Mass Spectrometry-Based Label-Free Quantitative Proteomics to study the effect of drugs at cellular level

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ABSTRACT: Human ECs (EA. hy926 cell lines) 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 TNFα induced inflamed ECs were incubated with and without 3PO at a concentration (20μM) 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, digestion by trypsin; b) peptides separation by nano-LC-MS/MS analysis by 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 have been employed by using mass spectrometry-based label-free quantification approach to explore the underlying mechanisms of 3PO at cellular level.

To get an improved understanding about the mechanism of action of small biologically active molecules, it is mandatory to categorize their cellular protein targets. Undoubtedly, this is a complex problem since 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 to gain 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 or 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 non-treated. From the past decade, many MS based approaches have been successfully developed to address the queries related to protein-protein interaction, post-translational modifications and protein expressions or abundances. In the 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 procedure and a limited number of sample analysis. Therefore, to overcome these complications, in the present study we focused on the development and application of 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 handling, 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 to analyse 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 PFKFB3 enzyme[5], an activator of a key glycolytic enzyme, 6-phosphofructo-1-kinase (PFK-1)[4]. Hence, identification and characterization of ECs proteins whose expression is modulated by the effect of 3PO will expand our understanding towards its mode of action. ECs relied on increased glycolysis activity instead of 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 ECs proliferation, migration, and vascular sprouting *in vitro*[6,7]. 3PO reduces vessel sprouting also in vivo (IP injection) in EC spheroids, zebrafish embryos, and the mouse retina by inhibiting EC proliferation and migration[4,8,9]. 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 intra-plaque angiogenesis and hemorrhages in mice, demonstrating its potential in preventing plaque rupture[10]. Therefore, PFKFBs play a crucial role in maintaining glucose homeostasis and controls 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 key factor

involved in the all the stages for disease progression of atherosclerosis, and novel findings provide an important link among EC dysfunction, angiogenesis, inflammation and EC metabolism in several diseases[11–14]. 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[15]. Notwithstanding the plethora of evidences indicating a key role of 3PO in reducing angiogenesis, the molecular mechanism of action of 3PO at ECs 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[16] 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 the understanding the quantitative modifications of protein profiles in 3PO-treated ECs, in inflamed ECs and in inflamed 3PO-treated ECs.

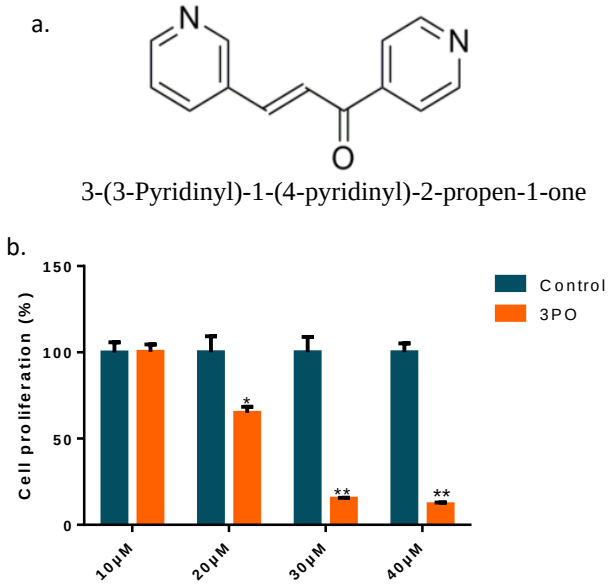

Figure 1. a) structure of 3PO, b) Effect of 3PO on EA.hy926 cells proliferation. ECs were treated with 3PO at various concentrations (10, 20, 30 and 40µM) for 24h and percentage of cell proliferation was assessed by using MTT assay. All data represented as mean ± S.E.M. *P<0.05, **P<0.001 compared with control.

To understand the 3PO molecular mechanism of action in the endothelium, in the presence or in absence of inflammatory agent, EA.hy926 cells, a permanent human endothelial cell line[17] 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 the EC proliferation, EA.hy926 cells were treated with different 3PO concentrations (10-40µM) for 24 hours, and cell proliferation percentages were determined using MMT assay (Experimental procedure in supplementary information). As shown in Figure 1b, we observed a significant cell proliferation inhibitory effect at 20µM concentration of 3PO. Therefore, we selected this concentration for further analysis. To induce the inflammation of endothelium, we treated the cells with 10ng/ml concentration of TNFα for 24 hrs) (experimental procedures mentioned in supplementary information)[18].

