

Naftidrofuryl alleviates LPS-mediated inflammatory-oxidative and fibrotic successions in a rat model of acute lung injury: An emerging mechanistic role of PPAR α /SIRT1/ATF3 axis

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ABSTRACT

Objective(s): Platelet-released serotonin (5-HT) is linked to lung inflammatory injury. Simultaneously, PPAR α activation increases SIRT1 activity, thereby regulating inflammatory damage in acute lung injury (ALI). Also, ATF3 can prevent ALI-related inflammation and oxidative injury. However, the relationship between 5-HT and PPAR α /SIRT1/ATF3 in ALI remains ambiguous. Hence, exploring the impact of naftidrofuryl (NAFT), a 5-HT_{2A} antagonist, on LPS-induced ALI in rats, particularly in combination with dexamethasone (DEXA), may be valuable, given the involvement of the PPAR α /SIRT1/ATF3 axis. Our investigation identified targets and pathways associated with the therapeutic benefits of NAFT using network pharmacology and molecular docking.

Materials and Methods: For *in vivo* validation, 24 rats were allocated to 8 groups. Lung W/D ratio and MPO activity were quantified. The BALF HO-1, MDA, TAC, PPAR α , SIRT1, ATF3, TNF- α , IL-6, and IL-1 β levels were analyzed. Further, expressions of JAK2, STAT3, HMGB1, AMPK, Nrf2, and MyD88 were assessed. Lung tissues were examined histologically and immunohistochemically to determine NF- κ B, caspase-3, and TGF- β 1 expression.

Results: Network pharmacology disclosed 176 intersecting targets between "NAFT-ALI" and "DEXA-ALI" with various KEGG pathways. Docking confirmed a strong affinity between NAFT and SIRT1, HMGB1, AMPK, and ATF3. Experimentally, NAFT inhibited JAK2/STAT3 and elevated PPAR α levels. PPAR α lowered TGF β 1-promoted fibroblastic proliferation and stimulated SIRT1-mediated HMGB1 deacetylation. Hence, HMGB1 repression prevented caspase-3-provoked apoptosis and enhanced the antioxidant role of AMPK/Nrf2/HO-1. Jointly, AMPK released ATF3, which consequently stopped TLR4/MyD88/NF- κ B-associated inflammatory cascade.

Conclusion: NAFT may alleviate inflammation, oxidative stress, apoptosis, and fibrosis in ALI by activating the PPAR α /SIRT1/ATF3 pathway. Moreover, NAFT may enhance DEXA treatment, particularly in resistant ALI patients.

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Introduction

Acute lung injury (ALI) is a deadly respiratory illness that happens when the endothelium and epithelial barriers are compromised, resulting in fluid and cellular exudation within the lungs. With a high incidence rate all over the world, ALI is significantly affecting public health. Despite advances in understanding ALI pathophysiology, more research is still required to identify prompt, targeted therapies that will minimize further complications and, ultimately, reduce its morbidity and mortality (1).

Medical research has demonstrated that the presence of pathogenic microbial structures, such as lipopolysaccharide (LPS), is frequently associated with ALI pathogenesis (2). As a "PAMP", LPS stimulates the secretion of crucial proinflammatory cytokines by initiating the TLR4-invoked

NF- κ B cascade (3). NF- κ B then triggers the release of TNF- α , IFN- γ , GM-CSF, and IL-6 by alveolar macrophages (AMs), promoting neutrophil and monocyte recruitment, activation, and infiltration into the lung parenchyma (4).

Remarkably, ATF3, a transcriptional repressor, can directly interact with p65 and thereby negatively regulate NF- κ B activity (5). In parallel, SIRT1, a histone deacetylase (HDAC), has been reported to mitigate LPS-induced lung injury. A large number of important regulatory proteins and transcription factors involved in DNA repair, inflammation, antioxidant responses, and cellular aging are deacetylated and down-regulated by SIRT1 (6). Surprisingly, boosting PPAR α has been found to improve SIRT1 expression and activity. Hence, regulating the PPAR α /SIRT1/ATF3 axis may be a recommended target to lessen the airway inflammatory-

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oxidative reactions associated with lung injury (7).

Dexamethasone (DEXA) has been universally reported to reduce cytokine production, pulmonary edema, and alveolar cell damage, thereby considerably enhancing the function of the alveolar epithelium-endothelial barrier (8)2022, including PUBMED, EMBASE, Cochrane Library, Wangfang, VIP, and CNKI. Other searches were also checked for dissertations/theses and the reference lists of the included studies. Two team members examined all citations and selected eligible articles. Randomized controlled trials (RCTs). Nevertheless, certain patients demonstrate resistance to DEXA, leading to unsatisfactory therapy outcomes (9). As a glucocorticoid, DEXA also elevates blood glucose levels and promotes insulin resistance. Both effects have been identified as contributing factors to lung damage (10). Therefore, DEXA itself may adversely affect the lungs and impair their physiological functions.

Intriguingly, during inflammation, platelets release serotonin (5-HT), which activates the vessel walls and promotes leukocyte adhesion and recruitment (11). Although 5-HT functions as a neurotransmitter and hormone, either centrally or peripherally, it is also linked to chronic lung diseases. In lung tissues, 5-HT induces oxidative stress, inflammation, protein and cell exudation, and up-regulation of fibrosis-related genes (12). Moreover, 5-HT_{2A} activation has been observed to increase dopamine and noradrenaline levels, thereby enhancing sugar release (13).

Thus, the use of 5-HT₂ antagonists, particularly those targeting 5-HT_{2A}, may improve airway remodeling and function, nominating them as a promising therapeutic strategy for ALI (14). Naftidrofuryl (NAFT), a vasodilator utilized for peripheral and cerebral vascular disorders, has been documented to selectively antagonize 5-HT₂ and specifically function as an inverse agonist of the 5-HT_{2A} receptor (15). Consequently, evaluating the therapeutic potential of NAFT, particularly in conjunction with DEXA against LPS-induced ALI, may be beneficial, considering its role in modulating the PPAR α /SIRT1/ATF3 pathway to alleviate lung injury.

However, recent related studies have shown that NAFT can mitigate LPS-induced pulmonary injury through macrophage polarization and NLRP3/TLR4 regulation, as well as through additional inflammatory pathways in an LPS-induced lung injury model (16, 17). The novelty of the present manuscript is that it extends this evidence by integrating network pharmacology, molecular docking, and *in vivo* validation to define a 5-HT_{2A}-linked PPAR α /SIRT1/ATF3 mechanism and by testing NAFT as an adjuvant to DEXA. Accordingly, the study does not merely replicate anti-inflammatory or macrophage-polarization findings; it proposes a mechanistic bridge among serotonergic blockade, PPAR α /SIRT1/ATF3 activation, HMGB1/AMPK/Nrf2 signaling, and potential improvement in DEXA responsiveness in ALI.

Materials and Methods

Network analysis

Finding the molecular targets attributed to naftidrofuryl

The SMILES format of the NAFT chemical structure was acquired from the PubChem database (Supplementary Table S1)(<https://pubchem.ncbi.nlm.nih.gov/compound/Naftidrofuryl>, accessed on 15 June 2024). The following five

datasets were analyzed to identify the targets for NAFT (Supplementary Table S2): (1) SwissTargetPrediction database (http://www.swisstargetprediction.ch/result.php?job=2130628990&organism=Homo_sapiens, accessed on 15 June 2024); (2) SuperPred database (https://prediction.charite.de/subpages/target_result.php, accessed on 15 June 2024); (3) Similarity ensemble approach (SEA, https://sea.bkslab.org/jobs/search_6e36a550-b077-4b22-b780-3d5c2037c152, accessed on 15 June 2024); (4) Comparative Toxicogenomic Database (CTD, <https://ctdbase.org/detail.go?type=chem&acc=D009257&view=ixn>, accessed on 15 June 2024); and (5) The TargetNet database (http://targetnet.scbdd.com/calcnnet/calc_text/, accessed on 15 June 2024). For additional investigation, the targets obtained from the five databases were transformed into standard gene symbols in accordance with the UniProt database (<https://www.uniprot.org/>, accessed on 15 June 2024). The UniProt IDs have been substituted with gene names utilizing the SynGO knowledge base (<https://www.synportal.org/convert>, accessed on 15 June 2024). Finally, the Jvenn tool (<https://jvenn.toulouse.inrae.fr/np/example.html>, accessed on 15 June 2024) was employed to exclude any duplicate targets from the last set of targets.

Obtaining the targets related to dexamethasone

The chemical structure of DEXA was acquired in SMILES format from the PubChem database (Supplementary Table S1)(<https://pubchem.ncbi.nlm.nih.gov/compound/Dexamethasone>, accessed on 15 June 2024). To determine the DEXA targets, the following five datasets were examined (Supplementary Table S3): (1) SEA (https://sea.bkslab.org/jobs/search_9fea80ad-1626-4273-ae57-5c45e39ee5b2, accessed on 15 June 2024); (2) SuperPred database (https://prediction.charite.de/subpages/target_result.php, accessed on 15 June 2024); (3) DrugBank (<https://go.drugbank.com/drugs/DB01234>, accessed on 15 June 2024); (4) CTD (<https://ctdbase.org/detail.go?type=chem&acc=D003907&view=gene>, accessed on 15 June 2024); and (5) TargetNet database (http://targetnet.scbdd.com/calcnnet/calc_text/, accessed on 15 June 2024). In order to facilitate further research, the targets from the five databases were converted into UniProt-valid gene symbols. The SynGO database was used to replace the UniProt IDs with gene names. Lastly, any recurring targets were eliminated from the final list using the Jvenn tool.

Determination of targets linked to acute lung injury

Using the keyword "Acute lung injury" (ALI), the molecular targets associated with the target illnesses were found in the following five databases (Supplementary Table S5): (1) GeneCards database (<https://www.genecards.org/Search/Keyword?queryString=ACUTE%20LUNG%20INJURY>, accessed on 15 June 2024); (2) Therapeutic Target Database (TTD, https://idrlab.net/ttd/search/ttd/target?search_api_fulltext=+ACUTE+LUNG+INJURY, accessed on 15 June 2024); (3) The Open Targets Platform database, (https://platform.opentargets.org/disease/EFO_0004610/associations?table=associations, accessed on 15 June 2024); (4) CTD, (<https://ctdbase.org/detail.go?type=disease&acc=MESH%3AD055371&view=gene>, accessed on 15 June 2024); and (5) MalaCards database (https://www.malacards.org/card/acute_lung_injury#Genes_Related_wrapper, accessed on 15 June 2024).

The five databases make it possible to find targets that are related to diseases. Using information from the UniProt database, all targets were changed to their Universal Gene Symbol, and then any duplicate targets were removed.

