

Figure 5. Quantitative RT-PCR was performed with inflammatory marker genes such as IL1B, ICAM-1, and IL8 and normalized with β -actin housekeeping gene. TNF α incubated ECs were compared with respective control. Inflamed ECs with 3PO were compared against untreated inflamed ECs (TNF α). The data represented as mean $\pm$ SEM (* $P < 0.05$, $N = 3$).

upregulated in inflamed ECs. However, after the 3PO treatment, the expressions of ICAM-1, IL1B, and IL8 were reduced. ICAM-1, which was also down-regulated in proteomics approach, is one of the plasma marker proteins for endothelial dysfunction or inflammation associated with myocardial infarction and other cardiovascular diseases.^{25,26} Altogether, the obtained results at proteomic and transcriptome levels clearly indicate the accuracy of network description and the inflammatory preventive character of 3PO by mass spectrometry-based proteomics.

In conclusion, in this study we have demonstrated an unbiased evaluation of cellular and molecular mode of action of 3PO. 3PO has multiple targets in the ECs, targeting mitochondrial inner membrane, and it inhibits the important cellular pathways including the TCA cycle, the mitochondrial respiratory chain, and vasculogenesis that may be useful for understanding an inhibitory effect of 3PO on EC proliferation and migration. Therefore, our present data suggests a potential application of this molecule as a starting point in designing novel molecules to prevent diseases where inflammatory reactions are involved, such as in atherosclerosis, cancer, or neurodegenerative diseases. Therefore, mass spectrometry-based label-free quantitative proteomics is a new powerful approach in medicinal chemistry, which can be used to describe protein network changes in cell or animal models upon drug treatments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00593.

Experimental procedures and additional figures as described in the text (PDF)

Supplementary tables (XLSX)

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S.B.N. performed all the experiments. G.B., S.B.N., and A.D. were involved in the data analysis and interpretation. S.B.N. and A.D. wrote the manuscript. G.A., M.C., and A.D. were involved in the designing of the work and final editing of manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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