Figure 2 shows the workflow of proteomics approach. In the first step, we incubated EA.hy926 cells as described before. After all the treatments, cells were collected and subjected to the following steps: a) protein extraction, reduction, alkylation, in-solution digestion by trypsin; b) peptides separation by nano-LC-MS/MS analysis by using an Orbitrap 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 the multi-scatter 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, indicated the high degree of reproducibility as shown in Figure S1.

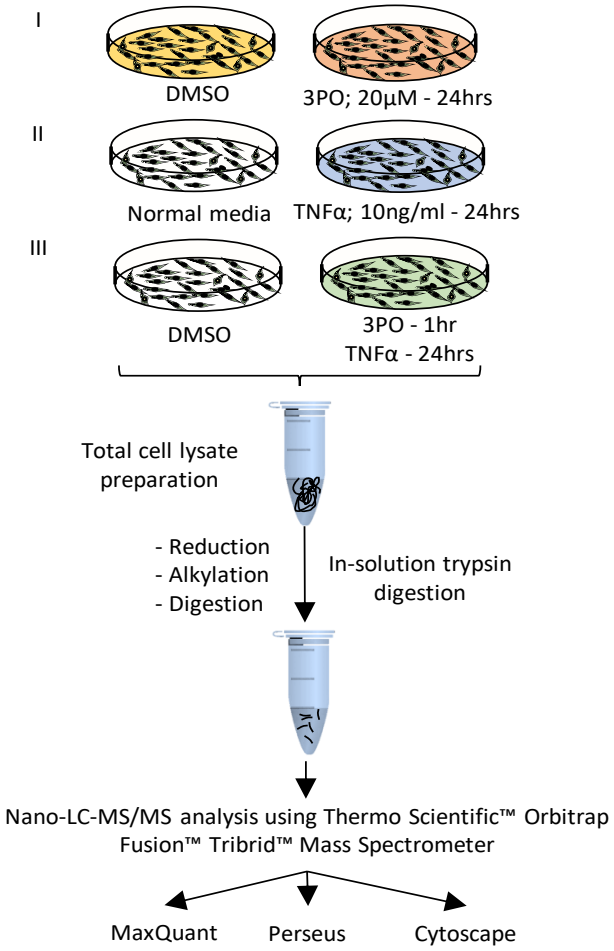

Figure 2. Work-flow employed for the MS-based label-free quantitative proteomic analysis.

2214 proteins and 18869 unique peptides were identified and quantified by Andromeda search engine in MaxQuant using Uniprot_*Homo Sapiens* database; searching parameters were: 10ppm tolerance on peptides, 0.8Da 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 group of samples. The following cri-

teria were applied: S0 value was set to 2 in both sides, permutation-based FDR value set it as 0.05. Proteins with a p-value less than 0.05 were considered statistically significant. As a result, we identified 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 was specified 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/TNFα) we used Clue-GO plug-in in the latest version of Cytoscape software (v 3.6.0) and Ingenuity Pathway Analyses (IPA, Quiagen). Both software interrogate 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 as cytochrome complex, mitochondrial respiratory chain complex I, III and IV, oxidoreductase complex, mitochondrial respiratory chain, mitochondrial membrane part and protein complex present in the mitochondrial inner membrane (Table S2). Most of the down-regulated proteins identified in the 3PO treatment originated from mitochondrion inner membrane: CYC1 and ATP5L respectively. CYC1 is a heme-containing component of the cytochrome b-c1 complex that shuttles electrons between the 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 proton gradient generated across the membrane by electron transport complexes of 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 proteins associated with multiplex complexes of 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 citric acid (TCA) cycle, ATP synthesis by 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 resulted NADH enters into the electron transport chain to produce ATP. Thus is essential for cell proliferation and cell metabolic activities. Studies have shown that increased levels of TCA intermediates increase anaerobic pathways and aerobic fatty acid oxidation for ATP production in macrophage-rich atherosclerotic arteries that furtherly potentiate the cell proliferation[19]. 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 shown down-regulation, which is crucial protein in the suppression of cancer cell motility, metastasis, differentiation, signal transduction, and cell adhesion[20]. It also involves in the platelet activation and aggregation.