Establishing disease-drug common targets, protein-protein interactions, and drug-target networks

The sets of targets were put into the Jvenn tool, which made a “Venn diagram” to display the overlapped targets between NAFT and ALI, DEXA and ALI, as well as NAFT-ALI and DEXA-ALI. For creating the protein-protein interaction (PPI) network, intersected targets were added to the “STRING database” Version 12.0 with a degree of confidence >0.4 (<https://string-db.org/>, accessed on 20 June 2024). The chosen organism for this investigation was Homo sapiens. Multiple merging targets were linked to ALI through a compound-target network. Network visualization was done in this study using “Cytoscape 3.10.2”, a program created by the “National Institute of General Medical Sciences (NIGMS)” in the United States. Using the “Cytoscape plugin CytoNCA”, the common overlapping targets were filtered according to the degree value and then ranked by Betweenness and Closeness indices. The hub targets were identified based on the ten highest-ranking proteins.

Functional enrichment analysis

Enrichments of pathways in the “Kyoto Encyclopedia of Genes and Genomes (KEGG)” and “Gene Ontology (GO)” were completed using the “ShinyGO program” (<http://bioinformatics.sdstate.edu/go/#tab-6415-3>, accessed on 20 June 2024). The cutoff value for the *P*-value was established at $P < 0.05$, regarded as a critical threshold. Consequently, the findings were illustrated using an enrichment bubble map for “Gene Ontology (GO)” and “Sankey and dot diagrams” to depict KEGG pathway analysis sourced from the “SRplot website” (<https://www.bioinformatics.com.cn/en?keywords=ENRICHMENT>, accessed on 20 June 2024).

Molecular docking

As cited by Torres *et al.* (2019), the elaborate methodology for molecular docking was followed accurately (18). The SDF file of the NAFT 3D chemical structural model was transformed into a PDB format using the “Open Babel GUI tool” (version 2.4.1). The NAFT SDF file was obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>, accessed on 25 June 2024). The three-dimensional (3D) structures of three target proteins, namely SIRT1 (PDB ID: 8anb), HMGB1 (PDB ID: 8i9m), and AMPK (PRKAA1; PDB ID: 7jjj), were obtained from the “Protein Data Bank (PDB) of the Research Collaboratory for Structural Bioinformatics (RCSB)” (<https://www.rcsb.org/>, accessed on 25 June 2024), in addition to the PDB file of ATF3 3D structure (AF-Q5VTZ4-F1-v4) obtained from the “AlphaFold Protein Structure Database” (<https://alphafold.ebi.ac.uk/entry/Q5VTZ4>, accessed on 25 June 2024).

All targets were initially processed for the docking process using the “ChimeraX 1.8 software”. Hence, for the computational docking, both the NAFT molecule and target files were separately loaded in the “PyRx 0.8 software”. Further, the optimum combination modes and the binding energies were determined. The measurement unit was kcal/mol, and the interaction strengths were sorted by binding affinity greater than -7 or less than -5 kcal/mol. Lastly, the

“BIOVIA Discovery Studio 2024 Client software” program was implemented to reveal and study the 3D and 2D force spatial environment images.

Chemicals and kits

Lipopolysaccharides from Escherichia coli (LPS; 297-473-0; L2880) and a colorimetric kit for MPO (MAK068-1KT) were received from “Sigma-Aldrich Co. Inc., St. Louis, MO, USA”. Calypsol[®] vial and Xyla-Ject[®] injectable solution were obtained from “Gedeon Richter Plc., Budapest, Hungary, and Adwia Pharmaceuticals Co., Egypt”, respectively. MDA (MD 2529) and TAC (TA 2513) spectrophotometric assay kits were obtained from “Bio-Diagnostics Co., Dokki, Giza, Egypt”. Rat ELISA kits for HO-1 (SEA584Ra), ATF3 (SEC303Ra), and TLR4 (SEA753Ra) from “Cloud-Clone Corp Co., Houston, USA”; for SIRT1 (E1145Ra), TNF α (E0764Ra), IL-6 (E0135Ra), and IL-1 β (E0119Ra) from “Bioassay Technology Lab, Shanghai, China”; as well as for PPAR- α (CSB-E09755r) from “Cusabio Co., Houston, USA” were purchased. In addition, the protein assay kit of Pierce[™] bicinchoninic acid (BCA) was purchased from “Thermo Fisher Scientific, Inc., USA”. Rabbit monoclonal anti-AMPK antibody (#5831) and phospho-AMPK antibody (Thr172) (anti-p-AMPK) (#2535) were supplied by “Cell Signaling Technology, Beverly, MA, USA”. As well, rabbit polyclonal anti-HMGB1 antibody (AF7020), anti-Nrf2 antibody (AF0639), and anti- β -Actin antibody (AF7018) were from “Affinity Biosciences LTD., USA”, while that of anti-MyD88 antibody (ab131071) was provided by “Abcam, Cambridge, UK”. Other analytical-grade compounds were available for commercial purchase or as specified.

Drugs

A commercial NAFT (Praxilene[®] 200 mg tablets) was obtained from the Minapharm company for pharmaceuticals & chemical industries as an oral film-coated tablet, under license of Merck Santé, France. Based on the drug label, each tablet contained naftidrofuryl hydrogen oxalate corresponding to 162 mg NAFT. The tablet contained a list of excipients including liquefied, weakly cationic acrylate and methacrylate copolymer; glycerol behenate; lactose; talc; magnesium stearate; aqueous suspension of neutral copolymerization of polymethacrylate acid esters; Hypromellose; as well as macrogol 4000. An injectable form of the commercial dexamethasone (Dexamethasone Sodium Phosphate[®] 2 mg sterile ampoules) was obtained from the Egyptian International Pharmaceutical Industries Company (EIPICO), Egypt. Each ampoule contained 4 mg DEXA (as disodium salt)/ml, according to the drug label.

Drug preparation

NAFT suspension was prepared daily by crushing one tablet containing 162 mg NAFT and suspending it in 5 ml of normal saline (NS) at 25 °C to produce a concentration of 32.4 mg NAFT/ml (19). The dose volume of NAFT was 1.5 ml/kg/day, corresponding to the NAFT dose (50 mg/kg/day) used effectively for 21 days to alleviate acute ischemia-reperfusion injury in rats (20). For DEXA, the present investigation administered 5 mg DEXA/kg/day, which was found to ameliorate LPS-induced lung injury after 28 days (21, 22).

Ketamine/xylazine solution preparation

To prepare the anesthetic solution of ketamine (12.5 mg/

ml)/xylazine (0.83 mg/ml), 3 ml of ketamine was added to 0.5 ml of xylazine and then diluted with 8.5 ml of NS (23). Therefore, the standard target dosages administered intraperitoneally (IP) were 100 mg Ketamine/kg and 6.7 mg Xylazine/kg.

Animals

Adult white male Wistar rats weighing 200–220 g, free of specific pathogens, were obtained and housed in plastic cages under standard laboratory conditions 10 days before the experiment. The animals were kept on a 12-hr day/night photoperiod with a standard diet and water *ad libitum*, based on the “Institutional Animal Care and Use Committee (IACUC)”, Faculty of Veterinary Medicine, University of Sadat City, Egypt (Ethical approval number: VUSC-001-1-23).

Experimental design and intervention

There were 64 rats, randomly split into 8 primary groups of 8 each. All rats received one dose (20 μ g lipopolysaccharide (LPS)/rat) intranasally (i.n.) dissolved in 50 μ L NS for ALI induction, except for those in the vehicle (V) and Naftidrofuryl (NAFT) groups, which received 50 μ L NS/rat (i.n.) instead (22). After 5 min, all medications were administered via intraperitoneal injection (IP) once daily for a duration of 28 days, as follows: (I) V group got 1.5 ml NS/kg/day; (II) NAFT group received 50 mg NAFT/kg/day; (III) Acute lung injury (ALI) group got 1.5 ml NS/kg/day; (IV) Dexamethasone (DEXA)-treated group received 5 mg DEXA/kg/day; (V) NAFT 25-treated group got 25 mg NAFT/kg/day; (VI) NAFT 50-treated group received 50 mg NAFT/kg/day; (VII) NAFT 25+DEXA group got 25 mg NAFT and 5 mg DEXA with 2 hr in-between; and (VIII) NAFT 50+DEXA group got 50 mg NAFT and 5 mg DEXA with 2 hr in-between (Figure 1).

After twenty-four hours, 4 ml of ketamine/xylazine anesthetic solution/kg was injected IP into each rat. An EDTA tube with blood samples collected by heart puncture was processed to count white blood cells (WBCs) through

a “hematology analyzer, model D6500, Diatek, India”. Then, sterile scissors were used to open the rat’s chest, and the lungs of 4 rats from each group were completely isolated to determine the lung wet-to-dry weight ratio. The bronchoalveolar lavage fluid (BALF) was taken from the left lung of the remaining rats, while the right lung was meticulously dissected and set aside using forceps. Following dissection, lung tissue samples were partitioned into two distinct parts. One portion was stored until used for assaying MPO activity, gene expression, and immunoblotting, while the other portion was evaluated for histopathologic analysis.

Consequently, sample size was determined based on the availability of tissues and assay-specific requirements. For conventional biochemical assays and quantitative immunohistochemistry, four independent animals ($n=4$) were selected for each group to minimize the inter-animal variation. For RT-qPCR and Western blot, at least 3 animals ($n=3$) were used in each group to assess biological variability and reproducibility, in accordance with methodological recommendations (24, 25).

Wet-to-dry weight ratio of the lung

Lung injury and pulmonary permeability were assessed using the lung wet-to-dry weight ratio as an indicator of pulmonary edema development. After being removed, the wet weight of lung tissues was measured using an analytical balance, and then baked for 48 hr at 60 °C in an oven. Then, the portion of dried lungs was reweighed (dry weight). Calculations were made to determine the lung weight before and after drying (26).

MPO activity assay

After homogenizing the lung tissues with the MPO assay buffer (using a tissue homogenizer), the homogenates underwent centrifugation at 13,000 $\times$ g for 10 min at 4 °C using a “Centurion centrifuge”. Then, MPO activity (as nmole/min/mg tissue) was quantified in the supernatant following the manufacturer’s guidelines.

