

1 **Protective Effect of Cannabinoid Type II Receptor Ligand** 2 **on Bleomycin-Induced Pulmonary Fibrosis in Mice**

3 Cheng Cao ^{1,2}, Run-Lin Wang¹, Chen-xin Guo³, Xiao-wei Xu¹, Jia-hui Wei¹, Wen-zhuo
4 Cheng¹, Yi-zhe Yu¹, Jia-jie Shi¹, Zhi-wei Feng^{4*}, Yan Chen ^{1,3*}

5 ¹College of Pharmaceutical Sciences, Zhejiang University of Technology, Zhejiang 310014,
6 China;

7 ²Department of Pharmacy, The Affiliated Suzhou Hospital of Nanjing Medical University, Jiangsu
8 215000, China;

9 ³Laboratory Animal Center of Zhejiang University of Technology, Hangzhou, P.R. China,
10 310014)

11 ⁴Faculty of Pharmaceutical Sciences, Shenzhen University of Advanced Technology, Shenzhen,
12 518107, P.R. China

13 *Corresponding Authors:

14 Yan Chen, Ph.D.

15 Associate professor of College of Pharmaceutical Science, Zhejiang University of Technology

16 18 ChaoWang Road, Hangzhou 310014, P.R. China

17 86(571)-88813480(Phone)

18 Email: chenyan2008@zjut.edu.cn

19 Zhi-wei Feng, Ph.D.

20 Professor of Faculty of Pharmaceutical Sciences, Shenzhen University of Advanced Technology

21 Shenzhen, 518107, P.R. China

22 86-18127854918(Phone)

23 Email:fengzhiwei@suat-sz.edu.cn

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29 **Abstract**

30 This study aimed to investigate the protective effects of the CB2R ligand
 31 compound COCA (N-(3-chloro-2-methylphenyl)-5-(4-hydroxyphenyl)-1,2-oxazole-3-
 32 carboxamide) against bleomycin (BLM)-induced pulmonary fibrosis in mice.
 33 Compound COCA was identified via AI-driven virtual screening from the ChEMBL
 34 database. Forty male C57BL/6J mice were randomly divided into five groups: control,
 35 model (BLM), low-dose COCA, high-dose COCA, and pirfenidone. Pulmonary
 36 fibrosis was induced by intratracheal BLM administration in the model and treatment
 37 groups. Pathological evaluation, ELISA, immunofluorescence, and Western blot were
 38 conducted. The results showed that COCA significantly alleviated BLM-induced
 39 inflammation and fibrosis. ELISA demonstrated decreased serum levels of TNF- α and
 40 IL-6 in the treated groups compared to the BLM group ($P < 0.01$). Immunofluorescence
 41 indicated reduced expression of Col-I and Col-III in the treated groups ($P < 0.01$).
 42 Western blot analysis revealed an upregulation of CB2R in the BLM group, and
 43 enhanced expressions of Nrf2 and Smad7 in the treated groups ($P < 0.01$). In conclusion,
 44 AI-driven virtual screening enabled the identification of the CB2R ligand COCA,
 45 which binds to CB2R, activates the Nrf2/Smad7 pathway, downregulates related
 46 cytokines, and plays a therapeutic and protective role in pulmonary fibrosis.

47 **Keywords:** Pulmonary fibrosis, CB2 receptor, Bleomycin, AI virtual screening

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50 Introduction

51 Idiopathic Pulmonary Fibrosis (IPF) is a chronic, progressive interstitial lung
52 disease with unknown etiology. Its annual incidence ranges from 0.9 to 13 cases per
53 100,000 individuals [1]. The prognosis of IPF is extremely poor; it significantly impairs
54 patients' quality of life and leads to substantial functional impairment within the first
55 few years after diagnosis [2, 3]. Currently, only two drugs-pirfenidone and nintedanib-
56 are approved for clinical use[4]. While these treatments can modestly improve lung
57 function and reduce the frequency of acute exacerbations, they do not halt disease
58 progression or improve long-term outcomes. Furthermore, the average daily cost of
59 these two drugs is relatively high, creating a pressing need for more effective and
60 economical therapeutic options.

61 The IPF research community has identified critical areas for future investigation,
62 including the molecular mechanisms underlying the pathogenesis of IPF. Potential
63 biomarkers for early diagnosis and innovative therapies targeting its progression remain
64 as key focus areas. Given the significant unmet medical needs and the growing burden
65 of this disease on patients worldwide, it is imperative to pursue innovative approaches
66 that can advance our understanding and improve treatment outcomes. The cannabinoid
67 type II receptor (CB2R), a core component of the endocannabinoid system (ECS), is
68 predominantly localized in peripheral tissues. Through its activity across various cell
69 types, CB2R exerts diverse regulatory effects, including inducing apoptosis, inhibiting
70 cell proliferation, suppressing pro-inflammatory cytokine production, promoting anti-

inflammatory cytokine release, and modulating T-cell activity. These mechanisms collectively contribute to immunomodulatory effects [5]. Studies have demonstrated that modulating CB2R may attenuate fibrotic processes in multiple organs, with particular relevance observed in the lungs across various experimental models [6-8].

Over the past few years, advancements in artificial intelligence (AI) technology have significantly transformed the fields of drug screening, preclinical research, and clinical studies [9]. Drug screening represents a critical foundational phase in the drug development process, primarily aimed at identifying compounds with specific pharmacological activities from vast chemical libraries. However, this step is often marked by substantial time investment and inherent risk due to the complexity of numerous factors that influence drug efficacy. AI technology stands out as a groundbreaking tool for accelerating drug discovery and development [10]. Its ability to model intricate, nonlinear pharmacological relationships while efficiently processing large datasets enables researchers to bypass traditional trial-and-error methods. This innovative approach not only expedites the identification of potential drug candidates but also enhances their therapeutic potential by minimizing reliance on exhaustive in vitro testing.

In this study, we leveraged AI-driven virtual screening methodologies to identify high-affinity ligands for the CB2R (Cannabinoid Type 2 Receptor) receptor. This cutting-edge approach allowed us to evaluate and rank compounds based on their predicted binding affinity, ultimately pinpointing a set of candidates with exceptional potential. The validated efficacy of these CB2R ligands was further corroborated

through preclinical animal experiments, underscoring the robustness and translational relevance of our findings. This innovative application of AI technology not only streamlines the drug discovery process but also opens new avenues for understanding receptor-mediated pathways in human health, paving the way for the development of more effective and precise therapeutic interventions.

Materials and Methods

AlphaFold 3 for Virtual Screening against ChEMBL Database

AlphaFold 3 Environment Setup for Protein-Ligand Complex Modeling

All protein-ligand structure predictions and virtual screening tasks were performed using AlphaFold 3 on a dedicated high-performance computing node equipped with an NVIDIA A100 GPU (80 GB VRAM), an AMD EPYC 7702P 64-core CPU, and 512 GB of RAM, running Ubuntu 22.04 LTS. The software environment was configured with CUDA 12.6, cuDNN v8.9, and JAX 0.4.20 with GPU support to enable AlphaFold 3's accelerated inference engine, particularly its FlashAttention-based, memory-efficient transformer implementation. A dedicated Conda environment named "af3" was created using Python 3.11, and all dependencies were installed following the official AlphaFold 3 GitHub repository instructions. A Docker container was built locally using the provided Dockerfile to encapsulate all required system libraries, Python packages, and alignment tools, ensuring reproducibility and minimizing conflicts across runs.

Database Configuration and Preparation

To support sequence alignment and structural template matching, all required databases were downloaded and organized according to AlphaFold-specific directory structures using the `fetch_databases.py` utility. The setup included the PDB mmCIF files (as of September 2022), UniRef90 and UniProt for sequence clustering and functional annotation, the BFD metagenomic database for broader sequence coverage, MGnify clusters (release 2022-05), RNACentral and Rfam databases for RNA recognition, and SEQRES-derived sequences from the PDB for template lookups. All data were verified with checksums to ensure integrity and consistency prior to use.

Model Weights and Licensing

AlphaFold 3 model weights are not publicly available by default. Access was granted upon submitting a license request to DeepMind. Once approved, the model parameters were downloaded, extracted, and placed in the local `alphafold3/model/` directory. These model files contain the trained neural network weights, structural templates, and scoring parameters essential for accurate complex structure predictions.

Input File Design and Ligand Specification

Each structure prediction task was defined through a structured JSON file adhering to the AlphaFold 3 schema. This input described the protein chain using a unique identifier (e.g., “A”) along with its full-length amino acid sequence. Ligands were specified using SMILES strings, which were converted into 3D conformations via RDKit, or by referencing CCD codes for standard ligands and cofactors from the PDB. Custom CCD entries could also be used for novel or modified ligands. Each input also included a random model seed for reproducibility, a dialect identifier (“alphafold3”),

and a version tag to ensure compatibility with future AlphaFold updates. The framework supported multi-chain proteins and multiple ligand entities, allowing for the modeling of complex assemblies.

