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Immunomodulatory Effects of Pentoxifylline: Profiling Data Based on RAW 264.7 Cellular Signaling

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Abstract: Pentoxifylline (PTX) is a methylxanthine derivative that has been developed as an immunomodulatory agent and an improvement of microcirculation. Osteoradionecrosis (ORN) is a serious complication of radiation therapy due to hypovascularity. Coronavirus disease 2019 (COVID-19) has spread globally. Symptoms for this disease include self-limiting respiratory tract illness to severe pneumonia and acute respiratory distress. In this study, the effects of PTX on RAW 264.7 cells were investigated to reveal the possibility of PTX as a therapeutic agent for ORN and COVID-19. To reveal PTX effects at the cellular level, protein expression profiles were analyzed in the PTX-treated RAW 264.7 cells by using immunoprecipitation high-performance liquid chromatography (IP-HPLC). PTX-treated RAW 264.7 cells showed increases in immunity- and osteogenesis-related proteins and concurrent decreases in proliferation-, matrix inflammation-, and cellular apoptosis-related proteins expressions. The IP-HPLC results indicate that PTX plays immunomodulatory roles in RAW 264.7 cells by regulating anti-inflammation-, proliferation-, immunity-, apoptosis-, and osteogenesis-related proteins. These results suggest that PTX may be used as supplement medications for ORN as well as for COVID-19.

Keywords: Pentoxifylline (PTX); immunoprecipitation high-performance liquid chromatography (IP-HPLC); RAW 264.7 cell; immunomodulation; osteoradionecrosis; COVID-19

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1. Introduction

Pentoxifylline (PTX) is a methylxanthine derivative (1-(5-oxohexyl)-3,7-dimethylxanthine) that has been used for the past several decades to improve the blood rheological properties and treat symptoms associated with impaired microcirculation [1]. Other methylated xanthine compounds, which include caffeine, theophylline, aminophylline, and theobromine, are plant components and have similar major pharmacologic actions [2]. The major enzymatic action of cyclic nucleotide phosphodiesterases (PDEs) is the degradation of cyclic adenosine monophosphate (cAMP). PTX works as a nonselective inhibitor of PDEs, and therefore upregulates the effects of cAMP and adenosine-5′-triphosphate and increases erythrocyte distensibility. PTX reduces leukocyte adhesion to endothelial cells, enhances prostacyclin synthesis, and diminishes platelets aggregation. The accumulation of the above effects leads to capillary dilatation, a reduction in blood viscosity, and an improvement in peripheral microvasculature [3].

In recent studies, attention has been given to the possibility of treating PTX as an immunomodulatory agent. PTX has shown the ability to down-regulate pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1). PTX inhibits TNF-α synthesis by blocking transcriptional activity [4,5]. A PTX-treated mice group showed significant TNF-α reduction in a radiation-induced lung injury model [6]. Zein et al. [7] performed a placebo-controlled randomized clinical trial in patients with non-alcoholic steatohepatitis. In comparison with the placebo-using group, PTX was reported to

significantly reduce steatosis, lobular inflammation, and liver fibrosis. In a study of neonatal sepsis, PTX inhibited the production of inflammatory cytokines by microorganisms, particularly when used in combination with antimicrobial agents [8]. In a randomized controlled trial involving 120 newborns with late-onset sepsis and mean gestational age of 30 weeks, PTX administration decreased TNF-α and C-reactive protein (CRP) levels, shortened the respiratory period and short-term hospitalization, and decreased incidence of disseminated intravascular coagulopathy [9]. However, there was no difference in mortality and short-term morbidity between PTX treated and untreated newborn sepsis [9,10].

PTX has effects on inhibiting dermal fibroblast proliferation and extracellular matrix synthesis [11,12] and increasing the activity of tissue collagenase [13]. Previous studies have shown the ability of PTX to reduce the fibrosis progress induced by external stress such as irradiation. PTX inhibits intracellular signaling in response to transforming growth factor-β (TGF-β), and connective tissue growth factor (CTGF). Lin et al. [14] reported that PTX suppresses CTGF expression as well as the promoter effects of CTGF on kidney cells. However, in another study, no significant changes were found in TGF-β expression at either the mRNA or protein level in the irradiated rat heart model [15]. In postoperative peritoneal adhesion studies, PTX may reduce intraperitoneal adhesion formation by increasing peritoneal fibrinolysis activity and inhibiting angiogenesis and collagen synthesis [16]. Western blot analysis of radiation-induced pulmonary fibrosis models showed that PTX treatment suppressed the expression of plasminogen activator inhibitor-1 and fibronectin in irradiated lung tissues as well as in epithelial cells [17].

Osteoradionecrosis (ORN) is one of the significant side effects of radiotherapy; the pathogenesis of this disease remains unclear [18]. Delanian and Lefaix [19] argued that ORN progression is related to the activation and deregulation of fibroblast activity, and atrophic tissue is formed at the irradiation site. It has been reported that PTX is effective in bone regeneration of ORN when used together with tocopherol [20]. Delanian et al. conducted a study involving 22 breast cancer patients with radiation-induced fibrosis; significant surface regression was observed for the combination of PTX and vitamin E [21]. In a subsequent study, they reported that long-term treatment with PTX and tocopherol is effective and curative for refractory osteoradionecrosis [22]. Taken together, PTX has effects on maintaining mitochondrial viability, reducing the level of the TNF-α, IL-6, interferon-γ, and IL-17 and upregulating the production of the anti-inflammatory cytokine IL-10, preserving microvasculature, preventing endothelial damage, and improving coagulation.

The world is facing a viral pandemic of coronavirus disease (COVID-19). The clinical symptoms of COVID-19 vary from asymptomatic or flu-like symptoms to serious conditions, including respiratory failure, acute respiratory distress syndrome, sepsis, or multiple organ dysfunction syndrome [23]. Methylxanthines have already shown improvement of the symptoms of adult respiratory distress syndrome, and PTX is a well-known anti-inflammatory and anti-oxidative molecule that has already been shown to suppress TNF-α as well as other inflammatory cytokines in pulmonary diseases, and this may be beneficial for better clinical outcomes in COVID-19 patients [24]. In the context of the COVID-19 pandemic, the diverse effect of PTX suggests that the drug can be used as an alternative treatment for patients with COVID-19 [25]. Even though there is no report about the antiviral activity against SARS-CoV-2, PTX is a potential treatment proposal for the SARS-CoV-2-induced complications including acute respiratory distress syndrome (ARDS) and dysregulated thrombosis [5,26]. In the treatment of ORN and COVID-19, control of the initial inflammatory response may affect disease severity and progression. In this regard, research on the cellular effects of PTX, one of the therapeutic agents for ORN, might be helpful in the treatment of COVID-19.

Immunoprecipitation high-performance liquid chromatography (IP-HPLC), a protein level detecting method, has been utilized in the determination of protein concentrations compared with reference controls. Protein samples and antibody-bound protein A/G agarose beads are mixed and incubated in a rotating stirrer. Then, the agarose beads are washed several times, and the target proteins are eluted by an IgG elution buffer and analyzed using UV spectroscopy in an automatic HPLC system. The advantage of IP-HPLC is that it is relatively accurate and reproducible among protein detection methods with a standard deviation of less than 5%. Simple experimental procedures can investigate the changes of expressions of multiple proteins in a relatively short time.

As mentioned earlier, there have been reports of using PTX for inflammation or infectious diseases, for example, in the treatment of osteoradionecrosis of the jaw [27]. Macrophages belong to the innate immune system representing the first line of defense against microorganisms, initiating a local inflammatory reaction. They are essential effector cells in inflammatory diseases and infectious diseases [28,29]. Therefore, in this study, RAW 264.7 cells derived from murine macrophages were used. To investigate the effects of PTX on RAW 264.7 cells, different protein (n = 132) expression profiles were analyzed using IP-HPLC.

2. Materials and Methods

2.1. Cell Culture

Unstimulated RAW 264.7 cells derived from murine macrophages (ATCC, Manassas, VA, USA) were used. Cell culture was performed using Dulbecco's Modified Eagle's Medium (WelGene Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea) with 10% fetal bovine serum (WelGene Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea), 100 units/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL amphotericin B (WelGene Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea) in a humidified incubator at 5% CO_2 and 37 °C. PTX (10 μg/mL) was included in the media in the test group. Cells were cultured for 12, 24, and 48 h with PTX, and control cells were treated with 1 mL of normal saline.

2.2. Protein Extraction and IP-HPLC

After treating RAW 264.7 cells with PTX at 10 μg/mL for 12, 24, and 48 h, the RAW 264.7 cells were harvested with protein lysis buffer (0.3% SDS, 50 mM Tris-HCl pH 8.0, 0.3% β-mercaptoethanol, 1 mL PMSF, 1 mL EDTA) containing a protein inhibitor cocktail (Sigma, Chicago, IL, USA). Protein extracts were kept in a −70 °C deep freezer until use to prevent further protein degradation [30].

For IP-HPLC, 100 μg of each protein extract was immunoprecipitated on a protein A/G agarose column (Amicogen Co., Jinju-si, Gyeongsangnam-do, Korea). The protein A/G agarose columns were pre-incubated with 1 μg of each antiserum, including 132 antibodies with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, α-tubulin (Table 1).