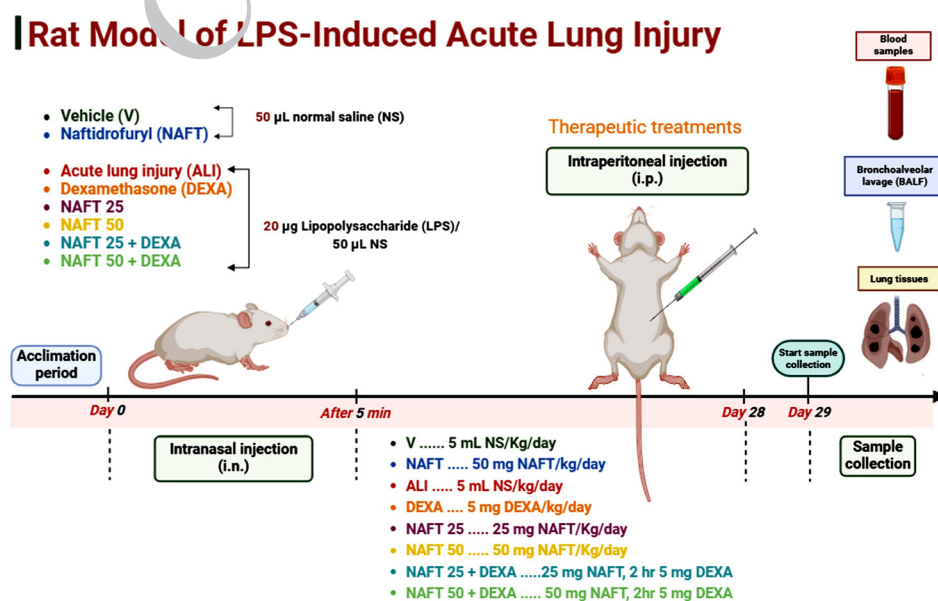


Figure 1. Schematic diagram of experimental study

ALI, Acute lung injury; BALF, Bronchoalveolar lavage; DEXA, Dexamethasone; LPS, Lipopolysaccharides; NAFT, Naftidrofuryl; NS, Normal saline; and V, Vehicle

Bronchoalveolar lavage fluid samples collection

After ligating the right main bronchus with 1-0 silk suture, all of the right lungs were taken out and kept separate. Next, all left lungs were washed out three to five times with 30 ml of sterile saline/kg (27). After that, the bronchoalveolar lavage fluid (BALF) was collected, centrifuged at 12,000 $\times$ g for 30 min, and the resulting supernatant was stored frozen at -20 °C until needed.

Gene expression of JAK2 and STAT3

Gene expression was studied using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The TRIzol[®] reagent was used to extract all ribonucleic acid (RNA) from the lung tissue. Then, complementary deoxyribonucleic acid (cDNA) was created by reverse transcription of the retrieved RNA. The primer sequences for JAK2 (101-bp), STAT3 (137-bp), and GAPDH (118-bp) genes were verified against the rat RefSeq transcripts NM_031514.1, NM_012747.2, and NM_017008.4, respectively, and displayed in Table 1. Afterward, the RT-qPCR procedures were completed using “Power SYBR[®] Green PCR Master Mix” and the “7500 Real-Time PCR System from Applied Biosystems, USA”. Thermal cycles lasted 4 min at 95 °C, 40 cycles of 10 sec each, followed by 30 sec at 60 °C, and finally 10 sec at 72 °C. The data were analyzed using the $\Delta\Delta C_t$ technique (28), which demonstrated the relative fold changes in gene expression in controls.

Measurement of lung inflammatory/anti-inflammatory markers

The BALF concentrations of TNF- α , IL-6, and IL-1 β in pg/ml, in addition to those of PPAR α , SIRT1, and ATF3 in ng/ml, were measured based on the manufacturer's ELISA kit instructions.

Analysis of lung oxidant/antioxidant status

Using spectrophotometric assay kits, the levels of MDA in nmol/ml and TAC in μ mol/ml were measured at 534 nm and 505 nm, respectively, in the BALF supernatant (31, 32). According to the manufacturer's instructions, HO-1 was measured in ng/ml using the “enzyme-linked immunosorbent assay (ELISA)” technology.

Western blotting analysis

The pulmonary tissues were quickly chopped and stored on ice to prevent protease breakdown. Total proteins were extracted using the guidelines outlined in the booklet of the total protein extraction kit. Before running on SDS-PAGE gels (4% stacking gel; 12% separation gel), an equal volume of 2X Laemmli buffer was added to 30 μ g of each protein

sample (after determining their concentration using the BCA Protein Assay Kit). Hence, the proteins were moved onto the PVDF membranes, which were then blocked with 5% skim milk in TBS and 0.05% Tween 20. The membranes were then incubated overnight at 4 °C with the primary antibodies, which were diluted 1:1000 and included anti-HMGB1, anti-AMPK, anti-p-AMPK, anti-NRF2, anti-MyD88, and anti- β -actin (as an internal loading control). After three washes with phosphate-buffered saline (PBS), the blotting membranes were incubated for 2 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. Next, the intensities of each target protein band were quantified using ImageJ densitometry software (National Institutes of Health, Bethesda, USA), and relative protein expression levels were determined.

Histopathological investigation

A microtome was used to cut the isolated lung tissue samples into 3 μ m-thick sections. The samples were then fixed in 10% neutral buffered formalin (pH 7.4) for 72 hr, washed, dehydrated, embedded in paraffin wax, and stained with hematoxylin and eosin (H&E)(33). The stained sections were then examined using a Leica microscope (CH9435 Hee56rbrugg). Histopathological quantitative scoring was assessed in lung tissue for epithelial thickness of bronchioles, alveolar thickness, collagen fiber density, inflammatory cell count, and the quantity of congested blood vessels per cross-sectional area using the image analysis system “Leica QWin DW3000, LEICA Imaging Systems Ltd., Cambridge, England”. For each segment in all groups, three typical fields were examined at x200 magnification by light microscopy, with the image reflected onto the screen.

Immunohistochemical detection of TGF- β 1, caspase-3, and NF- κ B

Immunohistochemistry was conducted on paraffin tissue slices fixed on positively charged slides utilizing the “avidin-biotin-peroxidase complex (ABC)” method. Rabbit anti-TGF- β 1 polyclonal antibody (Arigobio, Cat# ARG56429, dilution 1:50), rabbit anti-active caspase-3 monoclonal antibody, Clone [EPR18297], Unconjugated (Abcam Cat# ab184787, dilution 1:1000), and rabbit anti-NF- κ B-p65 polyclonal antibody (Elabscience, Cat# E-AB-32232, dilution 1:100) were tested. Samples from each analyzed group were incubated with the aforementioned antibodies, subsequently followed by the addition of the reagents necessary for the ABC method (Vectastain ABC-HRP kit, Vector Laboratories). Marker expression was assessed using peroxidase to detect the antigen-antibody complex, followed by staining with diaminobenzidine (DAB, Sigma). Non-immune serum was used as a negative control in place of primary or secondary antibodies. Using a Leica microscope with varying magnification powers (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland), immunostained sections were analyzed and recorded.

Quantitative morphometric analysis

Six high-power (x200) fields for each antibody/studied group that showed positive brown immunostaining were selected for examination. Areas displaying positive brown immunostaining were chosen for evaluation. Using “the Leica QWin 500 image analyzer computer system (England)”, the percentage (%) of stained sections for NF-

Table 1. Sequences of used primers (29, 30)

Gene name	Primer sequence (5'-3')
JAK2	F: TTTGAAGACAGGGACCCTACACAG
	R: TCATAGCGGCACATCTCCACA
STAT3	F: CACCCATAGTGAGCCCTTGGA
	R: TGAGTGCAGTGACCAGGACAGA
GAPDH	F: CAACTCCCTCAAGATTGTCAGCAA
	R: GGCATGGACTGTGGTCATGA

κ B-p65, caspase-3, and TGF- β 1 was calculated. All the necessary components for this image analyzer are a “Leica microscope”, a color video camera, a color monitor, and the hard drive of a “Leica IBM personal computer” that is connected to the microscope. The software used for this process is Leica QWin 500. The values represented the mean of six non-overlapping high-power fields collected from four animals (n=4) per group and averaged to generate a single animal-level value. Then, the mean and standard deviation (mean $\pm$ SD) as between-animal variability were used to describe the % quantitative scoring of antibody data statistically.

Statistical analysis

The data and statistical analysis were performed using SPSS version 25.0, a software produced by “SPSS, Inc. of Chicago, IL, USA”. The current study employed one-way analysis of variance (ANOVA) to evaluate the variation among parametric data. Several comparisons were performed based on “Tukey’s Honestly Significant Difference (HSD)”. All data were provided as mean $\pm$ S.D. Statistical significance thresholds were below 0.05 ($P<0.05$).

Results

Effect of naftidrofuryl on the lung wet/dry weight ratio in LPS-induced rat model of ALI

The ALI animals showed a significant increase in the lung wet/dry weight ratio compared to the V, NAFT, and all treatment groups. Furthermore, the lung wet/dry weight ratio in the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups was significantly increased versus that of the V and NAFT groups. On the other hand, the NAFT50+DEXA group showed a significant decrease in the wet/dry weight ratio of the lung relative to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated groups (Table 2).

Naftidrofuryl reduced the MPO activity and WBC count in a rat model of LPS-induced acute lung injury

Our data revealed that MPO activity and WBC count

Table 2. Effect of naftidrofuryl on the lung wet/dry weight ratio in LPS-induced rat model of acute lung injury

Animal group	Lung wet/dry (W/D) weight ratio
V	2.5400 $\pm$ 0.65238
NAFT	2.7302 $\pm$ 0.60740
ALI	8.4040 $\pm$ 0.78951 ^a
DEXA	6.4220 $\pm$ 0.52151 ^{a,b,c}
NAFT 25	6.0760 $\pm$ 1.28189 ^{a,b,c}
NAFT 50	5.6200 $\pm$ 0.59670 ^{a,b,c}
NAFT 25+DEXA	5.1980 $\pm$ 0.97669 ^{a,b,c}
NAFT 50+DEXA	3.4940 $\pm$ 0.81506 ^{c,d,e,f,g}

Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. Values (n=4) were expressed as mean $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P<0.05$; using One-way ANOVA followed by Tukey’s Honestly Significant Difference (HSD) *post hoc*. ALI, Acute lung injury; DEXA, Dexamethasone; LPS, Lipopolysaccharides; NAFT, Naftidrofuryl; V, Vehicle.

in the ALI group were significantly higher than in the V, NAFT, and all other treatment groups. Furthermore, there was a significant increase in MPO activity and WBC count in the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups relative to the V and NAFT groups. Conversely, the NAFT50 alone and NAFT25+DEXA groups showed a significant reduction in MPO activity and WBC count compared to the rats that were only given DEXA or NAFT25. The NAFT50+DEXA group showed a significant decrease in MPO activity and WBC count compared with DEXA-, NAFT25-, NAFT50-, and NAFT25+DEXA-treated rats (Figure 2; $P<0.05$).