High-Throughput Screening Using AlphaFold 3 Against the ChEMBL Database

To extend the virtual screening campaign to a broader chemical space, a large-scale evaluation was conducted using a curated library of approximately 500,000 ligands from the ChEMBL database. All entries were preprocessed to standardize molecular structures and remove duplicates. After initial filtering based on physicochemical properties and known liabilities, the remaining compounds were subjected to structure-based docking using AlphaFold 3-predicted target structures. The workflow prioritized ligands with high predicted binding affinities, novel scaffolds, and potential biological activity. Top-ranked hits from this screen were shortlisted for follow-up experimental assays and optimization cycles.

Experimental animals

Forty male SPF-grade C57BL/6J mice (6-8 weeks, 20 ± 2 g) were obtained from Hangzhou Qizhen Laboratory Animal Technology Co., Ltd. They were housed in an SPF facility at Zhejiang University of Technology (Animal Experiment License No. SCXK 2022-0026, Ethics Approval No. 202404270031). After acclimatization for three days, the mice were randomly divided into five groups ($n = 8$ per group): Control, BLM-induced model (BLM), low-dose COCA (COCA LD), high-dose COCA (COCA HD), and Pirfenidone (PF). All procedures followed the institution's guidelines and were approved by the Animal Ethics Committee.

Animal Modeling and Drug Intervention

The mice were anesthetized by intraperitoneal injection of 10% urethane (0.1 mL/10 g). Pulmonary fibrosis was induced by intratracheal instillation of bleomycin (2.5 mg/kg, 20 μ L/25 g). The control group underwent the same procedure but received saline instead of bleomycin. One day after modeling, intragastric administration was initiated as follows: ①COCA LD group: COCA 5mg/kg; ②COCA HD group: COCA 10mg/kg; ③ PF group: pirfenidone 50mg/kg; ④ BLM group and Control group: equal volume of saline. After 7 days of continuous administration, the mice were anesthetized with 10% urethane on the 8th day, and blood was collected from the orbital vein. Serum was stored at -80 °C for further analysis. Bronchoalveolar lavage fluid (BALF) was collected, centrifuged, and the cell pellet was resuspended in 1 mL PBS for cell counting. The right lung was placed in a cryotube and stored at -80 °C for subsequent experiments, while the left lung was fixed in 4% paraformaldehyde and stored at 4°C.

General Condition, Body Weight, and Lung Coefficient (LC)

Following the initiation of the experiment, the body weight, general activity status, and mortality of the mice were monitored and recorded on a daily basis. Prior to euthanasia, the body weight of each mouse was measured. Subsequently, the animals were humanely euthanized, and their lung tissues were carefully excised, rinsed, thoroughly dried, and weighed. The lung coefficient was then calculated using the formula: Lung coefficient = Lung wet weight (mg) / Body weight (g).

BALF Cell Counting

After securing the mice on a dissection board, the thoracic cavity was carefully opened to expose the trachea. The left main bronchus was ligated, and a needle was precisely inserted at the tracheal bifurcation. Bronchoalveolar lavage was performed three times, each time using 0.5 mL of pre-cooled (4 °C) saline. The collected bronchoalveolar lavage fluid (BALF) was centrifuged at 3000 rpm for 15 minutes. The resulting cell pellet was resuspended in 1mL of phosphate-buffered saline (PBS) and evenly distributed onto a counting chamber for microscopic examination.

HE and Masson Staining of Lung Tissues

The left lung tissues from the mice were fixed in formalin, embedded in paraffin, and sectioned for histological analysis. Hematoxylin and Eosin (HE) and Masson's trichrome staining were carried out in accordance with the manufacturer's instructions, and the stained sections were examined and documented under a light microscope.

ELISA for Serum TNF- α and IL-6 Levels

Serum supernatant was collected, and TNF- α and IL-6 concentrations were measured using ELISA kits according to the manufacturer's instructions. Absorbance was measured at 450nm, and concentrations were calculated based on the standard curve (pg/mL).

Immunofluorescence for Col-I and Col-III Expression:

Paraffin-embedded lung tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval using sodium citrate buffer. Endogenous peroxidase activity was blocked, and the sections were incubated with primary antibodies diluted in 5% BSA overnight at 4 °C . The following day, secondary antibodies diluted in 5%

BSA were applied, and the sections were incubated at room temperature for one hour in the dark. After thorough washing, nuclear staining was performed with DAPI for 10 minutes, and the sections were mounted using an anti-fade mounting medium. Fluorescent images were captured using a confocal microscope at an excitation wavelength of 520nm, where collagen type I exhibited green fluorescence and collagen type III exhibited red fluorescence.

Western Blot for CB2R, Nrf2, and Smad7 Expression

Frozen lung tissue (30 mg) was homogenized in 270 μ L of RIPA lysis buffer and subsequently sonicated for efficient protein extraction. The total protein concentration was quantified, and equal amounts of protein samples were loaded onto SDS-PAGE gels. Following electrophoresis, proteins were electrotransferred to PVDF membranes, which were then blocked with 5% skim milk for 90 minutes. The membranes were incubated with specific primary antibodies overnight at 4°C. After thorough washing, appropriate secondary antibodies were applied, and the immunoreactive signals were visualized using an enhanced chemiluminescence (ECL) detection system. Grayscale intensities of the bands were quantitatively analyzed using LANE-1D and ImageJ software, and the relative expression levels of the target proteins were normalized to GAPDH.

Statistical Analysis

Quantitative data were analyzed using SPSS version 27.0 and are expressed as mean $\pm$ standard deviation (SD). Inter-group comparisons were conducted via t-tests, with statistical significance defined as $P < 0.05$ and high significance as $P < 0.01$. Data

processing and quantitative analysis were carried out using GraphPad Prism version 9.5.0 and ImageJ software.

Results

Virtual Screening

Figure 1 illustrates the high-throughput screening workflow employed in this study using AlphaFold 3. The process is divided into four key stages: environment setup, database preparation, input file design, and screening. In the initial setup, AlphaFold 3 was deployed on a high-performance computing node with GPU acceleration and a Dockerized software environment to ensure reproducibility and efficiency. The database preparation step involved downloading and organizing multiple sequence and structural databases—including PDB mmCIF, UniRef90, UniProt, BFD, MGnify, RNAcentral, and Rfam—to enable accurate template matching and sequence alignment. Input file design was conducted through structured JSON schemas defining protein sequences, ligand specifications via SMILES or CCD codes, and additional parameters like dialect identifiers and version tags. Finally, the screening stage integrated structure-based docking across a large-scale ligand library (>500,000 compounds) to identify promising candidates, followed by experimental validation of top-ranked hits.

This high-throughput screening platform successfully identified six compounds with favorable docking profiles and predicted high-affinity binding poses. Among them, compound COCA (N-(3-chloro-2-methylphenyl)-5-(4-hydroxyphenyl)-1,2-oxazole-3-carboxamide) stood out as the most promising ligand based on binding energy, binding mode quality, and pharmacophoric complementarity to the CB2R binding site. As

illustrated in Figure 2, COCA exhibited a docking score of -6.772 kcal/mol, indicating strong predicted binding affinity within the orthosteric ligand-binding pocket of CB2R. Detailed molecular interaction analysis revealed that COCA is anchored through an extensive hydrophobic and aromatic interaction network involving multiple key residues. Specifically, it engages with PHE87, PHE91, PHE94, ILE110, VAL113, THR111, PHE106, PHE183, PRO184, ILE186, TYR190, and TRP194. Of particular note are three π - π stacking interactions: a single stacking interaction with PHE94 and two with TRP194, which likely contribute substantially to the compound 's stability and specificity within the binding pocket. Additionally, hydrogen bonding and van der Waals contacts further stabilize the COCA-CB2R complex, suggesting a well-optimized fit that may support functional modulation of receptor activity. Given its robust binding profile and favorable chemical properties, COCA was selected for downstream pharmacodynamic evaluation, including in vitro binding assays and functional activity studies. These findings not only underscore the effectiveness of the AlphaFold 3-based virtual screening framework but also demonstrate its potential to accelerate early-phase drug discovery by integrating high-accuracy structure prediction with large-scale ligand evaluation.

Animal Experiment Validation

General Morphological Observations

As shown in Figure 3, no statistically significant differences in initial body weight were found among groups ($P > 0.05$). After model establishment, all groups showed a general decline in body weight. In the Control group, weight began to recover slightly by day 2 post-surgery and returned to or exceeded baseline levels by days 3-4, followed

by gradual increase. These mice showed normal activity, quick feeding and drinking, glossy fur, and no respiratory distress. In contrast, the BLM group continued to lose weight during the first week, with only minor gains in a few mice that did not reach baseline. These mice had reduced feeding, poor condition, dull fur, slow responses, and significantly greater weight loss than the Control group ($P < 0.01$). The treatment groups (COCA LD, COCA HD, PF) also lost weight in the first week, but less than the BLM group. Mice in these groups showed better responses, improved feeding and drinking, normal behavior, and no signs of breathing issues. Their weight improvement was significantly higher than in the BLM group ($P < 0.01$). Notably, the COCA HD group improved more than the PF group ($P < 0.05$); however, none fully reversed the weight loss.