IP-HPLC is a fast and accurate method (±5% standard deviation) for analyzing protein levels; brief procedures of IP-HPLC could be referenced [28–30]. In the IP-HPLC results, the sample protein peak areas (mAU*s) obtained from HPLC analysis in the negative control were used to eliminate the antibody peak area (mAU*s). Proportions (%) of experimental and control groups were plotted, and each IP-HPLC analysis was carried out two to six times to achieve a mean standard deviation within 5%, and the mean value was used for all IP-HPLC data. The average data were handled with SPSS (Version 25.0, SPSS Inc., Chicago, IL, USA) using the simple proportions and the Chi-square tests. The expression of housekeeping proteins, β-actin, α-tubulin, and GAPDH were used as internal controls. Expression changes of housekeeping proteins were adjusted to <±5% using a proportional baseline algorithm. The final data were graphed according to the properties of the protein groups [29–31]. We summarized these important steps in Figure S1.

Table 1. All 132 antibodies used in this study.

Signaling Proteins	No.	Antibodies
Cellular proliferation	10	Ki-67 *, PCNA *, CDK4 *, PLK4 *, MPM2 *, p14 *, p16 *, p21 *, p27 *, cyclin D2 *
cMyc/MAX/MAD signaling	3	cMyc *, MAX *, MAD *
p53/Rb/E2F signaling	4 (2)	p53, Rb-1 #, E2F-1 *, MDM2 *, CDK4 *, p21 *
Epigenetic modification	6	DMAP1, histone H1 *, KDM4D $, HDAC-10 $, MBD4, DNMT1
Protein translation signaling	3	DOHH *, DHS *, elF5A-1 $
RAS signaling	7 (3)	NRAS $, KRAS $, HRAS, Rab, JNK-1 *, ERK-1 *, MEKK, (pAKT1/2/3 *, mTOR, PKC)
Growth factor signaling	9	TGF-β1 #, IGF-1 *, HER1 *, HER2 *, ERβ *, FGF-1, FGF-2, Met, CTGF
NFkB signaling	9 (3)	NFkB *, IKK *, NFATS *, PGC-1α, GADD45 *, GADD153 *, mTOR @, p38 *, MDR *, (ERK-1 *, pAKT1/2/3 *, TNFα *)
Immunity signaling	7	CD3 *, CD4, CD20 *, CD28 *, CD31 *, CD68 *, cathepsin K *
Inflammatory signaling	20	TNFα @, IL-1 *, IL-6 *, IL-10 *, IL-28 *, COX-2 *, lysozyme *, M-CSF *, MMP-1 *, MMP-2 *, MMP-3 *, MMP-9 *, MMP-10 *, MMP-12, MCP-1, LTA4H, CXCR4, TLR3, hepcidin, CRP-1 *
Cell protection	8 (3)	HSP-70 *, HSP-90 *, AP-1 *, SP-1 *, SP-3 *, p38 *, PKC *, pAKT1/2/3 *, (p38, JNK-1, TERT)
Cellular differentiation	6 (4)	PLC-β2, TGase-2, HXKII *, Jagged-2, Notch-1, GLI-1, (PKC, AP-1, SP-1, SP-3)
Antioxidant-related proteins	5	SOD-1 *, GST *, LC3 *, AMPK *, NOS-1,
p53-mediated cellular apoptosis	6 (1)	p53 *, MDM2 *, BAX *, BAK *, APAF-1 *, caspase 9 *, (PARP)
FAS-mediated cellular apoptosis	6	FASL *, FAS *, FADD *, FLIP *, caspase 8 *, PARP *
Oncogenic proteins	3 (3)	14-3-3 *, TERT *, YAP, (pAKT1/2/3, MBD4, mTOR)
Angiogenesis-related proteins	8 (4)	HIF &, VEGF-A *, VEGF-C *, VCAM, angiogenin *, CMG2 $, vWF $, ET-1 *(CD31, MMP-2, MMP-10, FGF-2)
Osteogenesis-related proteins	9 (2)	RANKL, OPG *, osteonectin, osteopontin, osteocalcin, RUNX2, ALP *, osterix *, BMP-2 * (cathepsin K, HSP-90)
Control cytoplasmic proteins	3	α-tubulin *, β-actin *, GAPDH *
Total	132 (25)	

* Santa Cruz Biotechnology, USA; # DAKO, Denmark; $ Neomarkers, CA, USA; @ ZYMED, CA, USA; & Abcam, Cambridge, UK. Abbreviations: ALP: alkaline phosphatase, AMPK: AMP-activated protein kinase, APAF-1: apoptotic protease-activating factor 1, AP-1: activating protein-1, BAK: BCL2 antagonist/killer, BAX: BCL2 associated X, BMP-2: bone morphogenic protein-2, CASP 3: caspase 3, CASP 8: caspase 8, CASP 9: caspase 9, c-CASP 9: cleaved-caspase 9, CD3: cluster of differentiation 3, CDK4: cyclin dependent kinase 4, CEA: carcinoembryonic antigen, cMyc: V-myc myelocytomatosis viral oncogene homolog, CMG2: capillary morphogenesis protein 2, COX-2: cyclooxygenase-2, CRP-1: C-reactive protein-1, CXCR4: C-X-C chemokine receptor type 4, DMAP1: DNA methytransferase 1 associated protein, DNMT1: DNA (cytosine-5)-methyltransferase1, DOHH: deoxyhypusine hydroxylase, DHS: deoxyhypusine synthase, E2F-1: transcription factor, elF5A-1: eukaryotic translation initiation factor 5A-1, ERβ: estrogen receptor beta, ERK: extracellular signal-regulated protein kinases, ET-1: endothelin-1, FAS: CD95/Apo1, FASL: FAS ligand, FADD: FAS associated via death domain, FGF-1: fibroblast growth factor-1, FLIP: FLICE-like inhibitory protein, vascular endothelial growth factor receptor 3 precursor (FLT4), GADD45: growth arrest and DNA-damage-inducible 45, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GST: glutathione S-transferase, HDAC-10: histone deacetylase-10, HIF: hypoxia inducible factor-1α, Histone H1, HER1: human epidermal growth factor receptor 1, hTERT: human telomerase reverse transcriptase, HSP-70: heat shock protein-70, HSP-90: heat shock protein-90, HXKII: hexokinase II, IKK: ikappaB kinase, IGF-1, IL-1: interleukin-1, JNK-1: Jun N-terminal protein kinase, KDM4D: Lysine-Specific Demethylase 4D, KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, LC3: microtubule-associated protein 1A/1B-light chain 3, LTA4H: Leukotriene A4 hydrolase, MAX: myc-associated factor X, MBD4: methyl-CpG-binding domain protein 4, MCP-1: monocyte chemoattractant protein 1, M-CSF: macrophage colony-stimulating factor, MDM2: mouse double minute 2 homolog, MEKK: MAP kinase kinase kinase, MPM2: mitotic protein monoclonal 2, MDR: Monoclonal Anti-P-Glycoprotein, Met: tyrosine-protein kinase Met, MMP-1: matrix metalloprotease-1, MMP-2: matrix metalloproteinase-2, mTOR: mammalian target of rapamycin, NFATS: nuclear factor of activated T-cells, NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells, NOS-1: NRAS: neuroblastoma RAS Viral Oncogene homolog, OPG: osteoprotegerin, OPN: osteopontin, OSX: osterix, pAKT: v-akt murine thymoma viral oncogene homolog, p-Akt1/2/3 phosphorylated (p-Akt, Thr 308), PARP: poly-ADP ribose polymerase, PCNA: proliferating cell nuclear antigen, PGC-1α: Peroxisome Proliferator-Activated Receptor-γ-Coactivator1α, PKC: protein kinase C, PLC-β2: 1-phosphoatidylinositol-4,5-bisphosphate phosphodiesterase beta-2, Rb-1: retinoblastoma-1, RANKL: receptor activator of nuclear factor kappa-B ligand, RUNX2: Runt-related transcription factor 2, SOD-1: superoxide dismutase-1, SP-1: specificity protein 1, SP-3: specificity protein 3, TGase-2: transglutaminase-2, TGF-β1: transforming growth factor-β1, TLR3: Toll like receptor 3, TNF-α: tumor necrosis factor-α, β-actin, 14-3-3, VEGF vascular endothelial growth factor, vWF: von Willebrand factor. The number of antibodies overlapped: ( ).

3. Results

Expression of proliferation-related proteins. CDK4 expression was reduced to 92.32% of baseline in 24 hour-PTX-treated RAW 264.7 cells and elevated back to 99.91% of baseline in the 48 hour-treated sample. The expression level of Ki-67 was decreased to 92.0% of baseline in the 12-hour-treated sample and increased with time to 105.2%. Significantly lower expression of MPM2 (93.9%), proliferation-inhibiting proteins, p21 (90.2%), p27

(90.9%), and cyclin D2 (88.7%) was expressed than untreated controls. Changes of PCNA, PLK4, p14, and p16 were measured within ±5% in response to PTX, which were analogous with control housekeeping proteins (Figure 1a). PTX inhibits the proliferation of RAW 264.7 cells.