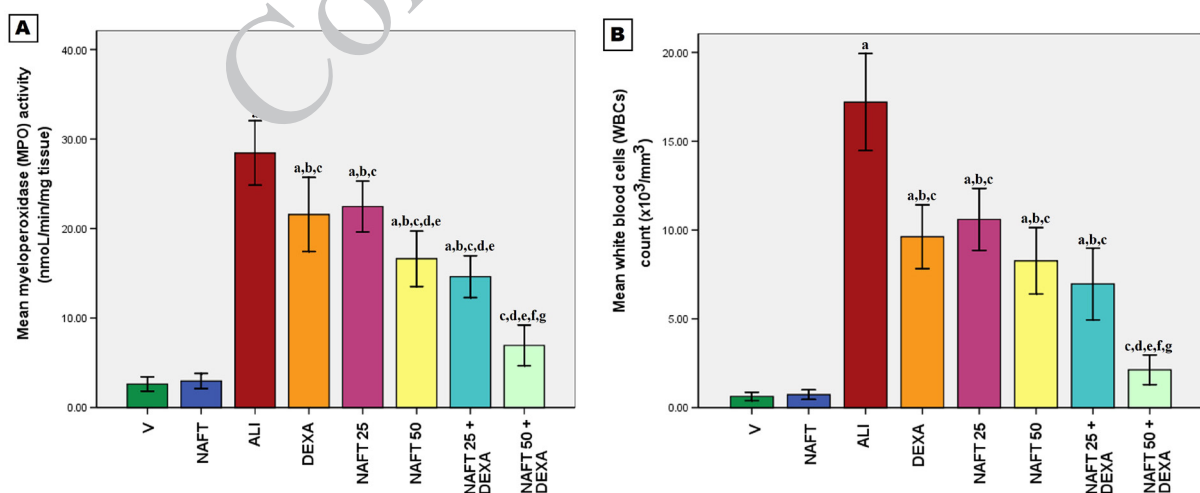


Figure 2. Effect of naftidrofuryl on MPO activity and WBCs count in a rat model of LPS-induced acute lung injury

Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) MPO activity (nmol/min/mg tissue); n=4 and (B) WBCs count (x10³/mm³); n=8. Values were expressed as mean $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P<0.05$; using One-way ANOVA followed by Tukey’s Honestly Significant Difference (HSD) *post hoc*. ALI, Acute lung injury; DEXA, Dexamethasone; MPO, Myeloperoxidase; NAFT, Naftidrofuryl; NS, Normal saline; WBCs, White blood cells; and V, Vehicle.

The network pharmacology analysis

Prospective targets associated with naftidrofuryl

During the analysis of the following five databases: SwissTargetPrediction (100), SuperPred (116), SEA (29), CTD (2), and TargetNet (29), there were 276 targets linked to NAFT. Consequently, the target count went down to 249 after duplicates were removed (Supplementary Table S4, Figure 3A).

Prospective targets associated with dexamethasone

The 7,995 targets were identified as associated with DEXA in the following five databases: SEA (13), SuperPred (116), DrugBank (27), CTD (7,809), and TargetNet (30). Therefore, once duplicates were removed, the target count was lowered to 7,883 (Supplementary Table S4, Figure 3B).

Prospective targets associated with ALI

The investigation of five databases was meticulously conducted to identify the targets linked with ALI. A total of 43,450 targets were identified: 10,448 are associated with the GeneCards database, 9 with TTD, 1,722 with the OpenTargets Platform, 31,251 with CTD, and 20 with MalaCards. After removing all duplicates, 31,847 targets

remained for the entire study (Supplementary Table S6, Figure 3C).

Intersecting targets among naftidrofuryl, dexamethasone, and ALI

Out of the 12 NAFT targets and 31,610 ALI targets, 237 were found, while among the 3 DEXA targets and 23,967 ALI targets, 7,880 were identified. Finally, it was found that 176 of the 61 NAFT-ALI targets and 7,704 of the DEXA-ALI targets were unique. (Supplementary Table S7, Figure 4A, B, & C).

Protein-protein interaction network of 176 drug-disease overlapped targets

Using the STRING database, a protein-protein interaction (PPI) diagram was created to visualize the connections between all 176 overlapping targets. The PPI network contained 176 nodes and 1417 edges after removing unconnected nodes (Figure 5A). Next, the targets were ranked by STRING protein node degree. According to the degree-median filtration ($\text{Degree} > 11$), 83 targets were identified. A network comprising 3 drugs and 83 targets was established using Cytoscape version 3.10.2 for additional investigation (Figure 5B). Furthermore, the top hub targets were ranked by Degree, Betweenness, and Closeness using the CytoNCA tool (Supplementary Table S7, Table 3). Subsequently, a PPI network of the top 10 overlapping targets (STAT3, AKT1, HSP90AA1, SRC, HSP90AB1, NFKB1, HIF1A, MTOR, PPARG, and TLR4) with the experimentally validated targets (JAK2, STAT3, PPAR- α (PPARA), SIRT1, HMGB1, AMPK, Nrf-2 (NFE2L2),

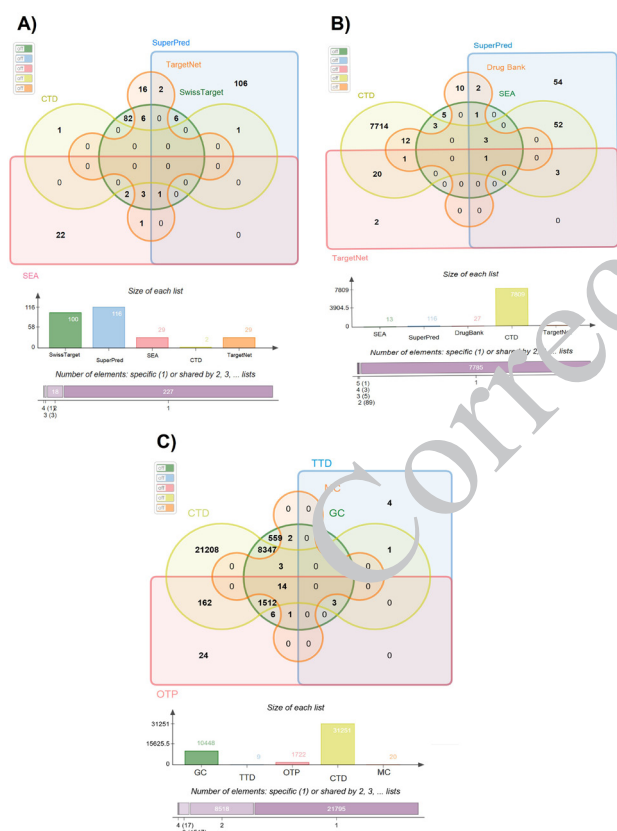


Figure 3. Targets linked to naftidrofuryl, dexamethasone, and acute lung injury

(A) The targets connected to “Naftidrofuryl (NAFT)”; collected from the five databases SwissTargetPrediction, SuperPred, Similarity ensemble approach (SEA), Comparative Toxicogenomics Database (CTD), and TargetNet; (B) The targets connected to “Dexamethasone (DEXA)”; collected from the five databases SEA, SuperPred, Drugbank, CTD, and TargetNet; and (C) the targets associated with “Acute lung injury (ALI)”; gathered from the five databases GeneCards, Therapeutic Target Database (TTD), OpenTargets Platform, CTD, and MalaCards. ALI, Acute lung injury; CTD, Comparative Toxicogenomics Database; DEXA, Dexamethasone; PO, Myeloperoxidase; NAFT, Naftidrofuryl; SEA, Similarity ensemble approach; and TTD, Therapeutic Target Database

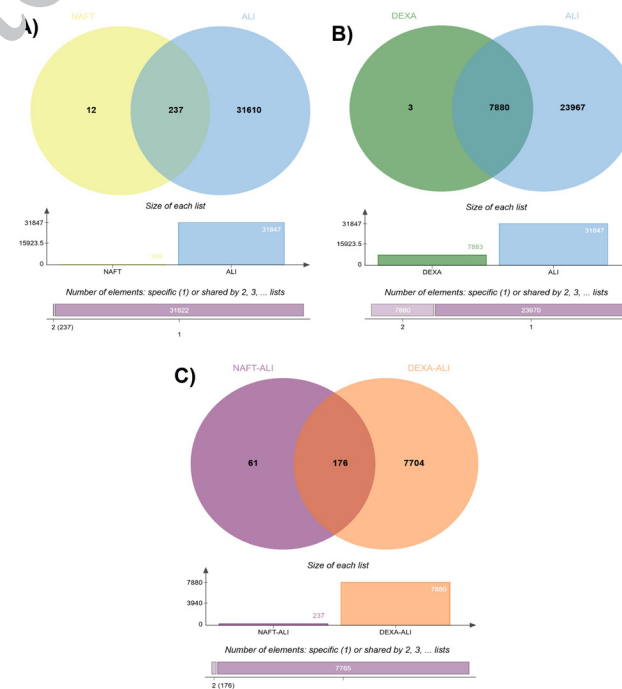


Figure 4. Overlapping targets between naftidrofuryl, dexamethasone, and acute lung injury

(A) The 237 intersection targets overlap with “Naftidrofuryl (NAFT)” and “Acute lung injury (ALI)”; (B) The 7880 intersection targets overlap with “Dexamethasone (DEXA)” and “ALI”; and (C) The 176 intersection targets overlap with “NAFT-ALI” and “DEXA-ALI”

ALI, Acute lung injury; DEXA, Dexamethasone; and NAFT, Naftidrofuryl

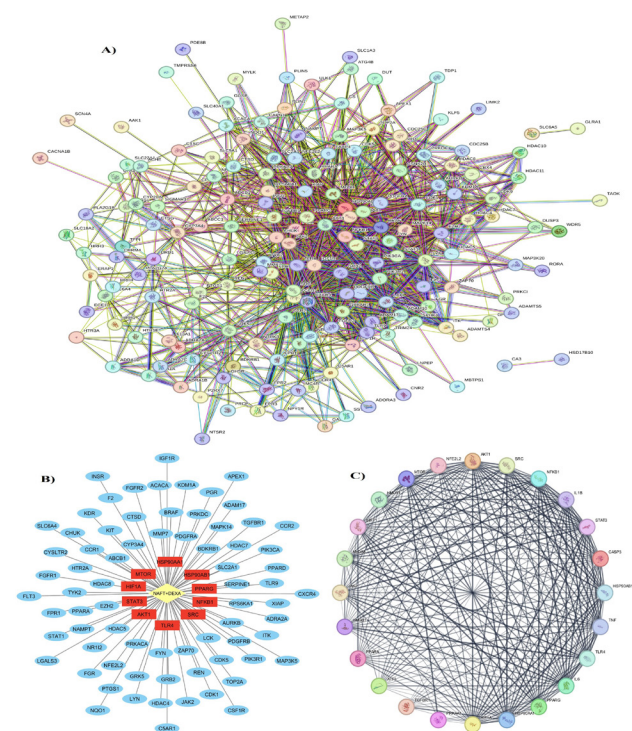


Figure 5. Network pharmacology analysis

(A) A protein-protein interaction (PPI) network consisting of 176 linked targets was constructed using 176 nodes and 1417 edges after excluding the unconnected nodes, with the average node degree of 16.1. Based on the confidence level, an interaction score of 0.4 was used as the medium confidence level and used as the baseline. (B) The drug-targets network, Diamond: "Naftidrofuryl (NAFT)" + Dexamethasone (DEXA), Rectangle: 10 hub targets (STAT3, AKT1, HSP90AA1, SRC, HSP90AB1, NFKB1, HIF1A, MTOR, PPARG, and TLR4), Ellipse: other overlapped targets, and (C) The PPI network of 10 hub targets and experimentally validated targets (JAK2, STAT3, PPARA, TGFBR1, SIRT1, HMGB1, caspase-3, AMPK, NFE2L2, HMOX1, ATF3, TLR4, MYD88, NFKB1, TNFa, IL6, and IL1B)