Alterations in Lung Tissue and Lung Coefficient in Mice

Lung tissue isolation revealed clear morphological differences across groups. The Control group had healthy lungs with a pinkish color, smooth texture, good elasticity, and soft consistency. In contrast, the BLM group showed lung tissues with nodules, blood streaks, dark pigmentation, and hemorrhagic spots. The COCA LD, COCA HD, and PF groups showed improved lung structure, including less rigidity, fewer blood streaks, moderate elasticity, and slightly rough surfaces. As shown in Figure 3, the BLM group had a significantly higher lung coefficient than the Control group ($P < 0.01$). COCA treatment reduced the lung coefficient, with both COCA LD and COCA HD showing significant decreases compared to BLM ($P < 0.01$). However, no significant difference was found between the two COCA doses ($P > 0.05$).

COCA Attenuates Bleomycin-Induced Pulmonary Inflammation and Fibrosis in Mice

Histopathological alterations in lung tissue sections from bleomycin-induced pulmonary fibrosis mice were assessed using hematoxylin-eosin (HE) and Masson's trichrome staining. As shown in Figure 4, HE staining demonstrated intact alveolar architecture and absence of significant inflammatory cell infiltration in the Control group at day 7. In contrast, the BLM group displayed severe structural damage to the alveoli, including disrupted alveolar organization, thickened septa, and prominent inflammatory cell infiltration compared to the Control group. The treatment groups exhibited mild serous exudation, disorganized alveolar structures, and inflammatory cell infiltration; however, these pathological changes were less severe, with more uniform connective tissue distribution and improved inflammatory response relative to the BLM group. Masson's staining revealed extensive collagen deposition (blue-stained fibers), marked alveolar structural disruption, widened alveolar septa, and increased fibroblast proliferation in the BLM group, consistent with advanced pulmonary fibrosis. Administration of COCA and pirfenidone significantly decreased collagen accumulation and mitigated alveolar structural damage compared to the BLM group. Among the treatment groups, the COCA HD group showed the most notable histological improvement, characterized by reduced architectural disarray and diminished collagen aggregation.

Modulatory Effects of COCA on Bronchoalveolar Lavage Fluid (BALF) in Mice

Analysis of bronchoalveolar lavage fluid (BALF) cell counts, as illustrated in Figure 5, revealed a significant elevation in total cell numbers in the lungs of the BLM group compared to the Control group ($P < 0.01$). Administration of COCA and

pirfenidone resulted in a marked reduction in total cell counts in the COCA LD, COCA HD, and PF groups relative to the BLM group ($P < 0.01$). Moreover, a statistically significant difference was detected between the COCA HD and PF groups ($P < 0.05$).

Modulatory Effects of COCA on Interleukin-6 and Tumor Necrosis Factor- α Levels in Pulmonary Fibrosis Mice

The regulatory effects of COCA on IL-6 and TNF- α levels in a pulmonary fibrosis mouse model were evaluated by quantifying serum cytokine concentrations using ELISA. As illustrated in Figure 5, the BLM group exhibited markedly elevated serum levels of IL-6 and TNF- α relative to the Control group ($P < 0.01$). Administration of COCA significantly reduced these cytokine levels, indicating effective suppression of pro-inflammatory mediator release from immune cells, particularly phagocytes, compared to the BLM group ($P < 0.01$). Furthermore, no statistically significant difference was observed between the COCA treatment group and the PF group ($P > 0.05$).

Effects of COCA on Col-I and Col-III Expression in Pulmonary Fibrosis Mice

Immunofluorescence analysis, as illustrated in Figure 6, demonstrated that green fluorescence corresponds to Collagen Type I (Col-I), while red fluorescence corresponds to Collagen Type III (Col-III). Compared to the Control group, the BLM group exhibited markedly increased fluorescence intensity of both Col-I and Col-III in lung tissues, indicating elevated expression levels. Conversely, the COCA LD and COCA HD groups showed reduced fluorescence intensity and significantly suppressed expression of Col-I and Col-III relative to the BLM group. Quantitative assessment using one-way ANOVA, as presented in Figure 6, revealed that Col-I and Col-III

expression levels in the BLM group were significantly higher than those in the Control group ($P < 0.001$). Administration of COCA and pirfenidone led to a significant decrease in the expression of these collagens compared to the BLM group ($P < 0.01$). However, no statistically significant differences were detected between the COCA treatment groups and the PF group ($P > 0.05$).

Western Blot Analysis of Protein Expression

The expression levels of CB2R and its downstream signaling pathways were evaluated by immunoblotting. As illustrated in Figure 7, CB2R expression in both the BLM group and the Control group was markedly elevated compared to that in the drug-delivered group. Administration of both low-dose and high-dose COCA resulted in significantly altered CB2R expression profiles relative to the BLM group ($P < 0.05$). Furthermore, protein expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and Smad7 were markedly upregulated in the COCA LD and COCA HD groups compared to both the model and control groups ($P < 0.01$).

Discussion

For centuries, cannabis-derived preparations have been employed for the treatment of various medical conditions, including cancer and neuropathic pain [11]. However, their therapeutic application has been limited by associated addictive properties. This limitation underwent a significant transformation in the 1980s and 1990s with the identification of cannabinoid receptors, particularly the cannabinoid type II receptor (CB2R), which is predominantly localized in immune cells and peripheral tissues. CB2R exerts critical regulatory functions in immune modulation, inflammatory responses, and neuroprotection [5]. Importantly, its activation does not induce

psychoactive effects, thereby positioning it as a major focus of pharmacological research [12]. Accumulating evidence indicates that CB2R modulators represent promising candidates for the treatment of idiopathic pulmonary fibrosis (IPF) [13]. For example, CB2R-deficient mice develop more rapid and severe lung fibrosis, whereas activation of CB2R attenuates bleomycin-induced pulmonary fibrosis in murine models [14, 15]. In contrast, inhibition of CB2R counteracts this protective effect [16], although certain studies suggest that CB2R antagonists may also offer therapeutic advantages [17]. Despite ongoing controversies concerning the relative efficacy of agonists versus antagonists, these findings collectively highlight the therapeutic potential of targeting CB2R in IPF.

The integration of artificial intelligence (AI) into drug screening has revolutionized the field by effectively modeling the nonlinear relationships inherent in pharmacological systems. AI's capability to process complex, high-dimensional datasets has substantially accelerated the drug discovery process. AI-driven drug screening predominantly utilizes two strategies: (i) ligand-based screening, which exploits the structural and physicochemical properties of known bioactive compounds to predict the biological activity of novel molecules; and (ii) structure-based screening, which leverages the three-dimensional architecture of target proteins to predict ligand binding and associated pharmacological effects [18, 19]. These complementary methodologies form the foundation of modern AI-based virtual screening. In this study, we constructed a library of drug-like small molecules sourced from commercial databases and performed pretraining to derive molecular descriptors. Simultaneously,

we extracted the binding pocket features of CB2R from a pretrained protein dataset. The preprocessed molecular and protein data were subsequently fed into a predictive screening platform to estimate protein-ligand binding affinities. Using the mean squared error (MSE) loss function, we ranked the binding potential of each molecule to CB2R, ultimately identifying COCA as the compound with the highest binding affinity, warranting its selection for subsequent pharmacological evaluation.

Pirfenidone, a broad-spectrum antifibrotic agent, inhibits fibroblast proliferation, suppresses collagen synthesis, and reduces extracellular matrix deposition, thus improving pulmonary fibrosis [20]. It was used as the positive control in this study. Intratracheal administration of bleomycin (BLM) is a standard method for establishing an idiopathic pulmonary fibrosis (IPF) model in mice [21, 22]. BLM-treated mice showed reduced food intake, weight loss, and increased lung coefficients due to inflammatory cell infiltration and ECM deposition; BALF cell counts were also elevated. COCA treatment reduced weight loss, inflammation, and lung coefficient increases. Histopathological analysis confirmed serous exudation, inflammation, and collagen buildup in the BLM group by HE and Masson's staining, indicating successful model induction. These changes were significantly reduced in the COCA group. IL-6, a pleiotropic cytokine, regulates cell growth, differentiation, immune responses, and hematopoiesis [6]. During acute inflammation, damaged cells undergo lipid peroxidation, triggering inflammatory cell activation and IL-6 secretion. IL-6 activates STAT3, MAPK, PKB/Akt, and NF- κ B pathways in turn, exacerbating inflammation. TNF- α , a pro-inflammatory cytokine produced by macrophages/monocytes during

acute inflammation, mediates intracellular signaling cascades that culminate in necrosis or apoptosis [23]. It is primarily secreted by macrophages and plays a pivotal role in amplifying inflammation while promoting cell proliferation and differentiation; thus perpetuating inflammatory responses in pulmonary fibrosis [24]. In this study, serum levels of IL-6 and TNF- α were significantly decreased in the COCA treatment group compared to the BLM group, indicating a reduction in inflammatory activity. This was supported by reduced bronchoalveolar lavage fluid (BALF) cell counts. Collagen type I (Col-I) and collagen type III (Col-III), primarily synthesized by myofibroblasts, are key components of the extracellular matrix (ECM). Upregulated expression of these collagens reflects ECM accumulation and serves as critical biomarkers for idiopathic pulmonary fibrosis (IPF) [25, 26]. In the COCA treatment groups, both fluorescence intensity and expression levels of Col-I and Col-III were markedly decreased, suggesting attenuation of fibrotic changes. Compared with pirfenidone, COCA exhibited comparable or enhanced therapeutic efficacy across most evaluated parameters, indicating that it mitigates pulmonary fibrosis through suppression of inflammatory responses, similar to pirfenidone.