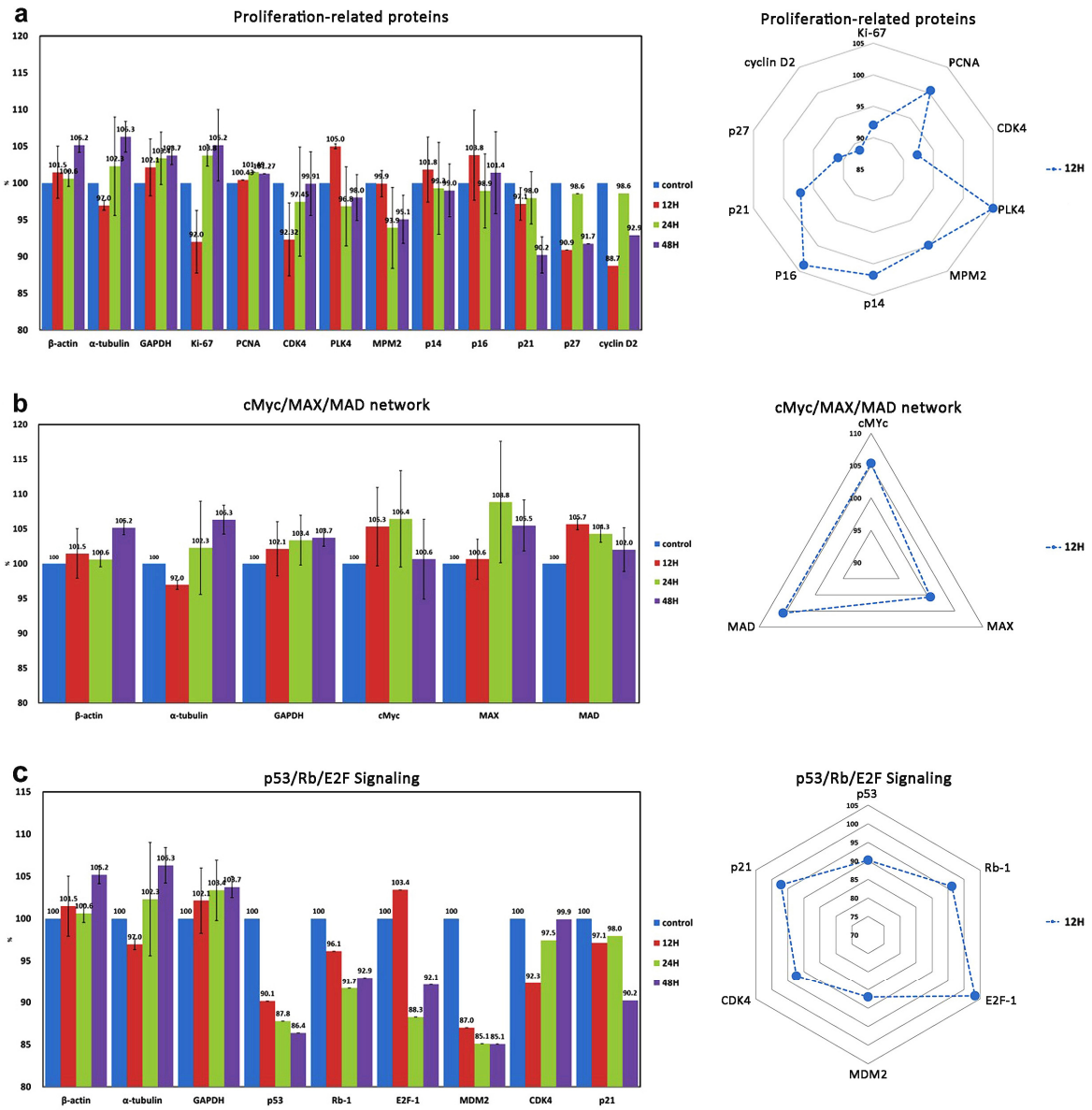

Figure 1. (**a**) Changes of proliferation-related proteins after PTX treatment in RAW 264.7 cells; (**b**) changes to cMyc/MAX/MAD signaling proteins after PTX treatment in RAW 264.7 cells; (**c**) changes to p53/Rb/E2F signaling-related proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes of protein expressions according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX.

Expression of cMyc/MAX/MAD signaling proteins. Slightly increased expression of cMyc (106.4%) and MAX (108.8%) is expressed in samples treated for 24 h. The expression level of MAD (105.7%) also increased slightly after treatment for 12 h. PTX slightly enhanced cMyc/MAX signaling and participated in transcriptional control in RAW 264.7 cells (Figure 1b).

Expression of p53/Rb/E2F signaling proteins. Expression of p53 (86.4%), Rb-1 (91.7%), E2F-1 (88.3%), MDM2 (85.1%), CDK4 (92.3%), and p21 (90.2%) is shown. PTX inhibits p53/Rb/E2F signaling leading to cellular proliferation (Figure 1c).

Expression of epigenetic modification-related proteins. Slightly increased expression of histone H1 (108.6%) and MBD4 (111.0%) but no significant change in the expression of KDM4D (95.1–103.6%) is found. A decrease in the expression of DMAP1 (88.0%), DNMT1 (94.5%), and HDAC-10 (94.9%) is also found. PTX may reduce DNA methylation and activate DNA transcription in RAW 264.7 cells. Therefore, PTX can be associated with epigenetic modifications and the regulation of gene expression. MBD4 may function to mediate the biological consequences of the methylation signal. MBD4 is similar in protein sequence to bacterial DNA repair enzymes and has been shown to perform some functions in DNA repair (Figure 2a).

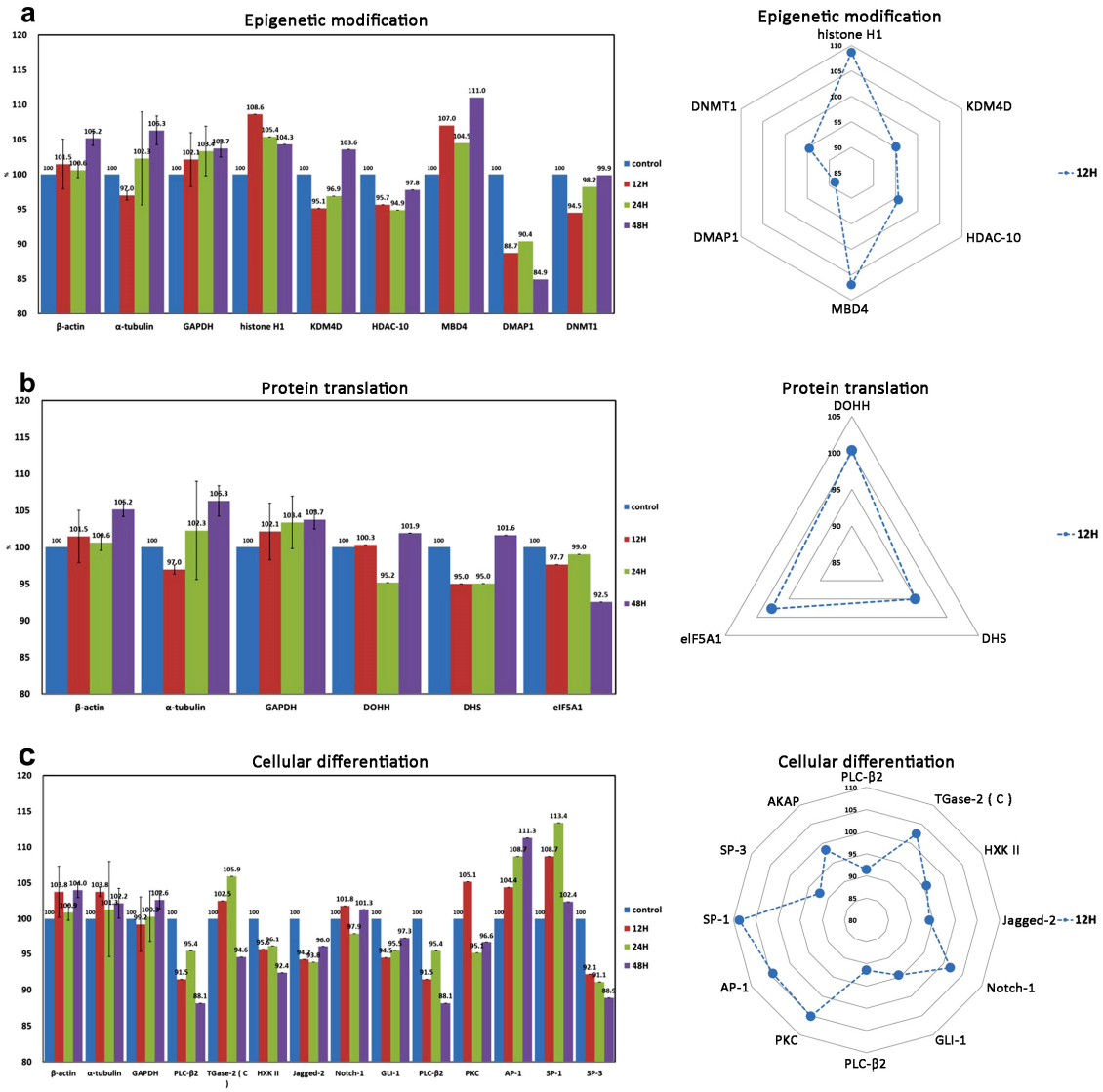

Figure 2. (**a**) Changes to epigenetic modification-related proteins after PTX treatment in RAW 264.7 cells; (**b**) changes to protein translation-related proteins after PTX treatment in RAW 264.7 cells; (**c**) changes in cellular differentiation-related proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes in protein expression according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX. (JPG 916 kb).