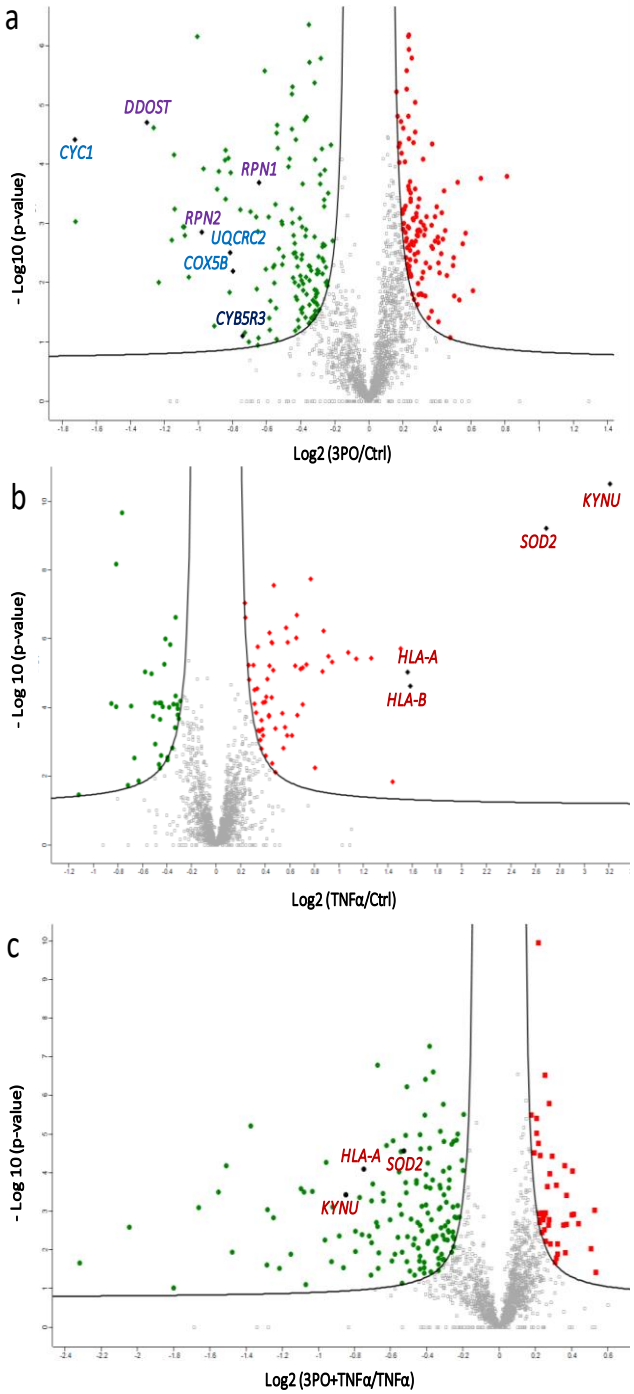

Figure 3. Distribution of differentially regulated proteins in ECs a) after 24h exposure with 3PO, b) after the induction of inflammation with TNFα in the absence of 3PO, c) induction of inflammation with TNFα in the presence of 3PO. Scatter plots of log2 fold change on x-axis against -log p-value on y-axis of significantly quantified proteins. Green colour indicates down-regulation, red colour represents upregulation.