CB2R expression was markedly upregulated in lung tissues of BLM-induced IPF mice, especially in fibrotic areas [16], and increased rapidly during the early inflammatory phase [27]. The control group also showed elevated CB2R levels, likely due to stress from sham surgery and saline treatment. How CB2R regulates IPF is still not fully understood. Studies suggest that the PI3K/Akt [28] and TGF- β 1/Smad [29] pathways are involved in CB2R's effects on immune regulation, cytokine suppression,

and ECM remodeling. CB2R activation also enhances the Nrf2/Smad7 pathway, which reduces TGF- β 1-induced EMT [30]. Western blot results showed higher Nrf2 and Smad7 expression in treatment groups, suggesting COCA protects against BLM-induced IPF by activating the Nrf2/Smad7 pathway after binding to CB2R.

In conclusion, AI-driven virtual screening serves as a powerful approach for the rapid identification of ligand candidates targeting specific receptor structures, holding great promise for accelerating drug discovery. Our experimental findings indicate that COCA, a CB2R ligand, exerts protective effects against BLM-induced pulmonary fibrosis (PF) in mice, likely through CB2R binding and activation of the Nrf2/Smad7 pathway. This mechanism contributes to the downregulation of inflammatory mediators, suppression of pulmonary inflammation, and reduction of alveolar wall damage and collagen deposition, thereby effectively inhibiting PF progression. These results support COCA as a promising therapeutic candidate for pulmonary fibrosis.

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Authors' contributions

Yan Chen, Zhi-wei Feng projected management, conceptualization, supervision, and contributed to the concept and design of the research and drafted the manuscript. Cheng Cao, Run-Lin Wang, Wen-zhuo Cheng contributed to the data acquisition. Jia-hui Wei, Xiao-wei Xu and Jia-jie Shi conducted the experiments. Chen-xin Guo and Yi-zhe Yu contributed to the analysis and interpretation of data.

Conflict of interest

No potential conflicts of interest are reported by the authors.

Ethics Approval

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at Zhejiang University of Technology, Hangzhou, China. Ethical approval was granted under number 202404270031, and the procedures adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996).

References:

- [1] MAHER T M, BENDSTRUP E, DRON L, et al. Global incidence and prevalence of idiopathic pulmonary fibrosis [J]. *Respir Res*, 2021, 22(1): 197.
- [2] WENG D, CHEN X Q, QIU H, et al. The Role of Infection in Acute Exacerbation of Idiopathic Pulmonary Fibrosis [J]. *Mediators Inflamm*, 2019, 7(3): 5160694.
- [3] GUI X, QIU X, XIE M, et al. Prognostic Value of Serum Osteopontin in Acute Exacerbation of Idiopathic Pulmonary Fibrosis [J]. *Biomed Res Int*, 2020, 2020: 3424208.
- [4] KEATING, GILLIAN, M., et al. Pirfenidone: A Review of Its Use in Idiopathic Pulmonary Fibrosis [J]. *Drugs: International Journal of Current Therapeutics and Applied Pharmacology Reviews, Featuring Evaluations on New Drugs, Review Articles on Drugs and Drug Therapy, and Drug Literature Abstracts*, 2015.
- [5] JORDAN C J, XI Z X. Progress in brain cannabinoid CB(2) receptor research: From genes to behavior [J]. *Neurosci Biobehav Rev*, 2019, 98: 208-220.
- [6] WEISKIRCHEN R, WEISKIRCHEN S, TACKE F. Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications [J]. *Mol Aspects Med*, 2019, 65: 2-15.
- [7] BARUTTA F, BRUNO G, MASTROCOLA R, et al. The role of cannabinoid signaling in acute and chronic kidney diseases [J]. *Kidney Int*, 2018, 94(2): 252-258.
- [8] WANG L L, ZHAO R, LI J Y, et al. Pharmacological activation of cannabinoid 2 receptor attenuates inflammation, fibrogenesis, and promotes re-epithelialization during skin wound healing [J]. *Eur J Pharmacol*, 2016, 786: 128-136.
- [9] VAMATHEVAN J, CLARK D, CZODROWSKI P, et al. Applications of machine learning in drug discovery and development [J]. *Nat Rev Drug Discov*, 2019, 18(6): 463-477.
- [10] GUPTA R, SRIVASTAVA D, SAHU M, et al. Artificial intelligence to deep learning: machine intelligence approach for drug discovery [J]. *Mol Divers*, 2021, 25(3): 1315-1360.
- [11] ARGUETA D A, AICH A, MUQOLLI F, et al. Considerations for Cannabis Use to Treat Pain in Sickle Cell Disease [J]. *J Clin Med*, 2020, 9(12):3902.
- [12] KESERU G M, MAKARA G M. The influence of lead discovery strategies on the properties of

485 drug candidates [J]. *Nat Rev Drug Discov*, 2009, 8(3): 203-212.

486 [13] TURCOTTE C, BLANCHET M R, LAVIOLETTE M, et al. Impact of Cannabis, Cannabinoids,
487 and Endocannabinoids in the Lungs [J]. *Front Pharmacol*, 2016, 7: 317.

488 [14] SERVETTAZ A, KAVIAN N, NICCO C, et al. Targeting the cannabinoid pathway limits the
489 development of fibrosis and autoimmunity in a mouse model of systemic sclerosis [J]. *Am J*
490 *Pathol*, 2010, 177(1): 187-196.

491 [15] FU Q, ZHENG Y, DONG X, et al. Activation of cannabinoid receptor type 2 by JWH133
492 alleviates bleomycin-induced pulmonary fibrosis in mice [J]. *Oncotarget*, 2017, 8(61): 103486-
493 103498.

494 [16] WAWRYK-GAWDA E, CHLAPEK K, ZAROBKIEWICZ M K, et al. CB2R agonist prevents
495 nicotine induced lung fibrosis [J]. *Exp Lung Res*, 2018, 44(7): 344-351.

496 [17] DU W, ZHANG T, YANG F, et al. Endocannabinoid signalling/cannabinoid receptor 2 is
497 involved in icariin-mediated protective effects against bleomycin-induced pulmonary fibrosis
498 [J]. *Phytomedicine*, 2022, 103: 154187.

499 [18] THAFAR M, RAIES A B, ALBARADEI S, et al. Comparison Study of Computational
500 Prediction Tools for Drug-Target Binding Affinities [J]. *Front Chem*, 2019, 7: 782.

501 [19] JABEEN A, RANGANATHAN S. Applications of machine learning in GPCR bioactive ligand
502 discovery [J]. *Curr Opin Struct Biol*, 2019, 55: 66-76.

503 [20] CONTE E, GILI E, FAGONE E, et al. Effect of pirfenidone on proliferation, TGF-beta-induced
504 myofibroblast differentiation and fibrogenic activity of primary human lung fibroblasts [J]. *Eur*
505 *J Pharm Sci*, 2014, 58: 13-19.

506 [21] JENKINS R G, MOORE B B, CHAMBERS R C, et al. An Official American Thoracic Society
507 Workshop Report: Use of Animal Models for the Preclinical Assessment of Potential Therapies
508 for Pulmonary Fibrosis [J]. *Am J Respir Cell Mol Biol*, 2017, 56(5): 667-679.

509 [22] CARRINGTON R, JORDAN S, PITCHFORD S C, et al. Use of animal models in IPF research
510 [J]. *Pulm Pharmacol Ther*, 2018, 51: 73-78.

511 [23] GHEBREMEDHIN A, SALAM A B, ADU-ADDAI B, et al. A Novel CD206 Targeting Peptide
512 Inhibits Bleomycin-Induced Pulmonary Fibrosis in Mice [J]. *Cells*, 2023, 12(9):1254.

513 [24] BOLOURANI S, BRENNER M, WANG P. The interplay of DAMPs, TLR4, and
514 proinflammatory cytokines in pulmonary fibrosis [J]. *J Mol Med (Berl)*, 2021, 99(10): 1373-
515 1384.

516 [25] LI Z, JIAO Y, WU Z, et al. The role of quercetin in ameliorating bleomycin-induced pulmonary
517 fibrosis: insights into autophagy and the SIRT1/AMPK signaling pathway [J]. *Mol Biol Rep*,
518 2024, 51(1): 795.

519 [26] WU Z, SHI R, YAN S, et al. Integrating network pharmacology, experimental validation and
520 molecular docking to reveal the alleviation of Yinhuang granule on idiopathic pulmonary
521 fibrosis [J]. *Phytomedicine*, 2024, 128: 155368.

522 [27] ATWOOD B K, STRAIKER A, MACKIE K. CB(2): therapeutic target-in-waiting[J]. *Prog*
523 *Neuropsychopharmacol Biol Psychiatry*, 2012, 38(1): 16-20.

524 [28] GUAN T, ZHAO G, DUAN H, et al. Activation of type 2 cannabinoid receptor (CB2R) by
525 selective agonists regulates the deposition and remodelling of the extracellular matrix [J].
526 *Biomed Pharmacother*, 2017, 95: 1704-1709.

527 [29] LIU A P, YUAN Q H, ZHANG B, et al. Cannabinoid receptor 2 activation alleviates septic lung
528 injury by promoting autophagy via inhibition of inflammatory mediator release [J]. *Cell Signal*,

2020, 69: 109556.