Expression of translation-related proteins. Slightly reduced expression of eIF5A1 (92.5%) is found. The expression levels of DHS (95%) and DOHH (95.2%) are similar to

control proteins. PTX may reduce protein translation for cellular proliferation and other functions (Figure 2b).

Expression of cellular differentiation-related proteins. Slightly reduced expression levels of the differentiation-related proteins including PLC-β2 (88.1%), TGase-2 (94.6%), HXK II (92.4%), Jagged-2 (93.8%), and GLI-1 (94.5%) is found. The expression of Notch-1 did not show significant changes, remaining similar to those of the housekeeping proteins. PTX treatment down-regulates cellular differentiation in RAW264.7 cells (Figure 2c).

Expression of RAS signaling proteins. PTX slightly reduced the expressions levels of V-Ki-ras2 KRAS (92.0%), NRAS (94.2%), and GTPase HRas (HRAS, 94.3%). MEKK (107.6%) expression was increased in the 12-hour-treated group but decreased with time similar to that of housekeeping proteins. The expression of ERK-1 (108.9%) increased in the 24-hour-treated group. The expression level of mTOR (94.0%) was decreased in the 12-hour-treated sample, while that of pAKT1/2/3 (89.1%) markedly decreased with time. Changes in JNK-1, Rab, and PKC expression were similar to housekeeping proteins. RAS signaling appeared to be reduced by PTX (Figure 3a).

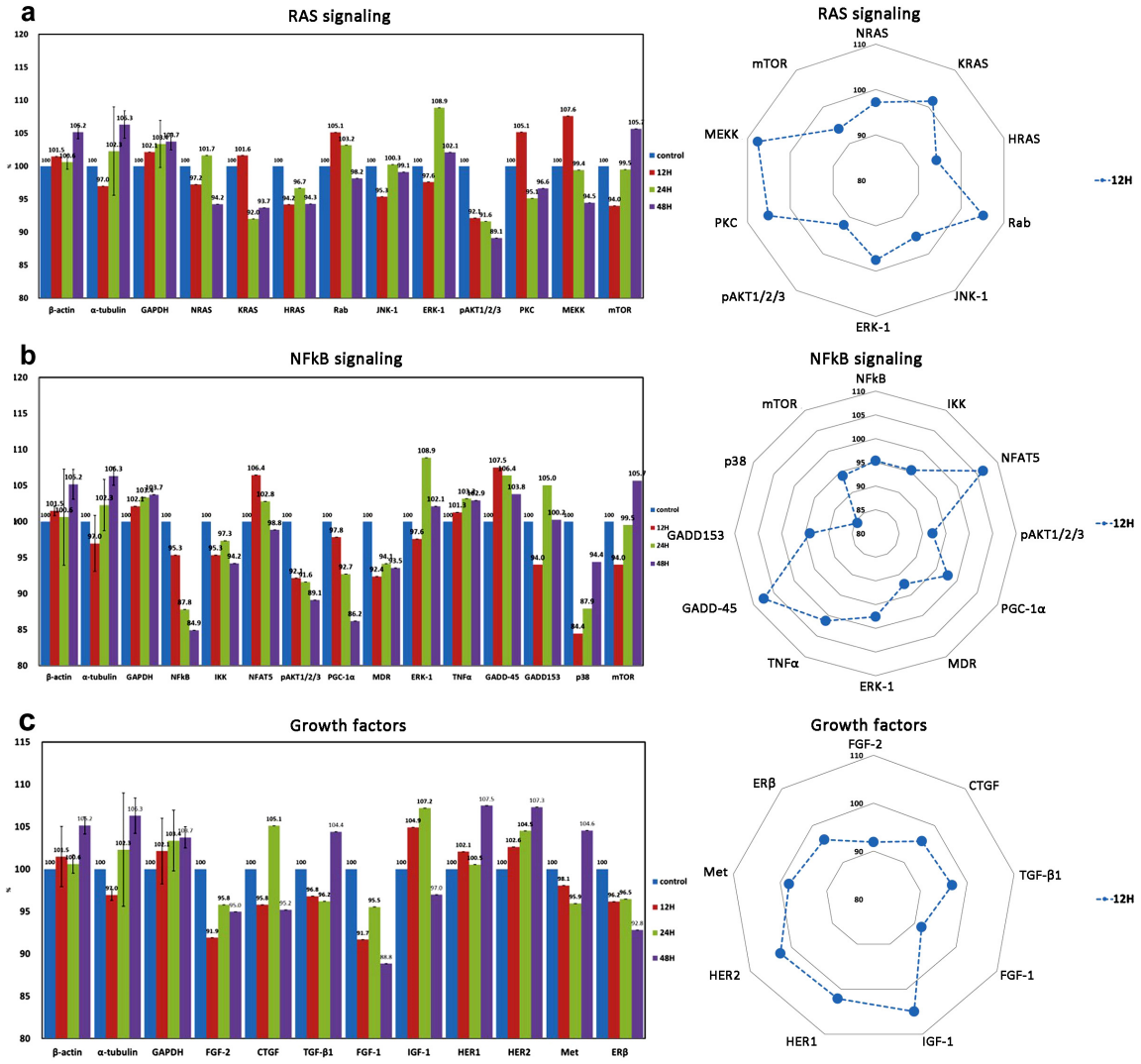

Figure 3. (**a**). Changes to RAS signaling-related proteins after PTX treatment in RAW 264.7 cells; (**b**) changes to NFκB signaling-related proteins after PTX treatment in RAW 264.7 cells; (**c**) changes to growth factor-related proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes of protein expressions according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX. (JPG 1.05 MB).

Expression of NFkB signaling proteins. A decrease in the expression of NFkB signaling proteins is shown. After PTX treatment, TNFα, GADD 153 decreased to a minimum of ±5%, but the expression levels of NFkB (84.9%), IKK (94.2%), pAKT (89.1%), PGC-1α (86.2%), and p38 (84.4%) were significantly reduced. RAW 264.7 cells treated with PTX showed a slight decrease in the expression of MDR (92.4%) and a slight rise in the expression of ERK1 (108.9%). The expression levels of GADD45 were slightly increased (105.0%) in the 12-hour-treated sample; however, levels decreased with time. NFκB is a key regulator of immune responses and inflammation. The NFκB signaling system is responsive to a number of stimuli. In our IP-HPLC result, protein expression related to NFκB signaling did not increase significantly.

These results indicated that NFkB signaling was affected by PTX treatment and that the cells were in a stress-free state (Figure 3b).

Expression of growth factor-related proteins. Slight increases in the expression of IGF1 (107.2%), HER1 (107.5%), and HER2 (107.3%) were observed. Conversely, the expression levels of FGF1 (88.8%), FGF2 (91.9%), and ERβ (92.8%) decreased in RAW 264.7 cells treated with PTX. The expression of TGF-β1 (96.2–104.4%), CTGF (95.2–105.1%), and Met (95.9–104.6%) changed only minimally to less than ±5% (Figure 3c).

Expression of inflammation-related proteins. A decrease in IL-6 (92.4%) and IL-1 (88.9%). IL-28 (97.7-105.9%) showed no significant change. The expression level of IL-10 significantly increased to 123.5% by PTX treatment. MMPs expression levels after PTX treatment varied; MMP-1, MMP-2, MMP-3, and MMP-9 were increased to 108.4%, 109.1%, 117.0%, and 117%, respectively, while MMP-10 revealed a minimal change of less than ±5%, and MMP-12 decreased in the 12-hour-treated sample to 93.9%. The expression levels of LTA4H, CXCR4, M-CSF, MCP-1, hepcidin, and cathepsin K were dramatically reduced by PTX to 91.2%, 82.3%, 87.7%, 93.8%, 94.2%, and 92.7%, respectively, but TNFα and TLR3 did not show significant changes. COX-2 (91.0%) expression was reduced by PTX treatment; however, that of CRP-1 was slightly increased in the 12-hour-treated sample (109.9%), which decreased to 101.4% with time. These results suggest that PTX down-regulates LTA4H, CXCR4, ILs, and MMPs and induces anti-inflammatory signaling in RAW 264.7 cells without affecting TNFα (Figure 4a,b).

Expression of immunity-related proteins. Significant increases in the expression of cluster of differentiation 4 (CD4, 112.1%) and CD20 (106.4%) were observed. The expression levels of CD31 and CD68 showed minimal changes of less than ±5%, which was analogous to that of the housekeeping proteins. The expression levels of CD3 and CD28 after PTX treatment were 87.4% and 91.6%, respectively. PTX is a phosphodiesterase inhibitor, the exact mechanism of various immunomodulatory functions has not been elucidated. Importantly, the expression levels of CD3 and CD28 were reduced by PTX, however, the exact mechanism could not be found. However, it is presumed that inhibition of these markers may reduce the T-cell mediated inflammatory response, thereby regulating the immune response. PTX can affect immunity-related proteins and, in turn, inhibit T-cell-associated immunity (Figure 4b).