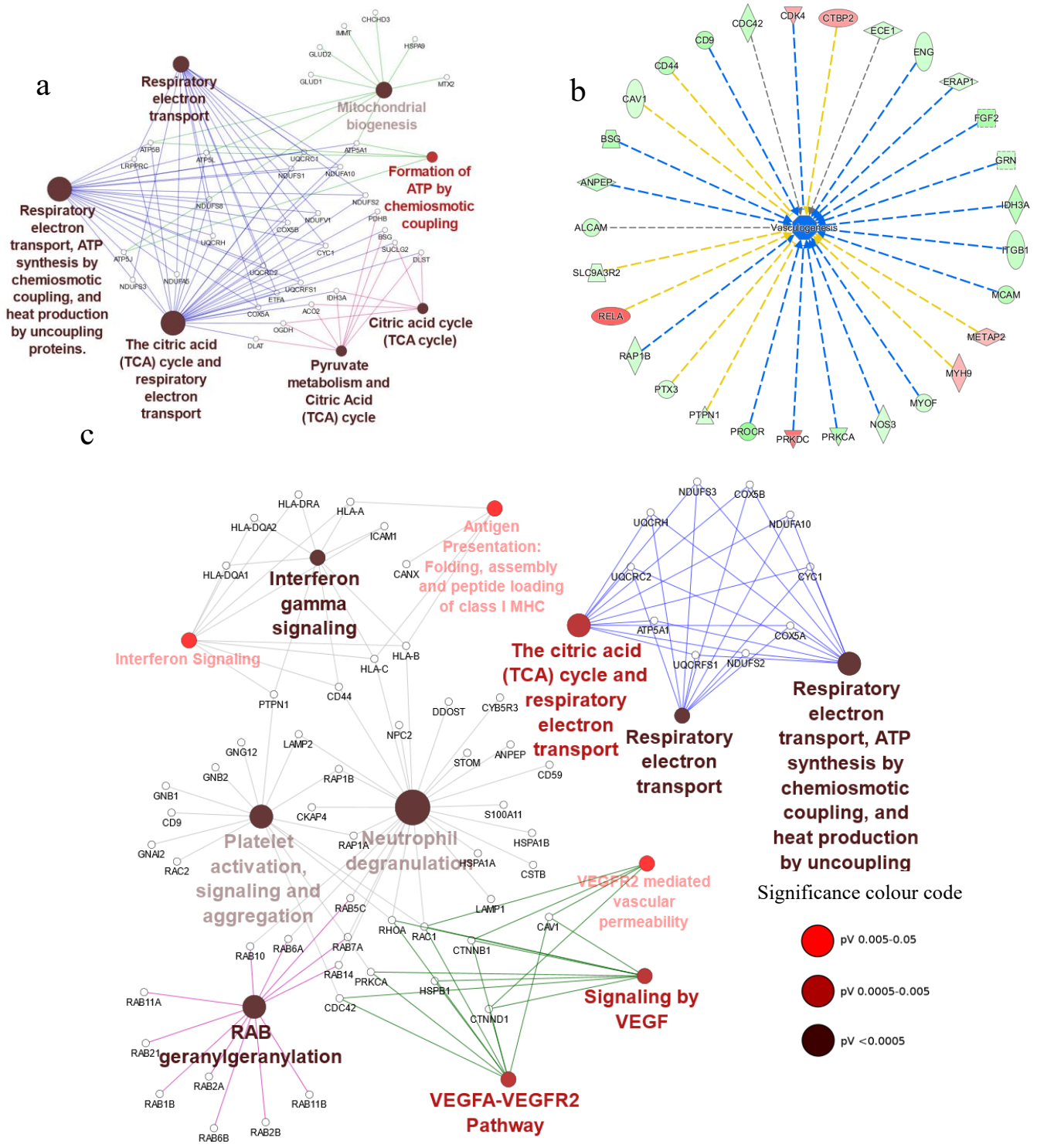

Figure 4. Main pathways and respective genes found in different treatments of ECs. ClueGo plugin for Cytoscape software was used for the Reactome network pathway analysis. a) and b) Key pathways of down-regulated proteins in ECs upon 3PO exposure for 24hrs c) Major pathways associated with down-regulated genes in inflammatory condition stimulated by using TNFα, in the presence of 3PO. Significance of the clustering is shown by colour code and the size of nodes.

The down-regulation of FGF2 by 3PO indicates the reduction of cell division, cell differentiation, cell migration, and angiogenesis[21]. In addition to this, 3PO reduced the expression of PROCR protein, which implicates in the blood coagulation, venous thromboembolism, myocardial infarction, and cancer[22]. Based on these results, we can hypothesize that 3PO might play a vital role in reducing the platelet aggregation, blood coagulation, angiogenesis, and tumour formation.