[30] LIU T, GU J, YUAN Y, et al. Discovery of a pyrano[2,3-b]pyridine derivative YX-2102 as a cannabinoid receptor 2 agonist for alleviating lung fibrosis [J]. J Transl Med, 2022, 20(1): 565.

Figure captions

Figure 1. The flowchart of high-throughput screening by AlphaFold 3. There are four steps for the high-throughput in the present work, including the environment setup, database preparation, input file design and the screening.

Figure 2. Results of AI Virtual Screening. **A.** Chemical structures of the top six candidate compounds identified from high-throughput virtual screening using AlphaFold 3 with CB2R receptor structures. Among these, COCA (N-(3-chloro-2-methylphenyl)-5-(4-hydroxyphenyl)-1,2-oxazole-3-carboxamide) exhibited the most favorable predicted binding affinity and was selected

for further analysis. **B.** The three-dimensional structure of the CB2 receptor (CB2R), highlighting the orthosteric ligand-binding pocket as the primary target site for docking. **C.** Predicted binding pose of COCA within the orthosteric site of CB2R, as determined through molecular docking. COCA is shown embedded in the binding cavity, forming multiple stabilizing interactions with surrounding amino acid residues. Key contacts include π - π stacking interactions with PHE94 and TRP194, and hydrophobic interactions involving PHE87, PHE91, ILE110, VAL113, THR111, PHE106, PHE183, PRO184, ILE186, and TYR190. The docking score of -6.772 kcal/mol indicates a high predicted affinity, supporting COCA's potential as a lead compound for further pharmacological evaluation.

Figure 3 Weight change curve(left) and Lung Coefficient(right) of mice in each group ($^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$)

Figure 4 HE staining and Masson staining of lung tissues of mice in each group (200 $\times$ & 1250 $\times$) the Control group had normal alveolar structure with no inflammation on day 7. The BLM group showed severe damage, including disorganized alveoli, thickened septa, and inflammation. The treatment groups had milder changes, with less inflammation and more uniform tissue structure. Masson's staining showed high collagen levels, structural damage, and fibroblast growth in the BLM group—signs of advanced fibrosis. COCA and pirfenidone reduced collagen buildup and structural damage. The COCA HD group showed the best improvement, with clearer lung structure and less collagen.

Figure 5 Cell count map of mouse BALF (left) and Expression of TNF- α (middle)、IL-6 (right) in lung tissues ($^{NS}P > 0.05$, $^{**}P < 0.01$)

Figure 6 Expression of Col- I and Col-III proteins in lung tissues of mice in each group ($\times 40$) **A.** Immunofluorescence analysis showed that green fluorescence corresponds to Collagen Type I (Col-I) and red fluorescence to Collagen Type III (Col-III). The BLM group had higher Col-I and Col-III fluorescence intensity in lung tissues than the Control group, indicating increased expression. In contrast, the COCA LD and COCA HD groups showed lower fluorescence and reduced collagen expression compared to the BLM group. **B.** Quantitative results confirmed that Col-I and Col-III levels were significantly higher in the BLM group ($P < 0.001$). COCA and pirfenidone treatment significantly reduced these levels ($P < 0.01$), with no significant difference between the COCA and PF groups ($P > 0.05$).

Figure 7 Representative Western blots and corresponding densitometric analyses of CB2R, Nrf2 and Smad7 in lungs from control (n=10) and COCA-treated IPF mice (n=10) ($^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$)



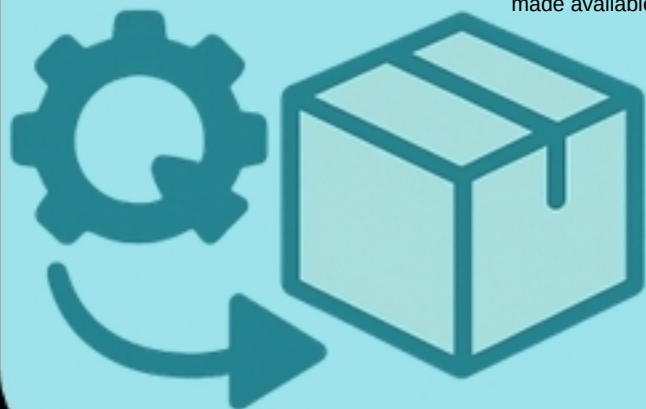
AlphaFold 3 Setup

- Download AlphaFold 3
- NVIDIA A100 GPU
- Ubuntu 22.04 LTS



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Database Preparation



- PDB mmCIF
- UniRef90 & Uniprot
- BFD, Mgnify, Rfam, etc.



Input File Design

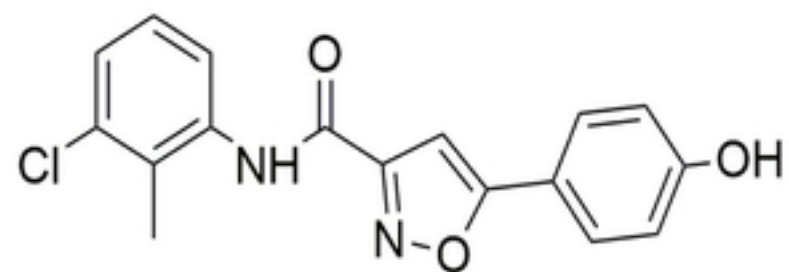
- Define JSON file
- Protein identifier
- Ligand by CCD or SMILES



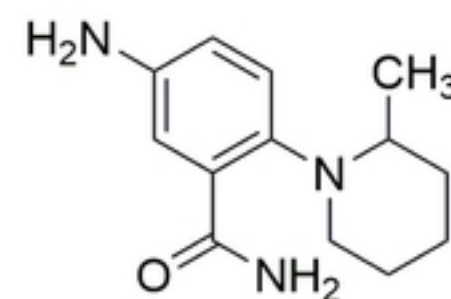
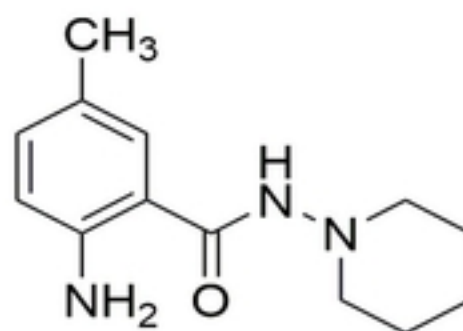
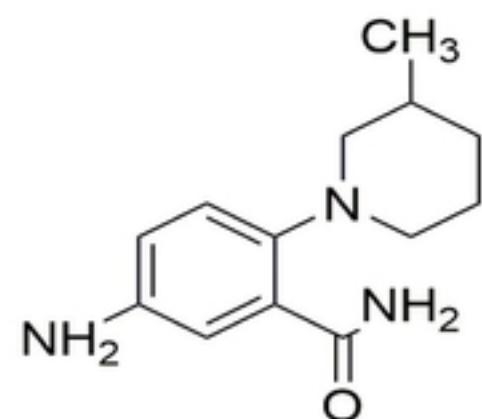
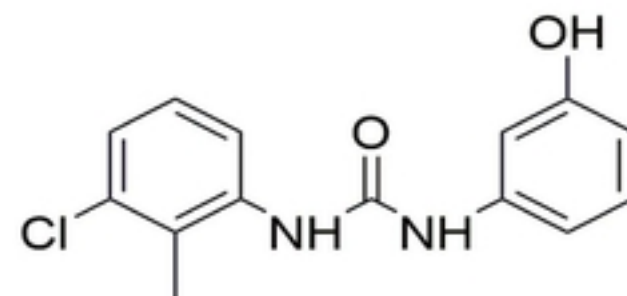
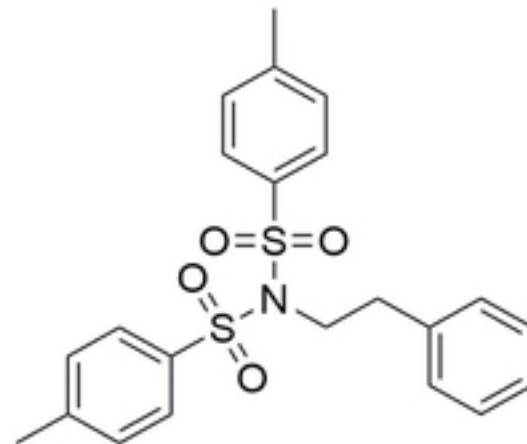
High-Throughput Screening