AKT1, AKT serine/threonine kinase 1; AMPK, AMP-activated protein kinase; ATF3, Activating transcription factor 3; DEXA, Dexamethasone; HIF1A, Hypoxia-inducible factor 1 subunit alpha; HMGB1, High mobility group box 1; HMOX1, Heme oxygenase 1; HSP90AA1, Heat shock protein 90 alpha family class A member 1; HSP90AB1, Heat shock protein 90 alpha family class B member 1; IL1B, Interleukin 1 beta; IL6, Interleukin 6; JAK2, Janus kinase 2; MTOR, Mechanistic target of rapamycin kinase; MYD88, Myeloid differentiation primary response 88; NAFT, Naftidrofuryl; NFE2L2, NFE2-like bZIP transcription factor 2; NFKB1, Nuclear factor kappa B subunit 1; SIRT1, Sirtuin 1; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; STAT3, Signal transducer and activator of transcription 3; PPARA, Peroxisome proliferator-activated receptor alpha; PPARG, Peroxisome proliferator-activated receptor gamma; TGFBR1, Transforming growth factor beta receptor 1; and TNFa, Tumor necrosis factor

HO-1 (HMOX1), TLR4, ATF3, MYD88, NF- κ B (NFKB1), TNF- α (TNFa), IL6, IL-1 β (IL1B), caspase-3, and TGF- β 1 (TGFBR1)) was constructed (Figure 5C).

Enrichment analysis of overlapping proteins

Utilizing ShinyGO, a web-based tool for GO enrichment analysis, and KEGG Pathways, we determined the essential functional and biological properties of the 83 relevant proteins. At a significance level of P -value < 0.05 , statistical enrichment analysis was performed on biological processes (BP), cellular components (CC), molecular functions (MF), and KEGG Pathways (Supplementary Table S8, Figure 6A-D).

Top BP included "regulation of phosphatidylinositol 3-kinase activity", "positive regulation of phosphatidylinositol 3-kinase activity", "positive regulation of lipid kinase activity", and "response to growth hormone"; Top CC included "receptor complex", "membrane microdomain, membrane raft", and "extrinsic component of cytoplasmic

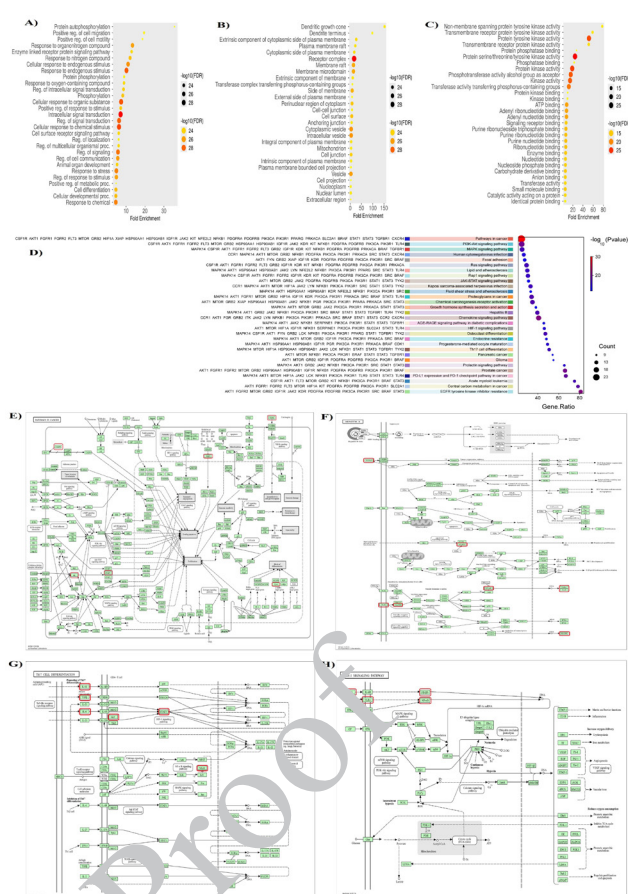


Figure 6. Enrichment analysis

The top 10 significantly enriched Gene Ontology (GO) analyses (sorted by Fold Enrichment), including (A) biological process (BP), (B) cellular component (CC), (C) molecular function (MF), (D) KEGG pathways (sorted by P value), (E) Pathways in cancer, (F) EGFR tyrosine kinase inhibitor resistance, (G) Chemokine signaling pathway, and (H) Hepatitis B pathway

BP, Biological process; CC, Cellular component; EGFR, Epidermal growth factor receptor; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; and MF, Molecular function

side of plasma membrane"; Top MF included "protein tyrosine kinase activity", "transmembrane receptor protein kinase activity", "transmembrane receptor protein tyrosine kinase activity", and "non-membrane spanning protein tyrosine kinase activity".

A KEGG pathway analysis was conducted to investigate the most likely pathways associated with the action of NAFT-DEXA against ALI. The 83 overlapping targets were included in this analysis with a significance level less than 0.05. Among the top 30 KEGG pathways, "Pathways in cancer", "EGFR tyrosine kinase inhibitor resistance", and "chemokine signaling pathway" were determined as molecular interactions between NAFT and ALI.

Notably, multiple confirmed targets in the experimental conditions, such as Nrf-2, HO-1, IL-6, NF- κ B, and caspase-3, were among those implicated in the detected KEGG pathways. Further targets, including MYD88 and TNF- α , were also found in the Hepatitis B pathway (Figure 6E-H).

Molecular docking confirmed the association between naftidrofuryl and experimentally validated targets

Using docking, the four targets (SIRT1, HMGB1, AMPK, and ATF3) were further confirmed to interact with the

Table 3. The 83 common targets between “NAFT-ALI” and “DEXA-ALI” filtered and ranked by Degree method

Rank	Target name	Target symbol	Degree	Betweenness	Closeness
1	Signal Transducer and Activator of Transcription 3	STAT3	64	400.4642766	0.82
2	AKT Serine/Threonine Kinase 1	AKT1	62	451.0057044	0.803921569
3	Heat Shock Protein 90 Alpha Family Class A Member 1	HSP90AA1	60	414.2807738	0.788461538
4	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase	SRC	59	700.2116409	0.780952381
5	Heat Shock Protein 90 Alpha Family Class B Member 1	HSP90AB1	57	362.1909834	0.76635514
6	Nuclear Factor Kappa B Subunit 1	NF- κ B1	53	206.2422771	0.738738739
7	Hypoxia Inducible Factor 1 Subunit Alpha	HIF1A	51	212.4717737	0.725663717
8	Mechanistic Target of Rapamycin Kinase	MTOR	47	166.3848727	0.700854701
9	Peroxisome Proliferator Activated Receptor Gamma	PPARG	45	220.256806	0.68907563
10	Toll Like Receptor 4	TLR4	44	189.8641301	0.67768595
11	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha	PIK3CA	44	82.26424217	0.67768595
12	Signal Transducer and Activator of Transcription 1	STAT1	42	74.60722003	0.666666667
13	Janus Kinase 2	JAK2	39	82.20136242	0.650793651
14	C-X-C Motif Chemokine Receptor 4	CXCR4	37	88.42078174	0.640625
15	Growth Factor Receptor Bound Protein 2	GRB2	36	44.00761941	0.635658915
16	KIT Proto-Oncogene, Receptor Tyrosine Kinase	KIT	35	40.57000163	0.635658915
17	Phosphoinositide-3-Kinase Regulatory Subunit 1	PIK3R1	34	38.00393205	0.625954198
18	Kinase Insert Domain Receptor	KDR	34	47.89068275	0.630769231
19	Insulin Like Growth Factor 1 Receptor	IGF1R	34	37.5401524	0.630769231
20	FYN Proto-Oncogene, Src Family Tyrosine Kinase	FYN	31	52.64155655	0.621212121
21	Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit	EZH2	31	59.13877272	0.611940299
22	Mitogen-Activated Protein Kinase 14	MAPK14	31	45.35171885	0.611940299
23	LYN Proto-Oncogene, Src Family Tyrosine Kinase	LYN	29	21.16855997	0.602941176
24	Progesterone Receptor	PGR	28	57.54855847	0.602941176
25	Colony Stimulating Factor 1 Receptor	CSF1R	28	36.11233606	0.598540146
26	B-Raf Proto-Oncogene, Serine/Threonine Kinase	BRAF	28	22.23293151	0.602941176
27	Platelet Derived Growth Factor Receptor Alpha	PDGFRA	27	18.02140309	0.594202899
28	Platelet Derived Growth Factor Receptor Beta	PDGFRB	27	15.39815017	0.594202899
29	Peroxisome Proliferator Activated Receptor Alpha	PPARA	25	42.3907353	0.589928058
30	Fms Related Receptor Tyrosine Kinase 3	FLT3	25	14.91501845	0.585714286
31	Fibroblast Growth Factor Receptor 1	FGFR1	24	19.98104333	0.581560284
32	Galactose-3	LGALS3	23	24.79935472	0.581560284
33	LCK Proto-Oncogene, Src Family Tyrosine Kinase	LCK	23	22.73589127	0.577464789
34	NFE2 Like 1/ NF- κ B Transcription Factor 2	NFE2L2	22	19.71584371	0.573426573
35	X-Linked Inhibitor of Apoptosis	XIAP	22	49.45260994	0.577464789
36	Cyclin Dependent Kinase 1	CDK1	22	32.01685589	0.573426573
37	Fibroblast Growth Factor Receptor 2	FGFR2	22	9.993592996	0.565517241
38	C-C Motif Chemokine Receptor 2	CCR2	22	21.16373323	0.573426573
39	Protein Kinase cAMP-Activated Catalytic Subunit Alpha	PRKACA	21	24.36178707	0.565517241
40	ATP Binding Cassette Subfamily B Member 1	ABCB1	21	127.7627928	0.573426573
41	Toll Like Receptor 9	TLR9	21	11.67594858	0.565517241
42	FGR Proto-Oncogene, Src Family Tyrosine Kinase	FGR	20	23.38239991	0.557823129
43	Transforming Growth Factor Beta Receptor 1	TGFBR1	20	7.87041556	0.554054054
44	Tyrosine Kinase 2	TYK2	19	2.415019192	0.561643836
45	Serpin Family E Member 1	SERPINE1	18	31.06202138	0.557823129
46	Solute Carrier Family 2 Member 1	SLC2A1	18	13.30699255	0.561643836
47	Renin	REN	18	50.5587164	0.561643836
48	C-C Motif Chemokine Receptor 1	CCR1	18	19.79991445	0.55033557
49	IL2 Inducible T Cell Kinase	ITK	17	6.168056336	0.543046358
50	Component of Inhibitor of Nuclear Factor Kappa B Kinase Complex	CHUK	17	5.895293903	0.55033557
51	Insulin Receptor	INSR	17	8.861291159	0.55033557
52	Zeta Chain of T Cell Receptor Associated Protein Kinase 70	ZAP70	17	1.84039878	0.554054054
53	Histone Deacetylase 5	HDAC5	17	19.79676068	0.55033557
54	Protein Kinase, DNA-Activated, Catalytic Subunit	PRKDC	16	11.20783128	0.525641026

Continued Table 3.