Expression of p53-mediated apoptosis-related proteins. Significantly reduced p53 expression (86.4%) with a concurrent decrease in MDM2 expression (85.1%) and PARP (92.2%)—but it increased p53-mediated apoptosis-related proteins, such as BAX (111.1%) and APAF-1 (106.3%)—was found. The expression levels of BAK (96.6%) and caspase 9 (97.5%) proteins showed slight decreases with time but were similar to housekeeping proteins within 5%. These results suggest that PTX significantly inhibited p53 but did not show any significant changes in down-regulated proteins (Figure 5a).

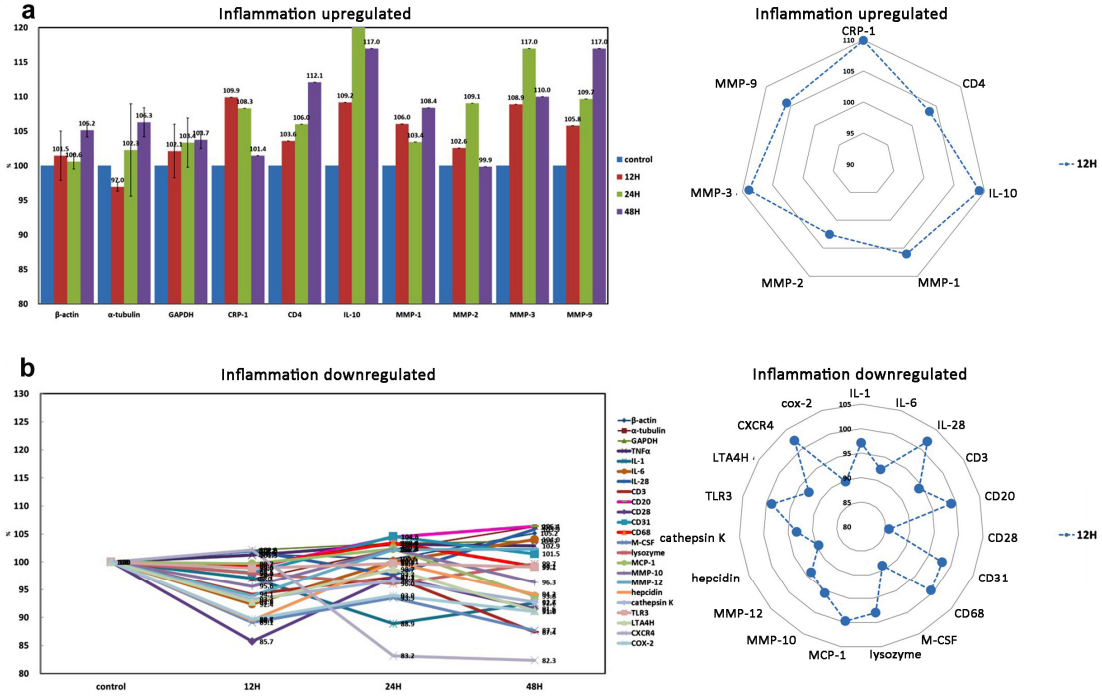

Figure 4. (**a**) Changes to immunity and inflammation-upregulated proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes to protein expression according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX. (**b**) Changes to immunity and inflammation down-regulated proteins after PTX treatment in RAW 264.7 cells. Left-sided line graft shows changes to protein expressions after PTX treatment over time. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX.

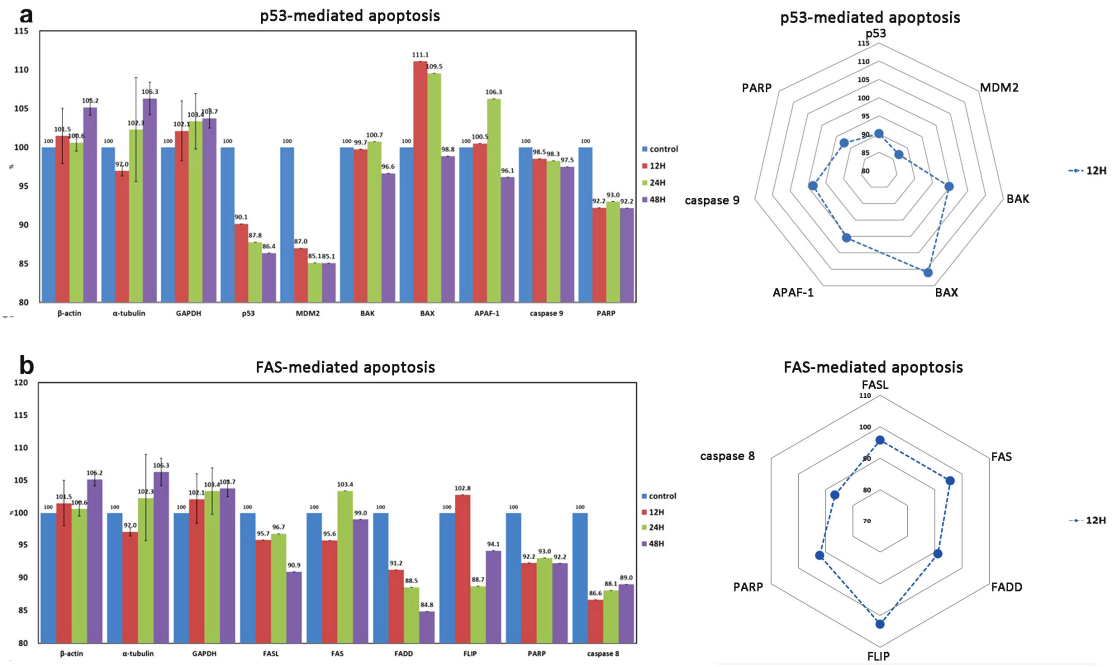

Figure 5. (**a**) Changes to p53 mediated apoptosis-related proteins after PTX treatment in RAW 264.7 cells; (**b**) changes to FAS-mediated apoptosis-related proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes to protein expression according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX.

Expression of F-mediated apoptosis-related proteins. A decreased expression of FASL (90.9%), FADD (84.8%), and FLIP (88.7%) is shown. Caspase 8 expression levels were consistently down-regulated by PTX to 89.0% of baseline. The expression of FAS (95.6%) showed only a slight change but was within 5% of the control housekeeping proteins. The expression of PARP slightly decreased to 92.2% by PTX. These results suggested that PTX tends to inhibit FAS-mediated cell death (Figure 5b).

Expression of cell protection-related proteins. A significant induction of some types of cytoprotective proteins: HSP-70 (110.2%), AP-1 (111.3%), SP-1 (113.4%), and PKC (105.1%) was observed. The expression levels of HSP-90 (93.2%) slightly decreased in 24-hour-treated cells; however, they were increased in the samples treated for 48 h. In the PTX treatment group, p38 (84.4% in the 12-h-treated sample), pAKT (89.1%), TERT (93.7%), and SP-3 (88.9%) were significantly decreased. JNK-1 showed minimal changes of less than ±5% after PTX treatment (Figure 6a). These findings suggest that the cytoprotective effect increased up to 12 h but was down-regulated as time progressed.