- Screening against library
- **Select the top candidates**

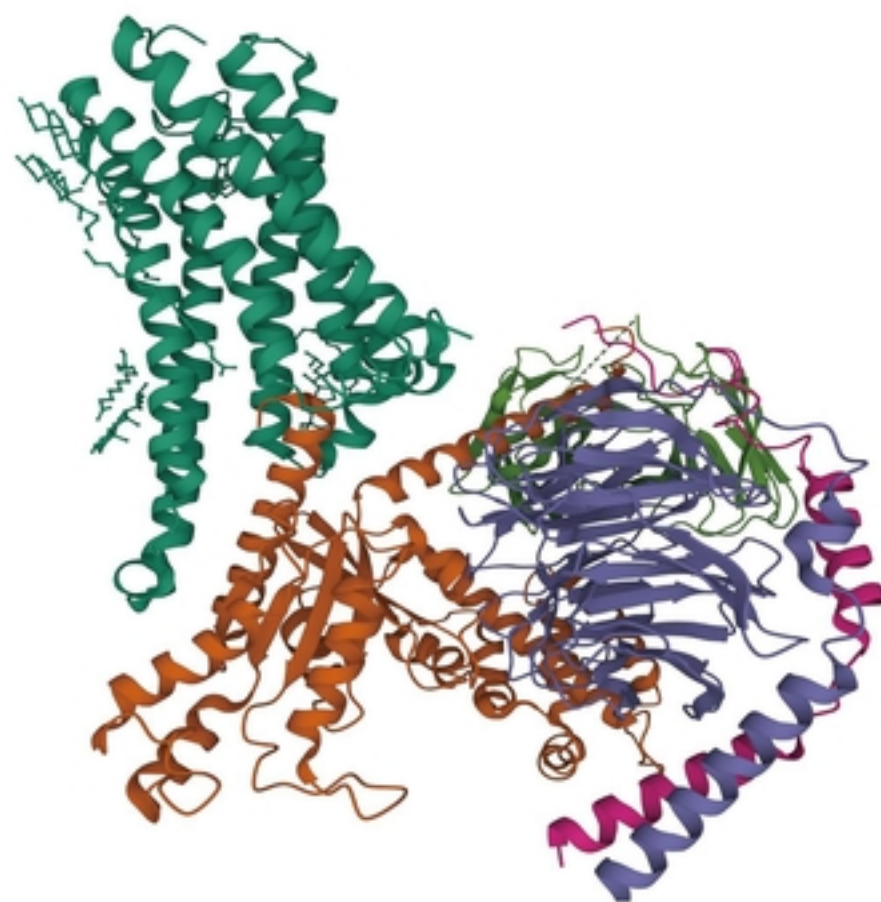
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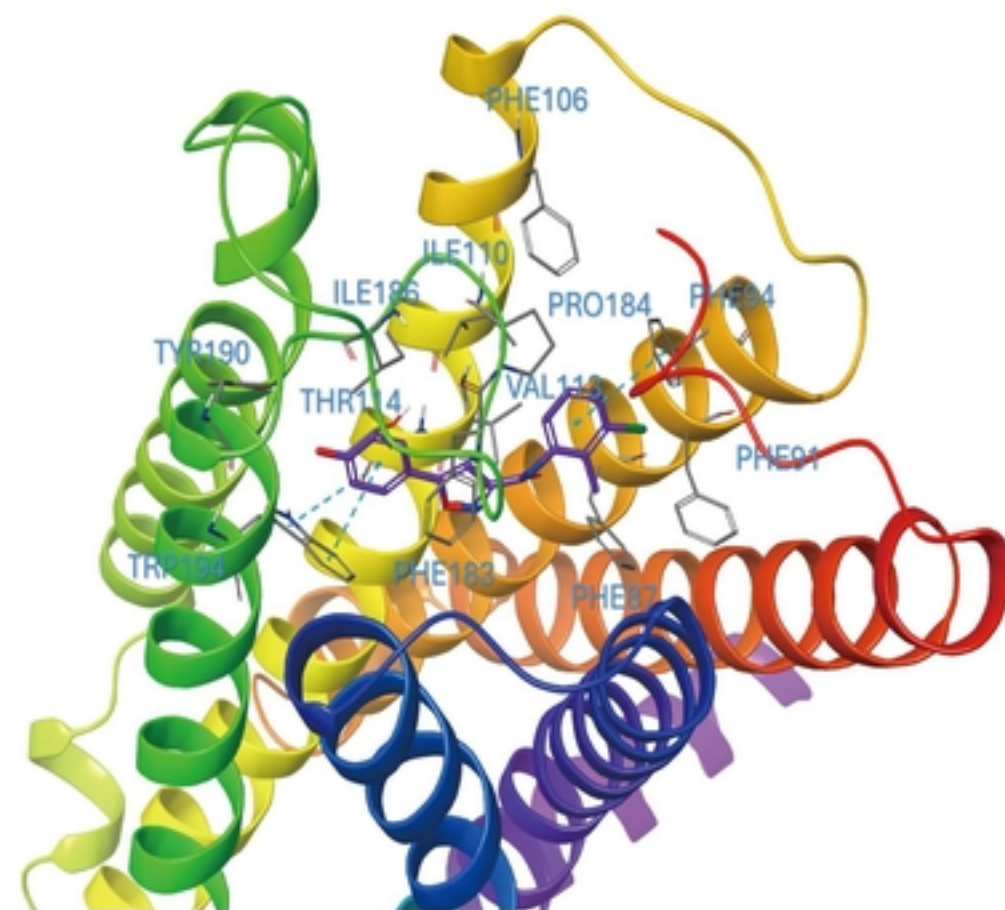
COCA (N-(3-chloro-2-methylphenyl)-5-(4-hydroxyphenyl)-1,2-oxazole-3-carboxamide)