55	Histone Deacetylase 4	HDAC4	16	10.79918356	0.529032258
56	Lysine Demethylase 1A	KDM1A	15	9.772911026	0.525641026
57	Mitogen-Activated Protein Kinase Kinase Kinase 5	MAP3K5	14	4.122570478	0.529032258
58	Cytochrome P450 Family 3 Subfamily A Member 4	CYP3A4	13	70.35014894	0.529032258
59	Matrix Metalloproteinase 7	MMP7	13	13.6991264	0.539473684
60	Ribosomal Protein S6 Kinase A1	RPS6KA1	12	7.967587786	0.532467532
61	Nicotinamide Phosphoribosyltransferase	NAMPT	12	3.78907563	0.51572327
62	Peroxisome Proliferator Activated Receptor Delta	PPARD	12	3.346295178	0.518987342
63	Formyl Peptide Receptor 1	FPR1	12	18.68774952	0.51572327
64	NAD(P)H Quinone Dehydrogenase 1	NQO1	12	6.542562108	0.529032258
65	Aurora Kinase B	AURKB	11	5.145867602	0.51572327
66	G Protein-Coupled Receptor Kinase 5	GRK5	11	55.108628	0.518987342
67	Cathepsin D	CTSD	11	10.90636773	0.51572327
68	DNA Topoisomerase II Alpha	TOP2A	11	5.697157948	0.50931677
69	Coagulation Factor II, Thrombin	F2	10	26.3625766	0.488095238
70	Apurinic/Apyrimidinic Endo deoxyribonuclease 1	APEX1	10	4.372492701	0.50931677
71	Nuclear Receptor Subfamily 1 Group I-Member 2	NR1I2	10	4.71682472	0.518987342
72	Histone Deacetylase 8	HDAC8	10	1.116703297	0.50931677
73	Histone Deacetylase 7	HDAC7	10	3.362121212	0.50617284
74	Prostaglandin-Endoperoxide Synthase 1	PTGS1	10	15.50550384	0.50931677
75	ADAM Metalloproteinase Domain 17	ADAM17	9	1.099350649	0.518987342
76	Complement C5a Receptor 1	C5AR1	8	10.06720443	0.488095238
77	Acetyl-CoA Carboxylase Alpha	ACACA	8	3.065620491	0.491017964
78	Bradykinin Receptor B1	BDKRB1	8	11.75144302	0.479532164
79	Cyclin Dependent Kinase 5	CDK5	7	2.052337202	0.491017964
80	5-Hydroxytryptamine Receptor 2A	HTR2A	7	21.01829479	0.482352941
81	Adrenoceptor Alpha 2A	ADRA2A	6	21.80123674	0.468571429
82	Cysteinyl Leukotriene Receptor 2	CYSLTR2	5	1.233259808	0.463276836
83	Solute Carrier Family 6 Member 4	SLC6A4	4	5.530852607	0.396135266

NAFT molecule. The results indicated that the binding affinities of the most effective and activated complexes of SIRT1, HMGB1, AMPK, and ATF3 were -6.1 Kcal/mol, -6.4 Kcal/mol, -8 Kcal/mol, and -5.8 Kcal/mol, respectively (Supplementary Table S9, Table 4; Figure 7A, B, C, & D).

Naftidrofuryl blocked JAK2/STAT3 axis in a rat model of LPS-induced ALI

According to the findings, JAK2 and STAT3 gene expression in the ALI group was significantly higher than in the V, NAFT, and other treatment groups. Likewise, as opposed to the V and NAFT groups, the JAK2 and STAT3 gene expression was significantly increased in the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups. Conversely, the expression of JAK2 and STAT3 genes was significantly decreased in the NAFT50 alone and

NAFT25+DEXA groups compared with that of the rats treated with DEXA or NAFT25 alone. With respect to rats treated with DEXA, NAFT25, NAFT50, or NAFT25+DEXA, the NAFT50+DEXA group showed a significant decrease in JAK2 and STAT3 gene expression (Figure 8A & B; $P < 0.05$).

Naftidrofuryl enhanced PPAR α levels and lowered TGF β 1-promoted fibroblastic proliferation in a rat model of LPS-induced ALI

In rats with ALI, PPAR α levels were markedly decreased, and TGF- β 1 protein expression was significantly higher in their lung tissues, indicating a favorable response compared to the V, NAFT, and all other treatment groups. Moreover, there was a significant increase in PPAR α levels, with a moderately increased TGF- β 1 protein expression in the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups,

Table 4. Details of molecular docking between Naftidrofuryl and measured targets

Molecule name	Targets	Gene name	PDB number / UniProt ID	Binding ability (kcal/mol)
Naftidrofuryl (NAFT)	Sirtuin 1 (SIRT1)	SIRT1	8anb	-6.1
	High mobility group box 1 (HMGB-1)	HMGB1	8i9m	-6.4
	AMP-activated protein kinase (AMPK)	PRKAA1	7jij	-8
	Activating transcription factor 3 (ATF3)	ATF3	AF-Q5VTZ4-F1-v4	-5.8

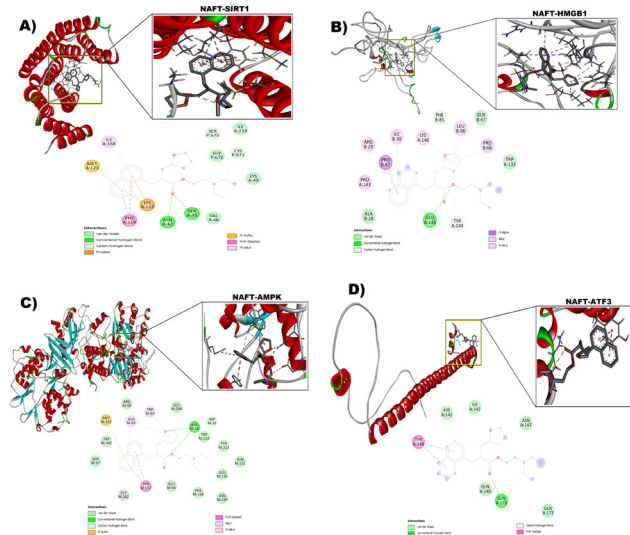


Figure 7. The predicted three- and two-dimensional binding models of naftidrofuryl (NAFT) binding to protein targets

Molecular docking results of NAFT with (A) SIRT1 (PBD ID: 8anb), (B) HMGB1 (PBD ID: 8i9m), (C) AMPK (PRKAA1) (PBD ID: 7jjj), and (D) ATF3 (Uniport ID: AF-Q5VTZ4-F1-v4).

AMPK, AMP-activated protein kinase; ATF3, Activating transcription factor 3; HMGB1, High mobility group box 1; NAFT, Naftidrofuryl; and SIRT1, Sirtuin 1

compared with the V and NAFT groups. In contrast, the NAFT50 alone and NAFT25+DEXA groups showed a significant increase in PPAR α levels, with a significant decrease in TGF- β 1 protein expression in their lungs relative to the rats administered DEXA or NAFT25 alone. The NAFT50+DEXA group showed a significant increase in PPAR α levels, with lower lung TGF- β 1 protein expression and reduced reactivity compared to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated rats. The V and NAFT groups showed no significant difference in TGF- β 1 protein levels as measured by immunohistochemistry and quantitative scoring (Figure 9A, B, & C; $P < 0.05$).

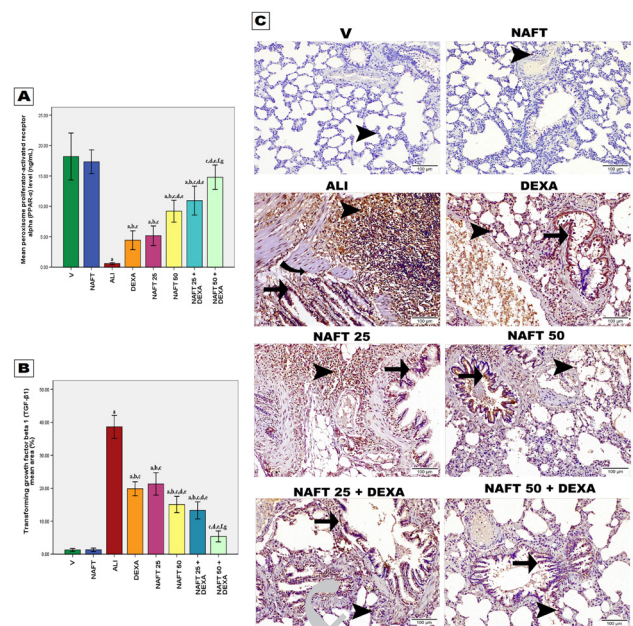


Figure 9. Effect of naftidrofuryl on PPAR α levels and TGF- β 1 expression in a rat model of LPS-induced acute lung injury

Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA with 2 hours in-between). All treatments were injected I.P. once daily for 28 days. (A) PPAR α levels (ng/mL), $n = 4$; (B) quantitative scoring of TGF- β 1 (%), $n = 4$, six fields examined. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$ using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) *post hoc*; and (C) photomicrographs showing TGF- β 1 immunostaining; Lung tissue; Rat TGF- β 1 Antibody, Magnification Power = $\times 200$. Lung Sections revealed positive cytoplasmic and nuclear expressions along bronchiole epithelium (arrow), alveoli and interstitial tissue (arrowhead), as well as muscle (arch arrow) with scarce reactivity along V and NAFT groups, highest in ALI group, moderate high in DEXA and NAFT 25 groups, moderate low in NAFT 50 and DEXA+NAFT 25 groups, while few reactivity in DEXA+NAFT 50 group