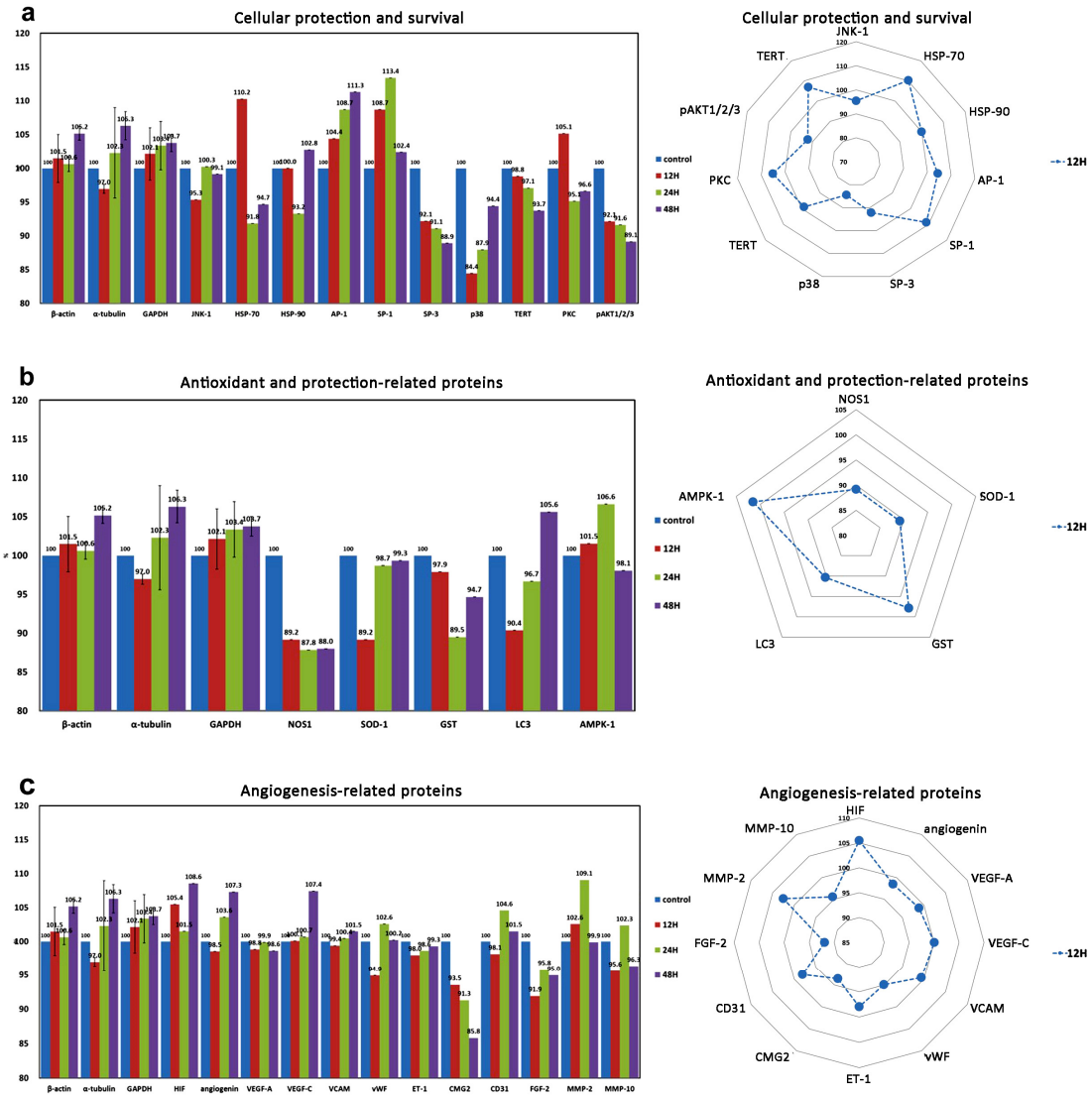

Figure 6. (**a**) Changes to cellular protection and survival-related proteins after PTX treatment in RAW 264.7 cells; (**b**) changes to antioxidant-related proteins after PTX treatment in RAW 264.7 cells; (**c**) changes to angiogenesis-related proteins in after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes to protein expressions according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX.

Expression of antioxidant-related proteins. A slight increase in AMPK (106.6%) is found in samples treated for 24 h. The expression of LC3 (90.4%) and SOD-1 (89.2%) were decreased in the samples treated for 12 h and increased with time to 105.6% and 99.3% of baseline, respectively. GST (89.5%) was down-regulated by PTX treatment. However, the expression of NOS1 decreased to 88.0% in the sample treated for 48 h. These results suggest that PTX inhibits NO synthesis and the action of other antioxidant enzymes (Figure 6b).

Expression of angiogenesis-related proteins. Increased expression of angiogenesis-related proteins such as HIF (108.6%), angiogenin (107.3%), and VEGF-C (107.4%) was found in the 48-hour-treated sample. MMP-2 (109.2%) was up-regulated in the 24-hour-treated sample; however, it was down-regulated in the 48-hour-treated sample. The expression levels of VEGF-A, vWF, ET-1, CD31, MMP-10, and VCAM showed a minimal change within 5%. CMG2 (85.8%) decreased significantly over time. These findings reveal that PTX had weak angiogenic properties (Figure 6c).

Expression of oncogenic proteins. A slight reduction in the expression level of TERT (93.7%) and 14-3-3 (94.3%) was observed. The expression of YAP (97.4%) and mTOR (94.0–105.7%) did not show significant changes. PTX treatment did not upregulate tumorigenic protein expression in RAW264.7 cells (Figure 7).

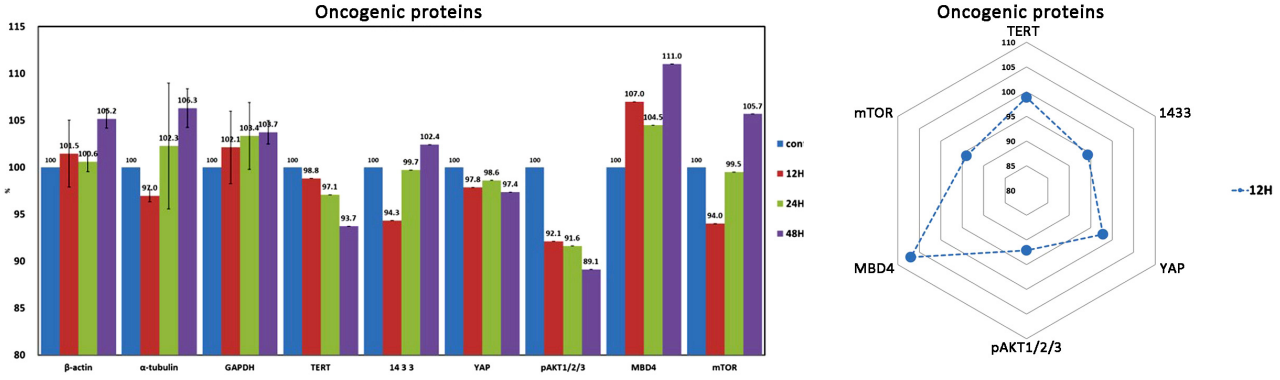

Figure 7. Changes to oncogenic proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes to protein expressions according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX. (JPG 342 kb).

Expression of osteogenesis-related proteins. A slightly increased expression of OPG (108.4%), osteopontin (115.8%), and osterix (110.5%) is shown compared with untreated controls. The expression level of RANKL was obviously increased to 117.1% after PTX treatment. The expression level of cathepsin K was down-regulated to 92.7%, and the expression of HSP-90 was reduced to 93.2% in the 24-hour-treated sample. The expression levels of BMP-2, osteonectin, and ALP demonstrated a small change similar to the control proteins. PTX slightly increased osteogenesis-related proteins in RAW 264.7 cells compared with the untreated control. However, RAW 264.7 cells are a macrophage cell line, which have the potential to become osteoclasts; after PTX treatment, the cells showed slight osteogenic properties compared with the untreated control. Thus, RAW264.7 cells treated with PTX may have an effect on bone formation (Figure 8).

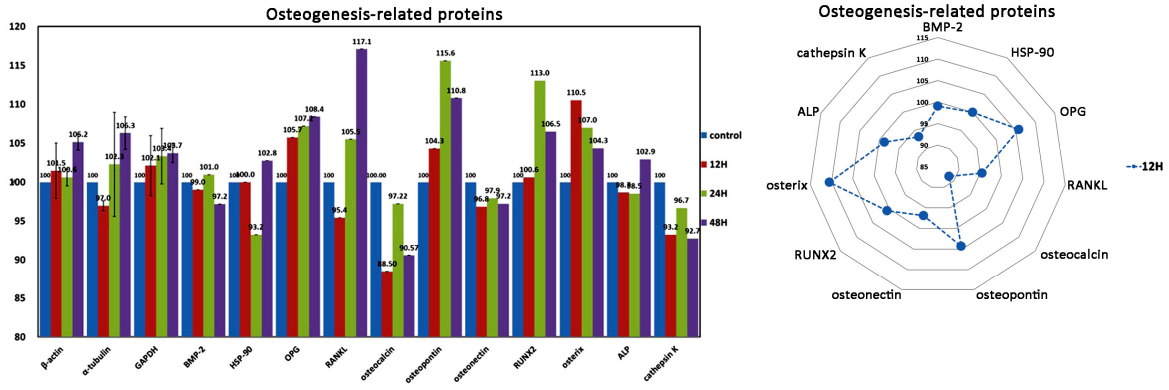

Figure 8. Changes to osteogenesis-related proteins after PTX treatment in RAW 264.7 cells. Left-sided graph shows changes to protein expressions according to the application time of PTX, blue bar; control group, red bar; 12 h treatment of PTX, green bar; 24 h treatment of PTX, purple bar; 48 h treatment of PTX. Right-sided polygonal graph shows protein expression profiles after 12 h treatment of PTX.

Effects of PTX on the global protein expressions. The results of this in vitro study are summarized in Figure 9. PTX was shown to inhibit cellular proliferation-, apoptosis-, and inflammation-related proteins in RAW 264.7 cells. Conversely, angiogenesis and antioxidant activities were not significantly affected. Although the origin of RAW 264.7 cells is a murine macrophage, PTX was expected to have an osteogenesis promoting effect (Table 2).