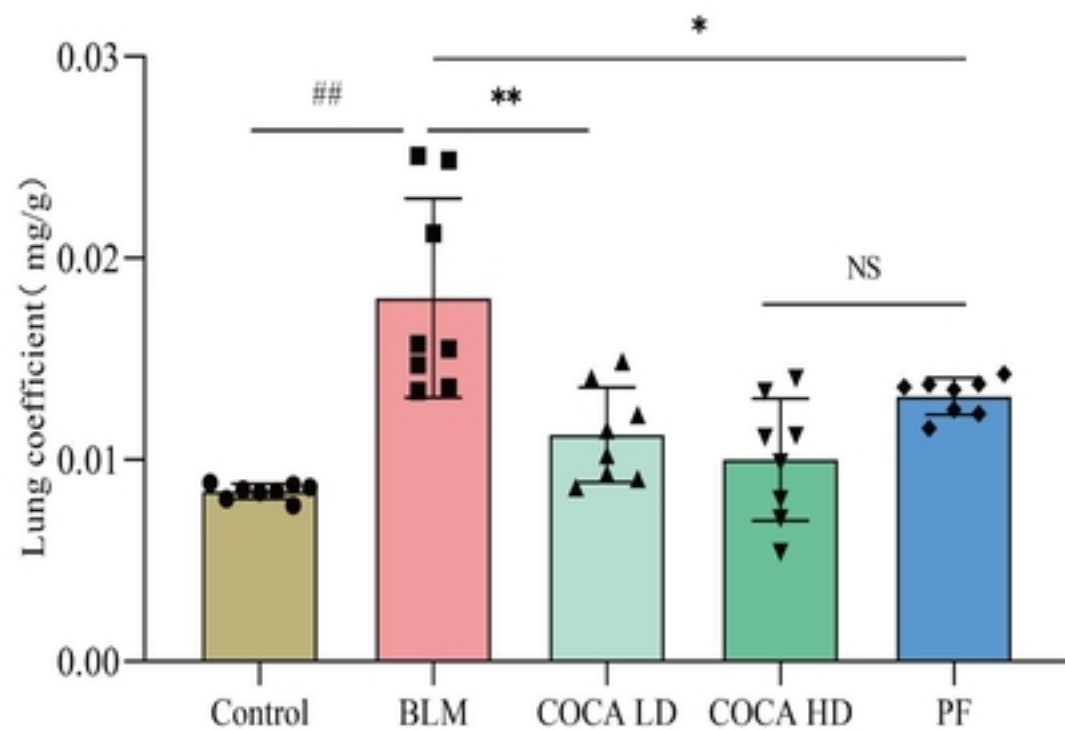
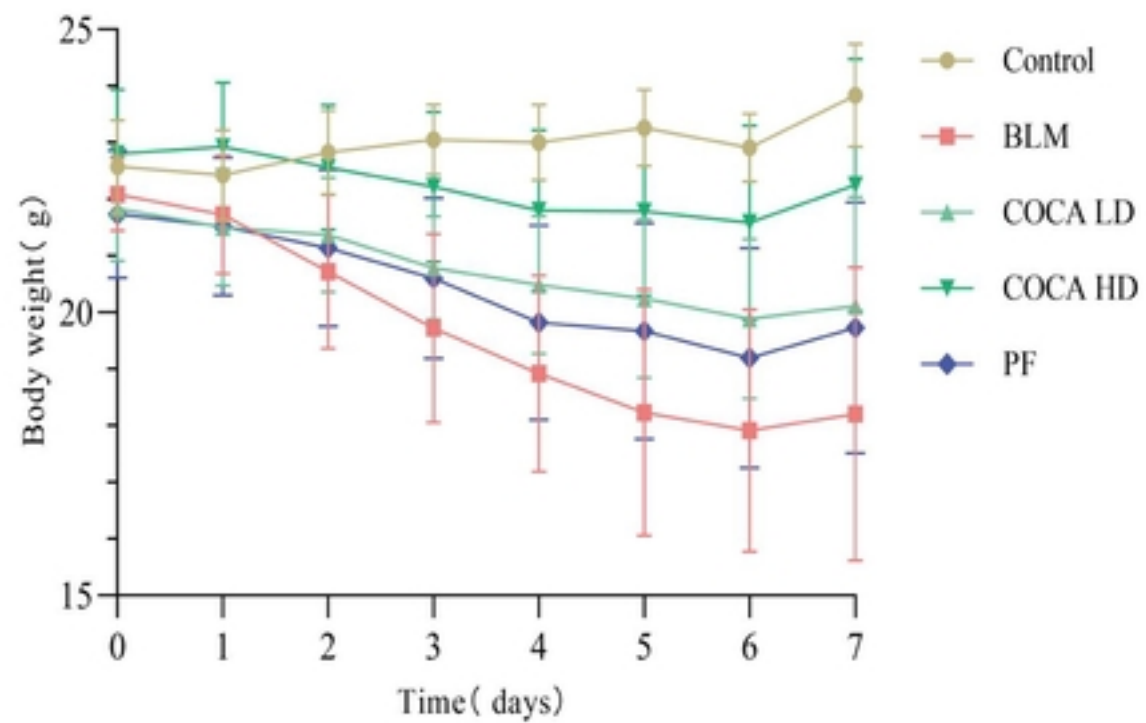
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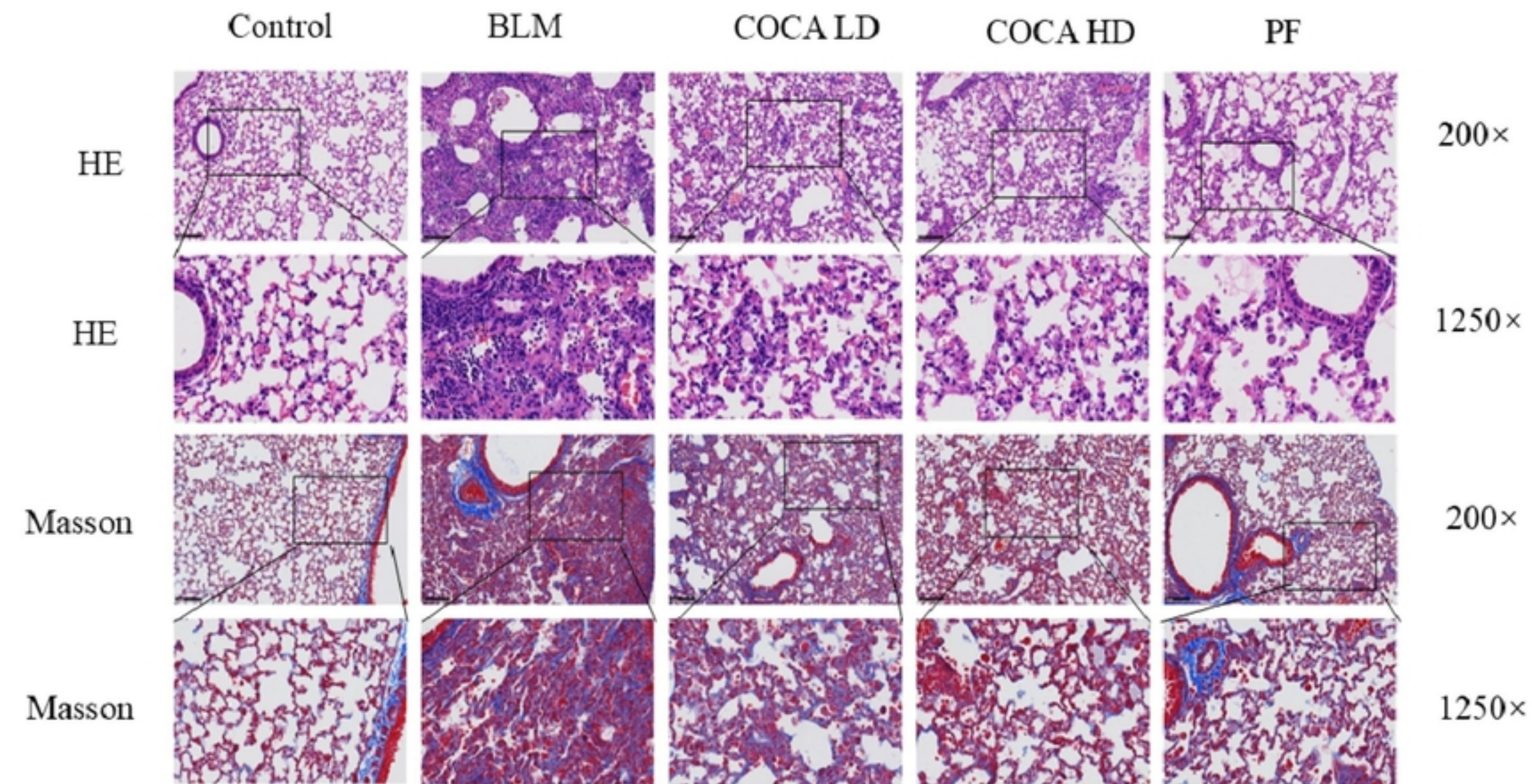
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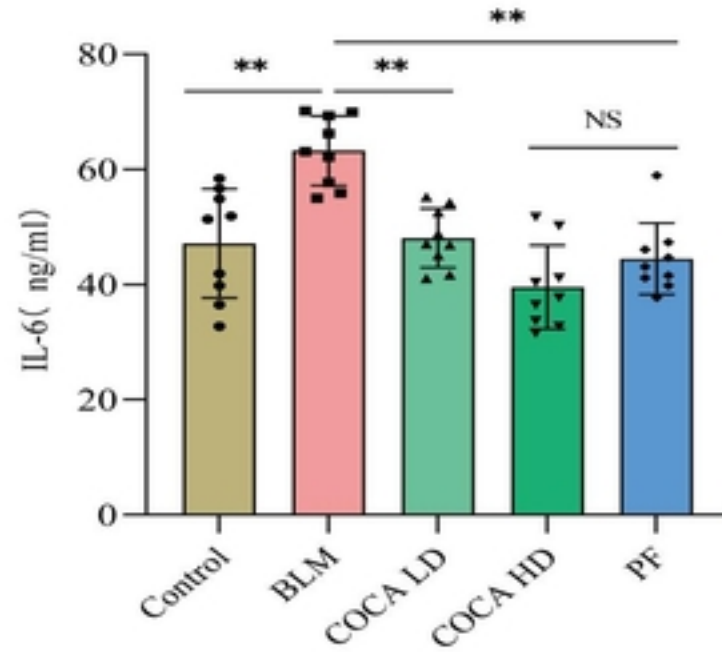
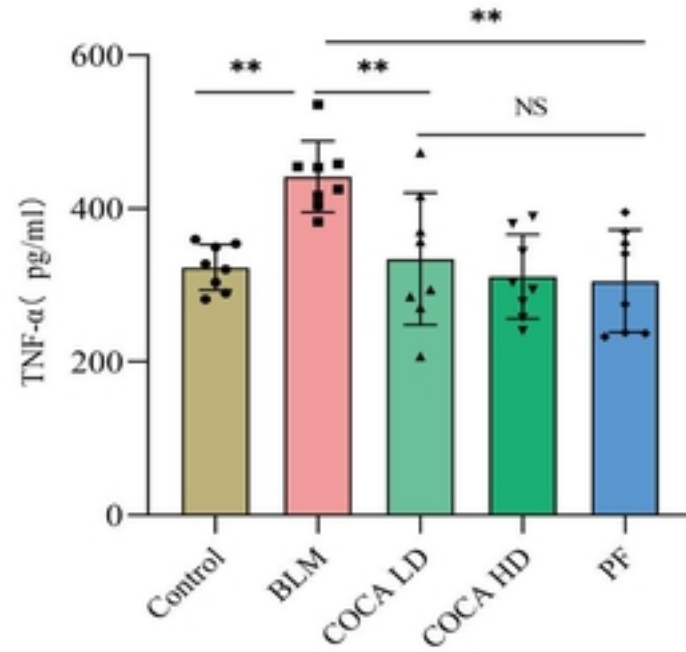
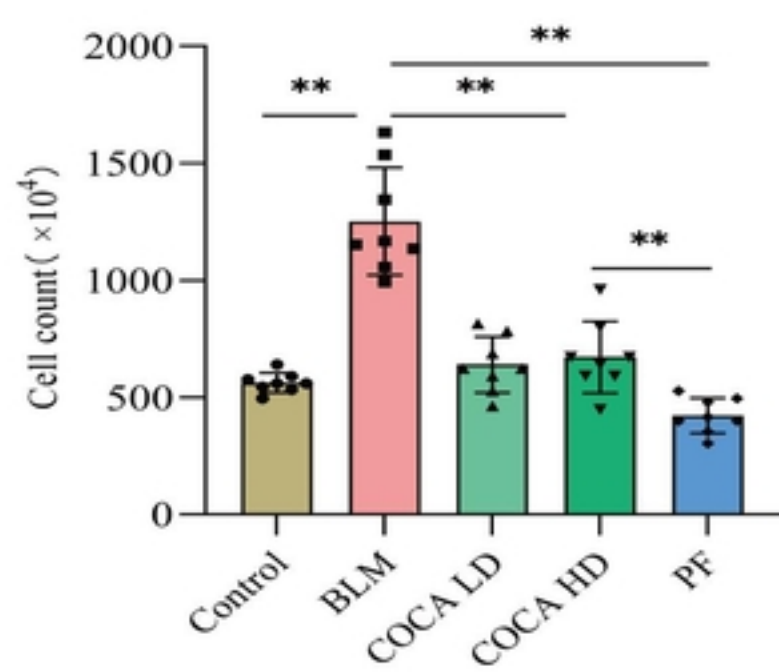
Figure