ALI, Acute lung injury; DEXA, Dexamethasone; NAFT, Naftidrofuryl; NS, Normal saline; PPAR α , Peroxisome proliferator-activated receptor α ; TGF- β 1, Transforming growth factor beta 1; and V, Vehicle

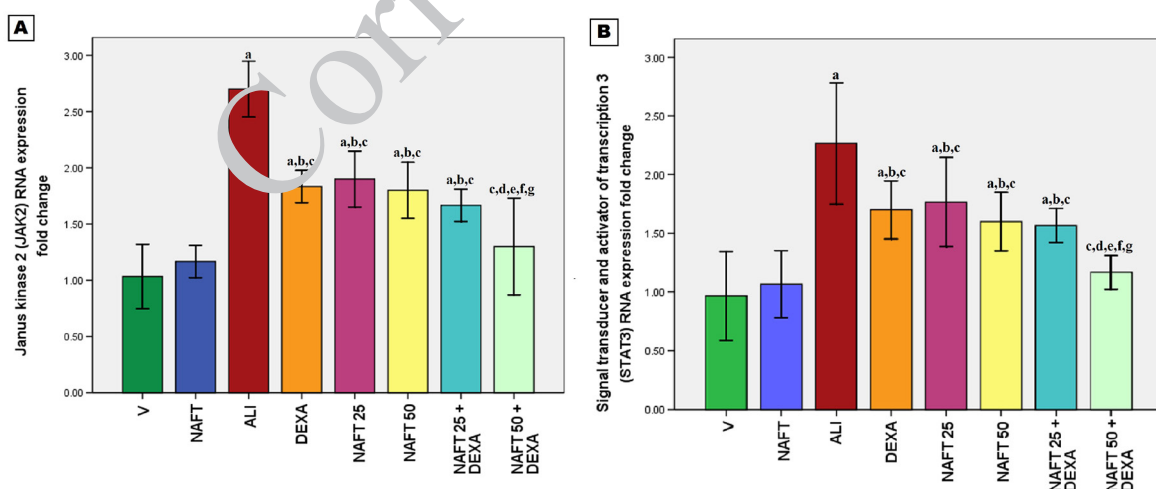


Figure 8. Effect of naftidrofuryl on the JAK2/STAT3 axis in a rat model of LPS-induced acute lung injury

Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) JAK2 gene expression and (B) STAT3 gene expression; $n = 3$. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) *post hoc*

ALI, Acute lung injury; DEXA, Dexamethasone; JAK2, Janus kinase 2; NAFT, Naftidrofuryl; NS, Normal saline; STAT3, Signal transducer and activator of transcription 3; and V, Vehicle

Naftidrofuryl stimulated SIRT1-mediated HMGB1 deacetylation and prevented caspase-3-provoked apoptosis in a rat model of LPS-induced ALI

The rats with ALI had significantly lower SIRT1 levels and higher HMGB1 protein expression, along with a significant increase in a positive caspase-3 protein reaction in their lung tissues compared with V, NAFT, and all other treatment groups. In the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups, SIRT1 levels were significantly decreased while HMGB1 protein expression increased, accompanied by a moderate caspase-3 reactivity in their lung tissues versus the V and NAFT groups. In contrast, NAFT50 alone and NAFT25+DEXA groups had higher SIRT1 levels and lower HMGB1 protein expression, with a milder caspase-3 reactivity in their lung tissues than rats that received DEXA or NAFT25 alone. The NAFT50+DEXA group had higher SIRT1 levels and lower HMGB1 protein expression, together with a significant reduction in caspase-3 reactivity in their lung tissues compared with the DEXA, NAFT25, and NAFT25+DEXA rats. The V and NAFT groups showed no significant differences in caspase-3 reactivity, as assessed by immunohistochemistry and quantitative scoring (Figure 10A, B, C, D, & E; $P < 0.05$).

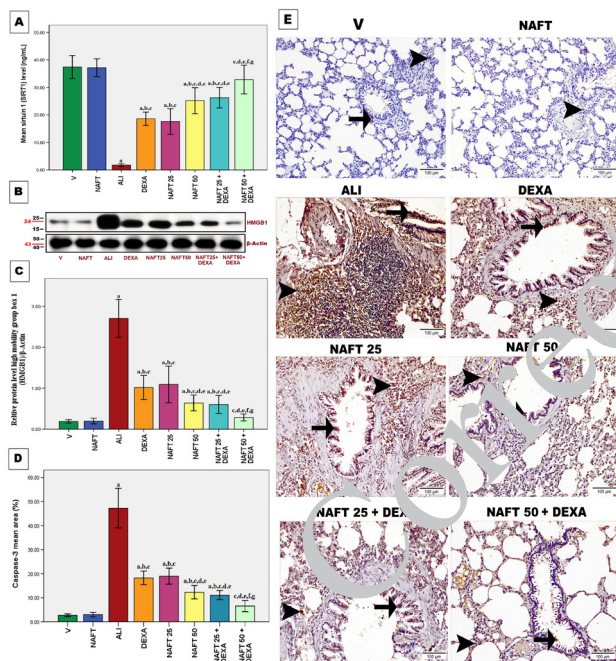


Figure 10. Effect of naftidrofuryl on SIRT1 levels, HMGB1, and caspase-3 expression in a rat model of LPS-induced acute lung injury. Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) SIRT1 levels (ng/ml), $n=4$; (B) Western blot showing HMGB1 protein expression. The expression level was normalized to β -actin; (C) HMGB1/ β -actin expression ratio, $n=3$; (D) quantitative scoring of caspase-3 (%), $n=4$, six fields examined. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc; and (E) photomicrographs showing caspase-3 immunostaining; Lung tissue; Rat (caspase-3 Antibody, Magnification Power= $\times 200$). Lung Sections revealed positive cytoplasmic and nuclear expressions along bronchiole epithelium (arrow), alveoli and interstitial tissue (arrowhead), as well as muscle (arch arrow) with scarce reactivity along V and NAFT groups, highest in ALI group, moderate high in DEXA and NAFT 25 groups, moderate low in NAFT 50 and DEXA+NAFT 25 groups, while few reactivity in DEXA+NAFT 50 group. ALI, Acute lung injury; DEXA, Dexamethasone; HMGB1, High mobility group box 1 protein; NAFT, Naftidrofuryl; NS, Normal saline; SIRT1, Sirtuin 1; and V, Vehicle

Naftidrofuryl activated the AMPK/Nrf2/HO-1 cascade and mitigated lung oxidative stress in a rat model of LPS-induced ALI

Relative to the V, NAFT, and all treatment groups, ALI rats showed a significant decrease in the expression of AMPK/pAMPK and Nrf2, as well as HO-1 and TAC levels, with a significant increase in MDA levels. Moreover, there was a significant decrease in the expression of AMPK/pAMPK and Nrf2, as well as in HO-1 and TAC levels, and a significant increase in MDA levels in the lungs of the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups, corresponding to the V and NAFT groups. By comparison to the rats given DEXA or NAFT25 alone, it was found that AMPK/pAMPK and Nrf2 expression, in addition to HO-1 and TAC levels, showed a significant increase, with lower MDA levels in the NAFT50 alone and NAFT25+DEXA groups. The NAFT50+DEXA group showed a significant increase in AMPK/pAMPK and Nrf2 expression plus HO-1 and TAC levels, along with a significant decrease in MDA levels as opposed to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated groups (Figure 11A, B, C, D, E, & F; $P < 0.05$).

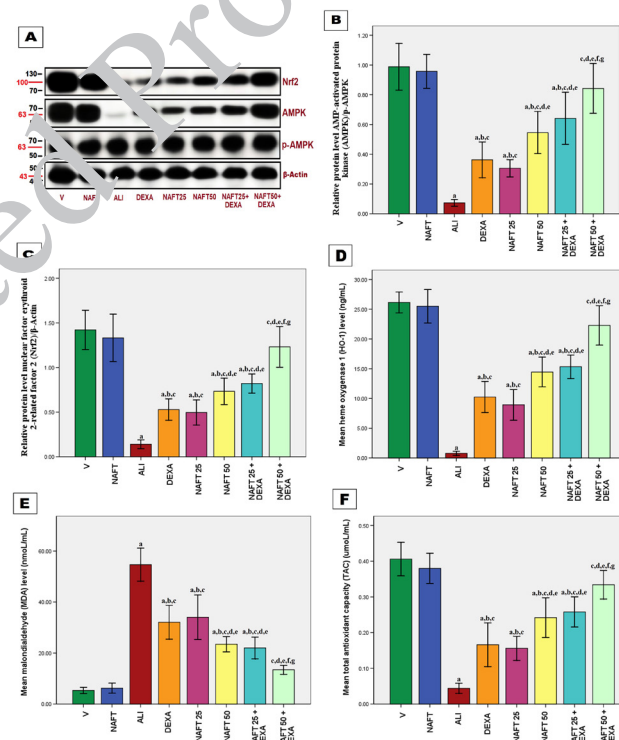


Figure 11. Effect of naftidrofuryl on oxidants/antioxidants mediators in a rat model of LPS-induced acute lung injury. Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) Western blot showing pAMPK, AMPK, and Nrf2 protein expressions. The expression levels were normalized to β -actin; (B) AMPK/pAMPK expression ratio; (C) Nrf2/ β -actin expression ratio, $n=3$; (D) HO-1 levels (ng/ml); (E) MDA levels (nmol/ml); and (F) TAC (umol/ml), $n=4$. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc. ALI, Acute lung injury; AMPK, Adenosine monophosphate-activated protein kinase; DEXA, Dexamethasone; HO-1, Heme oxygenase-1; MDA, Malondialdehyde; NAFT, Naftidrofuryl; Nrf2, Nuclear factor erythroid 2-related factor 2; NS, Normal saline; SIRT1, Sirtuin 1; TAC, Total antioxidant capacity; and V, Vehicle

Naftidrofuryl enhanced ATF3 levels and obstructed TLR4/MyD88 pathway in a rat model of LPS-induced ALI

In contrast to the V, NAFT, and all treated groups, ALI rats showed a significant decrease in ATF3 levels with a significant increase in TLR4 levels and MyD88 expression. TLR4 levels and MyD88 expression significantly increased, and ATF3 levels significantly decreased in the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups when compared to the V and NAFT groups. Unlike the rats given DEXA or NAFT25 alone, the NAFT50-alone and NAFT25+DEXA groups showed significant increases in ATF3 levels and significant decreases in TLR4 and MyD88 expression. With respect to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated groups, the NAFT50+DEXA group showed a significant increase in ATF3 with a significant decrease in TLR4 levels and MyD88 expression (Figure 12 A, B, C, & D; $P < 0.05$).