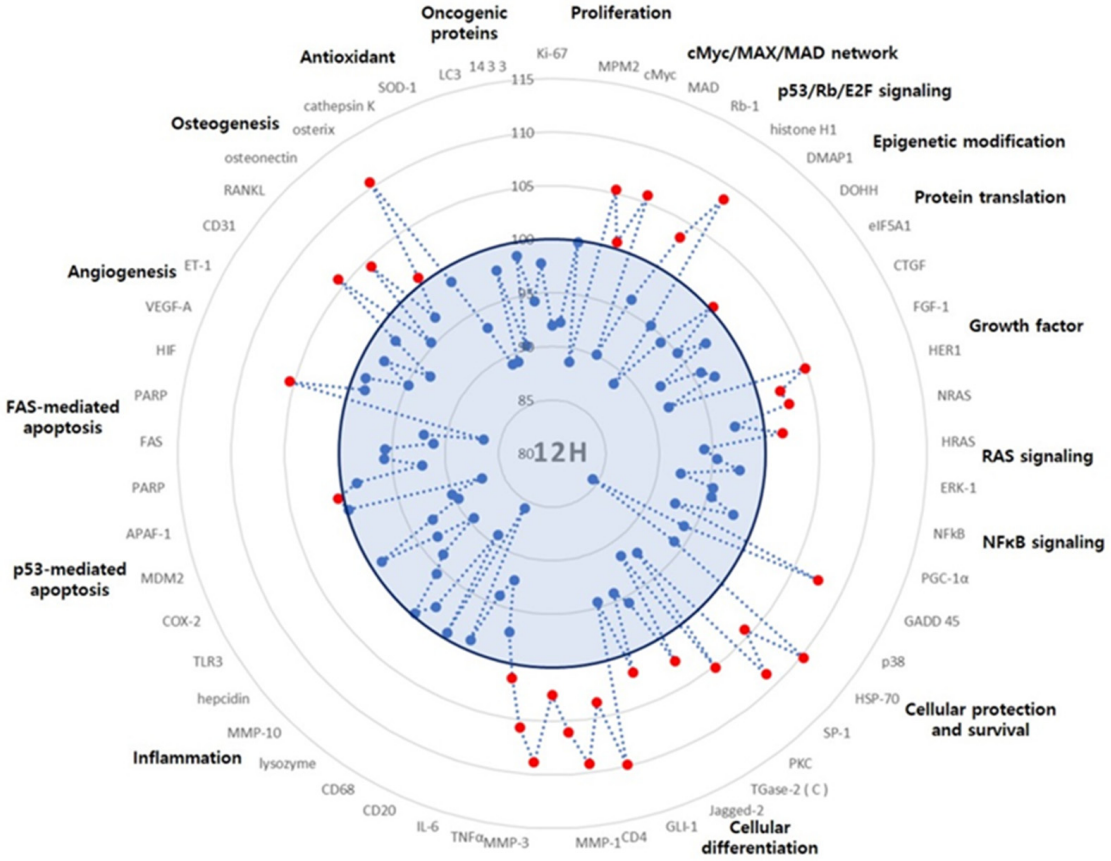

Figure 9. Global protein expression diagrams showing the effects of PTX on RAW 264.7 cells after 12 h treatment. Up-regulated (red dots) and down-regulated (blue dots) proteins are displayed in this diagram. PTX down-regulated the proliferation-, apoptosis-, inflammation-, oncogenic-related proteins and up-regulated the osteogenesis-related proteins.

Table 2. Representative cellular signaling affected by Pentoxifylline in RAW 264.7 cells.

Signaling	Up-Regulated	Unchanged	Down-Regulated
Proliferation ↓		PCNA	Ki-67, CDK4, PLK4, MPM2, cyclin D2, Rb-1, E2F-1, MDM2
Apoptosis ↓	BAX, APAF-1	BAK, caspase 9	p53, PARP, FASL, FADD, caspase 8
Antioxidant and cellular protection	AP-1, SP-1, AMPK-1		NOS-1, SOD-1, GST
Angiogenesis	HIF, angiogenin, VEGF-C	VEGF-A, vWF, ET-1, CD31	CMG2
Osteogenesis ↑	OPG, RANKL, osteopontin, RUNX2, osterix	osteonectin, ALP	Cathepsin K
Inflammation ↓	CD4, IL-10, MMP-1, MMP-2, MMP-3, MMP-9,	TLR3, CD31, CD68, lysozyme, TNF-α	IL-1, IL-6, CD3, CD20, CD28, M-CSF, cathepsin K, LTA4H, CXCR4, COX-2
Growth factor	IGF-1, HER1, HER2	TGF-β1	FGF-1, FGF-2, ERβ

Abbreviations: PCNA: proliferating cell nuclear antigen, Ki-67, CDK4: cyclin dependent kinase 4, PLK4: polo-like kinase 4, MPM2: mitotic protein monoclonal 2, Rb-1: retinoblastoma-1, MDM2: mouse double minute 2 homolog, BAX: Bcl-2 associated X, APAF: apoptotic protease activating factor 1, BAK: Bcl-2 homologous antagonist killer, PARP: poly-ADP ribose polymerase, FASL: FAS ligand, FADD: FAS-associated via death domain, AP-1: activating factor-1, SP-1: specific protein-1, AMPK-1: AMP-activated protein kinase, NOS1: nitric oxide synthase 1, SOD-1: superoxide dismutase-1, GST: glutathione S-transferase, HIF: hypoxia inducible factor, VEGF-A: vascular endothelial growth factor-A, VEGF-C: vascular endothelial growth factor-C, vWF: von Willebrand factor, ET-1: endothelin-1, CD31: cluster of differentiation 31, CMG2: capillary morphogenic protein 2, OPG: osteoprotegerin, RANKL: receptor activator of nuclear factor kappa-B ligand, RUNX2: RUNT-related transcription factor 2, ALP: alkaline phosphatase, CD4: cluster of differentiation, IL-10: interleukin-1, MMP-1: matrix metalloproteinase-1, MMP-2: matrix metalloproteinase-2, MMP-3: matrix metalloproteinase-3, MMP-9: matrix metalloproteinase-9, TLR3: Toll like receptor 3, CD68: cluster of differentiation 68, TNF-α: tumor necrosis factor-α, IL-1: interleukin-1, IL-6: interleukin-10, CD3: cluster of differentiation 3, CD30: cluster of differentiation 20, CD28: cluster of differentiation, M-CSF: macrophage-colony stimulating factor, LTA4H: leukotriene-A4 hydrolase, CXCR4: C-X-C chemokine receptor 4, COX-2: cyclooxygenase-2, IGF-1: insulin growth factor-1, HER1: human epidermal growth factor receptor 1, HER2: human epidermal growth factor receptor 2, TGF-β1: transforming growth factor-β1, ERβ: estrogen receptor β, ↑: upregulation, ↓: downregulation.

4. Discussion

We treated RAW 264.7 cells (a murine macrophage cell line) with PTX and investigated global protein expressional changes in cells by IP-HPLC using 132 antisera. As PTX could be primarily engulfed by macrophages, after treating RAW 264.7 cells with PTX at 10 μg/mL for 12, 24, and 48 h, the RAW 264.7 cells were harvested with protein lysis buffer (0.3% SDS, 50 mM Tris-HCl pH 8.0, 0.3% β-mercaptoethanol, 1 mL PMSF, 1 mL EDTA) containing a protein inhibitor cocktail (Sigma, Chicago, IL, USA). Protein extracts were kept in a −70 °C deep freezer until required. In spite of there being a direct characterization between major inflammatory diseases such as COVID-19 and different protein expression profiles of unstimulated murine RAW 264.7 cells, we have tried to reveal and clarify the basic characteristics of PTX profiling. From these repeated and statistical data, the basic effects of PTX could be useful as antiviral and immunomodulatory functions, which will be appropriate for disease control, such as ORN and COVID-19.

In comparison with Western blot data of β-actin, a known cytoplasmic housekeeping protein, IP-HPLC exhibited a small error range of less than ±5.0% statistical significance [31–33]. In the IP-HPLC, housekeeping proteins, standard β-actin, α-tubulin, and GAPDH, were used as internal controls. Expression changes of housekeeping proteins were adjusted to ≤±5% using a proportional baseline algorithm [34]. PTX seemed to suppress proliferation-related proteins, up-regulated cMyc/MAX signaling in 12 and 24 h samples; however, decreased in the 48 h sample and is thought to promote cellular proliferation up to 24 h and suppress it more. These data suggest that PTX inhibited the p53/Rb/E2F signaling pathway which is associated with cellular proliferation. IP-HPLC may show a relative protein expression value compared to reference controls.

Methylxanthines have already shown improvement in the symptoms of adult respiratory distress syndrome, and PTX is a well-known anti-inflammatory and anti-oxidative molecule that has already been shown to suppress TNF-α as well as other inflammatory cytokines in pulmonary diseases, and this may be beneficial for better clinical outcomes in COVID-19 patients [24].

PTX reduced the expression of KRAS, NRAS, HRAS, and pAKT. The expression of ERK-1 was slightly up-regulated, while JNK-1, Rab, PKC levels were changed less than 5% in response to PTX. These results suggest that PTX slightly reduced RAS signaling compared to non-treated controls. PTX is known to inhibit TNF-α and the synthesis of other pro-inflammatory cytokines [35]. Inhibition of NFκB may be associated with the reduction of pro-inflammatory cytokines [36]. In this study, PTX showed gradual decreases in NFkB signaling-related proteins, however, TNF-α was not significantly affected by PTX treatment in this study. Inflammation-related protein, IL-1, IL-6, CD28, M-CSF, MCP-1, MMP-10, hepcidin, cathepsin K, LTA4H, CXCR4, and COX-2 were significantly downregulated. This feature is expected to reduce the cytokine storm that can occur in COVID-19 patients. PTX activates the adenosine A2A receptor, which is expressed by many types of immune cells, endothelial cells, and platelets, thereby up-regulating anti-inflammatory and anti-thrombotic molecules, and finally inhibiting the cytokine storm syndrome, which is believed to be the main pathogenic mechanism of COVID-19's lethal complications [26].