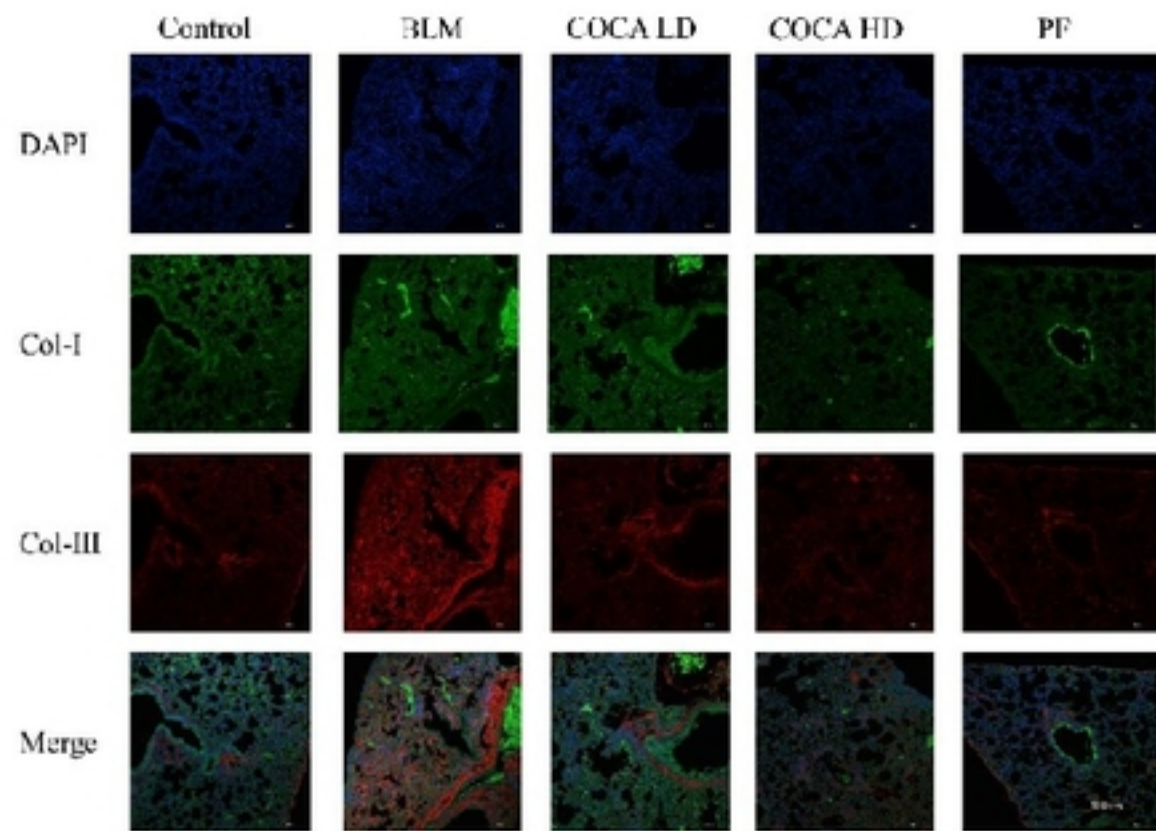
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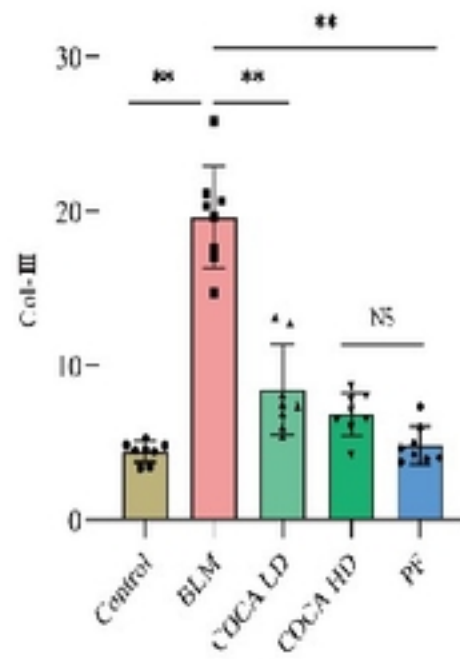
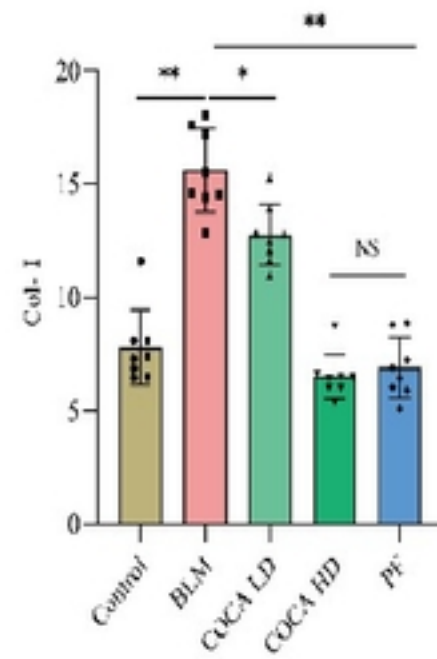
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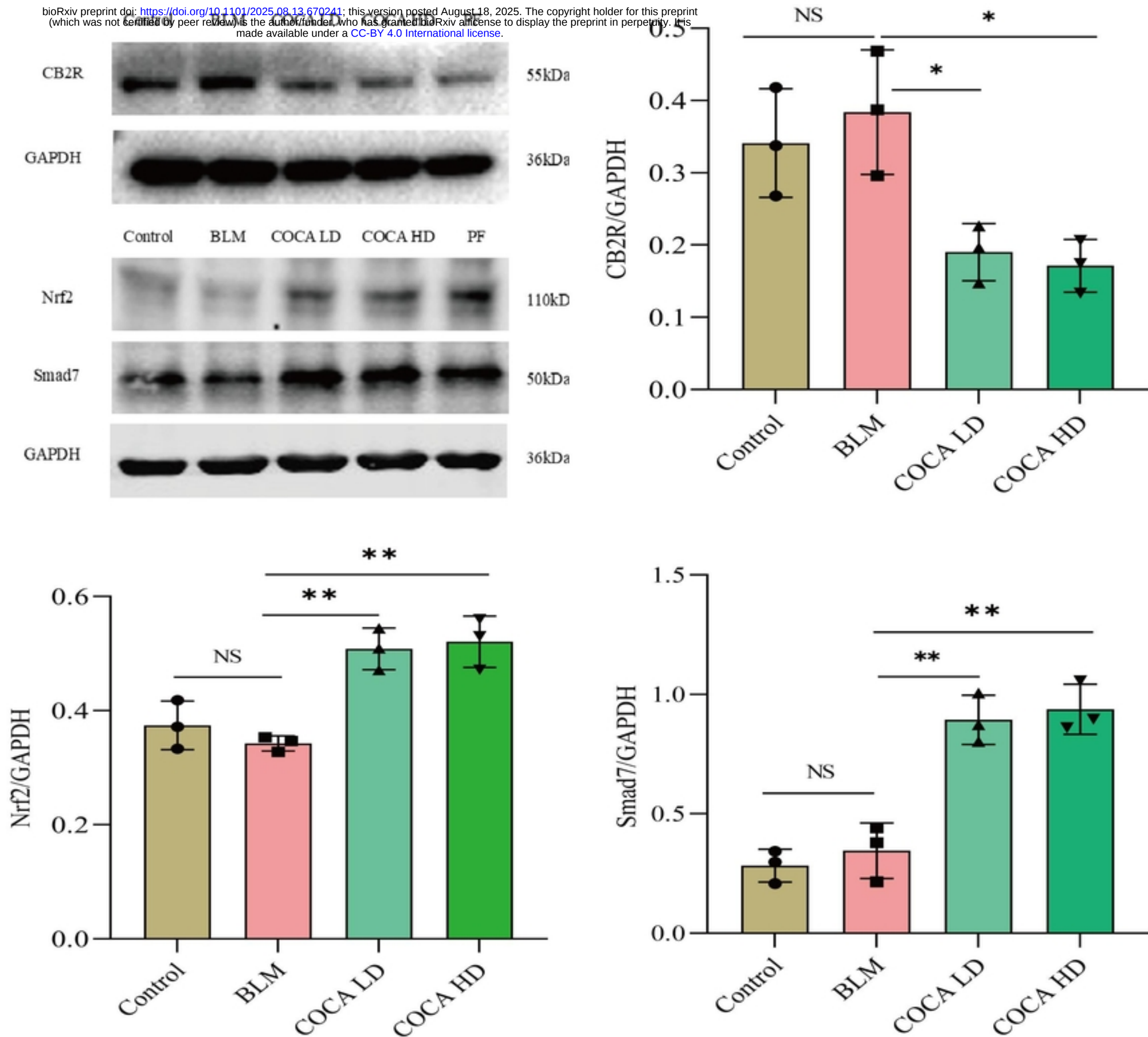


A



B

Figure



Figure