In the second step, under the TNFα induced inflamed ECs in the absence of 3PO, we identified 75 up and 48 down-regulated proteins. The distribution of differentially regulated proteins are 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 also 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%). On the other hand, we found more than 88% of cellular components belong to MHC class I protein complex. Moreover, network analysis of upregulated proteins revealed the upregulation of pathway represented by enriched Kegg terms: interferon signaling, antigen presentation process, activation of NF-kappa B and regulation of Hipoxia-inducible factor by Oxygen (Suppl table???) These results significantly explain the successful induction of inflammation in ECs with the use of TNFα.

In the third step, we evaluated the 3PO anti-inflammatory property. ECs were treated with 20μM of 3PO for 1hr, followed by 24hrs incubation with 10ng/ml concentration of TNFα (experimental procedures mentioned in supplementary information). In this analysis, we identified 40 and 140 up and down-regulated proteins, respectively. The distribution of differentially regulated proteins in the presence of 3PO in TNFα induced inflamed ECs were reported in Figure 3c, and their precise log2 fold change variations are reported in Table S4. Interestingly, the inflammatory and oxidative stress-related proteins expression completely reversed (Table S5), thanks to the 3PO action that minimize the effect of subsequent TNF-alpha treatment. For example HLA-A showed a log2 fold change of 1.56 in inflamed cells and -0.75 in inflamed cells previous treated with 3PO (Fig. 3 b and c) and SOD 2 showed a log2 fold change of 2.69 in inflamed cells and -0.53 in inflamed cells previous treated with 3PO (Fig. 3 b and c, Table S????). 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 S4). Moreover we also found an inhibition of TCA cycle, 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 were reported in Figure 4c. Several studies have been reported that 3PO reduces angiogenesis by inhibiting the activity of vascular endothelial growth factor (VEGF)[4,23]. Similarly, in our study 3POshowed an inhibition of VEGFA-VEGFR2 and VEGF signaling pathways (Figure 4c). Interestingly, in the presence of 3PO in TNFα 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 geranylgeranylation, 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 up-regulated in inflamed ECs. However, after the 3PO treatment, the expression of ICAM-1, IL1B, and IL8 were reduced. ICAM-1, resulted 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[24,25] Altogether, the obtained results at proteomic and transcriptome levels clearly indicate the accuracy of network description and inflammatory preventive character of 3PO by mass spectrometry-based proteomics.

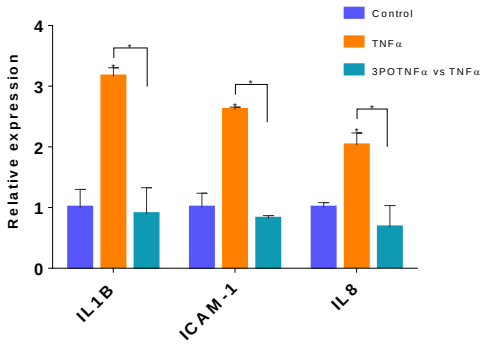

Figure 5: Quantitative RT-PCR was performed with inflammatory marker genes such as IL1B, ICAM-1 and IL8 and normalized with β-actin housekeeping gene. TNFα incubated ECs were compared with respective control. Inflamed ECs with 3PO were compared against untreated inflamed ECs (TNFα). The data represented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

In conclusion in this study, we 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 inhibits the important cellular pathways including TCA cycle, mitochondrial respiratory chain, and vasculogenesis that may be useful for understanding an inhibitory effect of 3PO on ECs proliferation and migration. Therefore, our present data suggesting a potential application of this molecule as a starting point in designing novel molecules in preventing the diseases where inflammatory reactions are involved as in atherosclerosis or cancer or neurodegenerative diseases. Therefore, mass spectrometry-based label-free quantitative proteomics is a new powerful approach in medicinal chemistry to describe protein network changing in cell or animal model upon drug treatments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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

Supplementary Tables (XLSX)

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Author Contributions

S.B.N performed all the experiments. A.D involved in the data analysis and interpretation. S.B.N and A.D written the manuscript. G.A and M.C 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

The authors declare no conflict of interest.

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