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an RNA virus genetically located within the genus Betacoronavirus that uses a glycoprotein (spike protein) to bind to the angiotensin-converting enzyme 2 (ACE2) receptor. After binding, the serine protease TGRBBS2 facilitates virus entry into the cell [37]. Currently, no therapeutic agent is effective in treating the SARS-CoV-2 infection, but the classes of drugs that are used include antiviral agents, neuraminidase inhibitors, and anti-inflammatory drugs [38]. As an antiviral agent, Lopinavir/ritonavir is an antiretroviral of the protease inhibitor class and is mainly used in COVID-19 patients with less severe symptoms and also in the early stage of the disease. Remdesivir, which belongs to the class of nucleotide analogs, was previously used in the Ebola virus epidemic in Africa and is currently used in moderate and severe COVID-19 [39].

As immunomodulatory drugs, an abnormal release of pro-inflammatory cytokines such as interleukin-6 (IL-6), interferon-gamma, and tumor necrosis factor-alpha was observed in moderate to severe stage COVID-19 patients. Anti-inflammatory drugs (particularly monoclonal antibodies) are used in COVID-19 patients. In this category, anti-IL6 (Tocilizumab), anti-IL-1 (Anakira), JAK inhibitors (Baricitinib), corticosteroids, antimalarials (Chloroquine/Hydroxychloroquine), heparins, and immunoglobulins. Antiviral agents are useful to inhibit the clinical progression and complications of COVID-19. Clinical and survival improvements were found in patients treated with plasma and hyperimmune immunoglobulins. Inflammation inhibitors are candidates for the treatment of advanced-stage COVID-19 [39].

Most patients with severe COVID-19 show elevated serum levels of pro-inflammatory cytokines including IL-6, IL-1β, IL-2, IL-8, IL-17, G-CSF, GM-CSF, MCP1, and TNF, characterized as cytokine storm [40]. A number of studies have trialed plans to reduce inflammatory responses. As already mentioned, PTX is derived from a methylxanthine derivative that inhibits phosphodiesterase-4 (PDE-4). PTX functions as a hemorrheologic agent and affects immunomodulation, inflammation, and oxidative stress [41]. In this study, the expression levels of IL-1, IL-6, M-CSF, MCP-1, MMP-10, and COX-2 were significantly down-regulated by PTX treatment in RAW 264 cells. Inhibition of proinflammatory cytokines by PTX treatment shares a context to other immunomodulatory agents used in COVID-19. The levels of CD4 and IL-10 were increased and PTX was found to increase immune function. In addition, the proliferation of macrophages is inhibited by PTX treatment, the macrophage-related acute immune response can be suppressed (Figure S2).

The expressions of IGF1, HER1, and HER2 in growth factor-related protein expressions were slightly up-regulated. The expression levels of FGF-1 and FGF-2 were significantly reduced after PTX treatment. When PTX was applied to a renal fibroblast cell line, it inhibited fibroblast proliferation and suppressed FGF-2 synthesis [42]. Clinical studies on radiation-induced fibrosis have also shown that circulating the FGF-2 level was reduced after eight weeks of PTX treatment [43]. Tissue hypoxia can induce macrophage infiltration,

which is a source of pro-fibrotic mediators, including TGF-β1 [44]. In this study, TGF-β1 expression was not significantly affected by PTX treatment.

The expression of CRP-1, CD4, IL-10, MMP-1, MMP-2, MMP-3, and MMP-9 was upregulated in inflammation-related protein profiles. The expression levels of IL-1, IL-6, CD3, CD28, M-CSF, MCP-1, MMP-10, hepcidin, cathepsin K, LTA4H, CXCR4, and COX-2 were all down-regulated by PTX treatment. In this study, immune-related markers were increased, and matrix inflammation-associated markers were decreased. The level of cytokines and T-lymphocytes in COVID-19 patients was studied, and it was suggested that a decrease in T cells in COVID-19 patients may be associated with a high serum concentration of TNF-α, IL-6 negatively regulating T cell survival or proliferation. [45] The severity of a cytokine storm and decreased T cells are associated with exacerbation of diseases such as pulmonary damage and respiratory distress [46,47]. The expression of CD4 is slightly increased by PTX treatment, in which it can be presumed that PTX contributes to macrophage immune function.

PTX downregulated the expression of p53, MDM2, and PARP. However, BAX expression was increased in the 12 and 24 h PTX treatment group and decreased slightly in the 48 h PTX treatment group to 98.8% of baseline. The expression of BAK was decreased in the 48 h sample, APAF-1 increased at 24 h and decreased at 48 h. The expression of caspase 9 decreased with time; however, the changes were not significant. PTX seems to down-regulate p53, but downstream proteins associated with the intrinsic apoptotic pathway require further study. PTX also down-regulated FAS-mediated apoptosis-related proteins, FASL, FAS, FADD, PARP, and caspase 8 were down-regulated by PTX treatment. PTX inhibited the extrinsic apoptotic pathway, which is in contrast to a previous study [48]. In this in vitro study, it was found that PTX suppressed the proliferation of RAW 264.7 cells and also suppressed the expression of proteins related to cellular apoptosis. However, we did not perform the cellular proliferation and apoptosis assay. In a previous animal study, an experiment irradiated the mandible of rats with radiation. It was shown that PTX had an effect of inhibiting cellular apoptosis [20]. Therefore, protein expressions related to proliferation and apoptosis were analyzed through the IP-HPLC in this study.

Referring to the antioxidant-related proteins, NOS1, SOD-1, GST, and LC3 were downregulated by PTX in contrast to the control group. This suggests that the antioxidant effect of PTX in RAW 264.7 cells is unclear. Luo et al. [49] studied the protective effects of Pentoxifylline on acute liver injury; the levels of SOD and glutathione (GSH) in liver tissue were elevated by PTX treatment compared to that of the control group. In a study of methotrexate-induced damage in the livers and kidneys of rats, immunostaining of iNOS and TNF-α was decreased in the PTX administered group, while SOD levels decreased in liver tissue. These results are similar to the result of our in vitro study [50].

The oncogenic protein expressions, TERT, and 14-3-3 were down-regulated; PTX did not elevate tumorigenic protein expression but actually showed some anti-cancer effects by up-regulating the expression of MBD4, which has some function in DNA repair. PTX not only has anti-tumor activity, but it also increases the susceptibility of cancer cells to radiation therapy [51].

Mild increases in cell protection-related proteins, such as HSP-70 (110.2%), AP-1 (111.3%), SP-1 (113.4%), and PKC (105.1%) also occurred. Most proteins associated with cellular protection increased in cells treated with PTX for 12 h and decreased for the next 24, and 48 h back to baseline, with the exception of the transcription factor. This means that the PTX has no deleterious effect on RAW 264.7 cells.

The expressions of OPG, osteopontin, and osterix, were slightly increased, and RANKL expression was markedly up-regulated by PTX treatment, which was not surprising since RAW 264.7 cells originate from murine macrophages and are likely to differentiate into osteoclasts. The advantage of IP-HPLC research is that it can investigate the expression of multiple proteins in one experiment. Therefore, when investigating osteogenesis, we tried to observe as many related proteins as possible. Very few studies have been published on

PTX and osteogenesis; Horiuchi et al. [52] reported that PTX promotes rh-BMP-induced bone formation.

In the angiogenesis-related proteins, VEGF-A, vWF, ET-1, and CD31 did not show significant changes compared to the housekeeping proteins. The expression of HIF (108.6%), angiogenin (107.3%), and MMP-2 (109.1%) were slightly elevated by PTX treatment. PTX showed weak angiogenic effects. The effect of PTX on angiogenesis is controversial. In a study of segmental cortical bone defects of the radius in a rat model, PTX appeared to improve angiogenesis [53]. Conversely, PTX treatment significantly inhibited angiogenesis in a melanoma model [54].

The results of this study are summarized in Figure S3. PTX was shown to inhibit cellular proliferation, apoptosis, and inflammation-related proteins in RAW 264.7 cells. Conversely, angiogenesis and antioxidant activity were not significantly affected. PTX was expected to have an osteogenesis-promoting effect, which is expected to provide an advantage in the treatment of osteoradionecrosis with anti-inflammatory effects.

Moreover, having a potential role in suppressing inflammation and immune modulation, PTX has the potential to serve as an adjuvant therapeutic agent in COVID-19 pandemic situations, but additional preclinical and clinical studies are needed to prove the effect of PTX.

Besides, it may be possible to glimpse the potential as an adjuvant treatment in COVID-19 pandemic situations, but additional preclinical and clinical studies are needed.

5. Conclusions

PTX showed decreased expressions of proliferation-, extrinsic apoptosis-, and inflammation-related proteins and increased expressions of osteogenesis-related proteins in this in vitro study (Figure S3). The anti-inflammatory effect, cytoprotective effect, and osteogenesis-promoting effect are thought to be helpful in the treatment of osteoradionecrosis. In addition, the results showed the possibility of using PTX as a therapeutic adjuvant for COVID-19. Additional animal studies or clinical studies are needed to verify these results.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11178273/s1. Figure S1. Schematic flows of IP-HPLC procedures with data analysis. Figure S2. Our suggested summary of PTX as the immunomodulatory drugs in the COVID-19 management. Figure S3. Summary of differential protein expressions in RAW 264.7 cells treated with PTX focused on the proliferation, inflammation, angiogenesis, and osteogenesis.

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Conflicts of Interest: The authors declare no conflict of interest.

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