Naftidrofuryl minimized the NF- κ B-dependent outflow of inflammatory cytokines in a rat model of LPS-induced ALI

The immunohistochemical analysis and quantitative assessment of NF- κ B-p65 proteins across the studied groups showed no significant variations between the V and NAFT groups. The ALI rodents showed a significant increase in NF- κ B-p65 expression within their lung tissues, as shown by a strong affirmative response, with a significant increase in the BALF levels of TNF- α , IL-6, and IL- β as opposed to the V, NAFT, and all treated groups. Additionally, the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups revealed a moderately high NF- κ B-p65 expression, with a significant increase in the BALF concentrations of TNF- α , IL-6, and IL- β versus the V and NAFT groups. In contrast

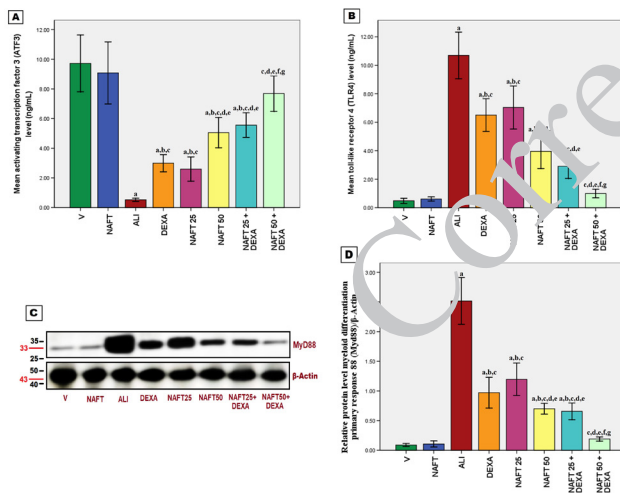


Figure 12. Effect of naftidrofuryl on ATF3 and TLR4 levels, in addition to MyD88 expression in a rat model of LPS-induced acute lung injury. Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) ATF3 levels (ng/ml); (B) TLR4 levels (ng/ml), $n=4$; (C) Western blot showing MyD88 protein expression. The expression level was normalized to β -actin; and (D) MyD88/ β -actin expression ratio, $n=3$. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) *post hoc*.

ALI, Acute lung injury; ATF3, Activating transcription factor 3; DEXA, Dexamethasone; MyD88, Myeloid differentiation primary response 88; NAFT, Naftidrofuryl; NS, Normal saline; TLR4, Toll-like receptor 4; and V, Vehicle

to the rats that were administered DEXA or NAFT25 alone, the NAFT50 alone and NAFT25+DEXA groups showed a significant decrease in NF- κ B-p65 expression, with a reduction in the levels of TNF- α , IL-6, and IL- β . Similarly, the NAFT50+DEXA group showed a significant decrease in lung expression of NF- κ B-p65, along with a significant decrease in the levels of TNF- α , IL-6, and IL- β as compared to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated groups (Figure 13A, B, C, & D; $P < 0.05$).

Naftidrofuryl corrected the pathological changes in the lung tissues of LPS-induced rat ALI

Lung specimens from the V and NAFT groups showed preserved lung architecture, with typical bronchioles and intact alveoli. The ALI group displayed substantial degenerative changes, evidenced by noticeable thickening and hyperplasia of the bronchiole lining epithelium, vacuolated and apoptotic cells, increased alveolar epithelial thickness, and a significant increase in collagen. Moderate improvements were detected in the lung sections from the DEXA group, with decreased bronchiole epithelium thickness, a significant decrease in alveolar epithelium, a moderate number of inflammatory cells, few congested

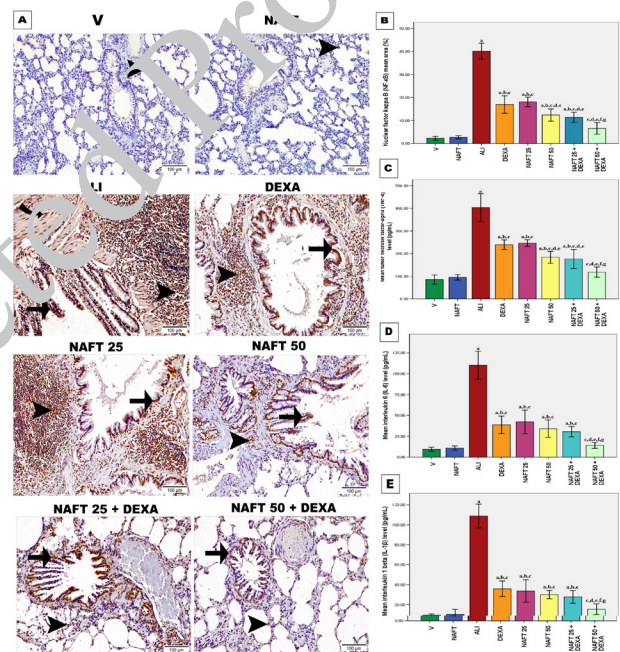


Figure 13. Effect of naftidrofuryl on NF- κ B expression and inflammatory cytokines levels in a rat model of LPS-induced acute lung injury. Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. (A) Photomicrographs showing NF- κ B immunostaining; Lung tissue; Rat (NF- κ B Antibody, Magnification Power= $\times 200$). Lung Sections revealed positive cytoplasmic and nuclear expressions along bronchiole epithelium (arrow), alveoli and interstitial tissue (arrowhead), as well as muscle (arch arrow) with scarce reactivity along V and NAFT groups, highest in ALI group, moderate high in DEXA and NAFT 25 groups, moderate low in NAFT 50 and DEXA+NAFT 25 groups, while few reactivity in DEXA+NAFT 50 group; (B) quantitative scoring of NF- κ B (%), $n=4$, six fields examined; (C) TNF- α levels (pg/ml); (D) IL-6 levels (pg/ml); and (E) IL-1 β levels (pg/ml), $n=4$. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at $P < 0.05$; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) *post hoc*.

ALI, Acute lung injury; DEXA, Dexamethasone; IL-1 β , Interleukin 1 beta; IL-6, Interleukin 6; NAFT, Naftidrofuryl; NF- κ B, Nuclear factor kappa B; NS, Normal saline; TNF- α , Tumor necrosis factor; and V, Vehicle

blood vessels, and few interstitial hemorrhages. Generally, some cells were normal, but others were still degenerating. The NAFT 25 group showed a few improvements, with a thinner bronchiole epithelium, more intact lining cells, and fewer vacuolated apoptotic cells, along with a significant decrease in alveolar epithelium. However, other areas still had high thickness, a moderate number of inflammatory cells, and high congestion along the blood vessels. Lung sections from either the NAFT 50 alone or the NAFT 25+DEXA group showed significant advancement, defined by reduced bronchiole epithelium thickness, with most lining cells remaining intact and only a few showing vacuolated apoptosis. There was a significant decrease in alveolar epithelium, a minimal presence of inflammatory cells, sparse interstitial hemorrhage, and restricted congestion along the blood vessels. Regarding NAFT 50+DEXA, lung tissue showed the most significant improvement, as detected by the preservation of bronchiole lining epithelium and alveolar epithelium, with only localized regions displaying moderate thickness, a weak presence of inflammatory cells, minimal interstitial hemorrhages, and restricted congestion along the blood vessels (Figure 14).

Histopathological assessment indicated no significant disparities between the V and NAFT groups regarding bronchiolar epithelium thickness, alveolar wall thickness, inflammatory cell count, collagen fiber quantity, and congested blood vessel count. The ALI group showed a statistically significant increase compared with the V, NAFT, and all treatment groups. At the same time, the DEXA, NAFT25, NAFT50, and NAFT25+DEXA groups showed a significant increase relative to the V and NAFT groups. However, a significant decrease was observed in the NAFT50-alone and NAFT25+DEXA groups compared with the DEXA and NAFT25 groups. Meanwhile, the NAFT50+DEXA group showed a significant decrease in all quantifiable parameters with regard to the DEXA, NAFT25, NAFT50, and NAFT25+DEXA-treated groups (Figure 14 A, B, C, D, & E).

Discussion

Within the ALI paradigm, platelets may release serotonin (5-HT) to stimulate the vascular endothelial cells, hence allowing leukocytes to adhere and be recruited to inflamed tissues (11). This process is crucial for an adequate immune response, but excessive leukocyte infiltration can disrupt the alveolar-epithelial barrier (34). Concurrently, activated platelets and neutrophils engage the inflamed endothelium, releasing MPO, thereby increasing endothelial cell permeability and provoking edema and inflammation (35). Elevated alveolar permeability and edema may then contribute to the emergence of alveolar-epithelial dysfunction, as evidenced by an elevated lung wet/dry weight ratio (36).

Therefore, antagonizing the serotonergic receptor may decrease alveolar permeability, leukocyte adherence, and migration to inflamed lung tissue, effectively alleviating inflammation and protecting lung tissue (37). Given this, it is imperative to investigate the prospective pharmacological contribution of the 5-HT_{2A} receptor antagonist NAFT in mitigating LPS-associated inflammation and tissue injury in ALI rats.

In accordance with the previously provided data, this study affirms that all groups treated with NAFT, either alone

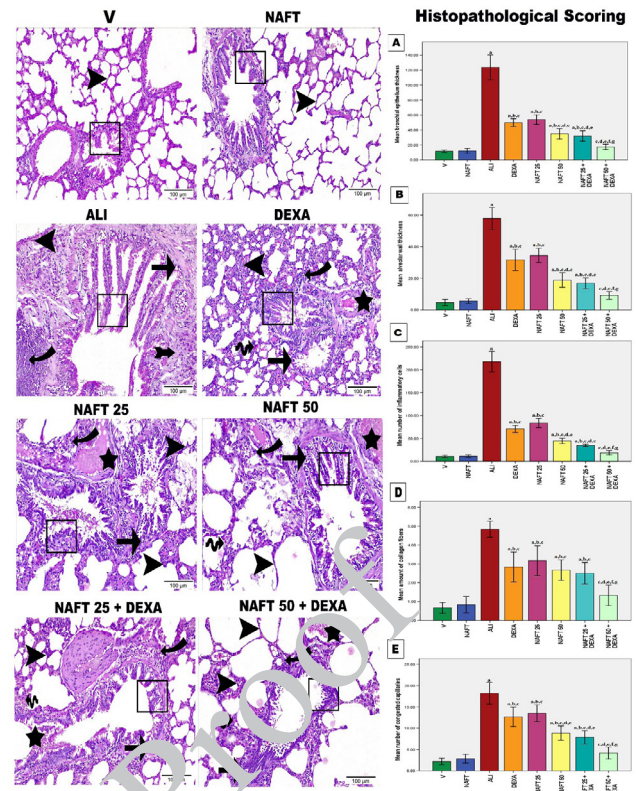


Figure 14. Photomicrographs showing the effect of naftidrofuryl on histopathological changes in a rat model of LPS-induced acute lung injury (Hematoxylin and Eosin, H&E stain, Magnification Power= x200, Scale bar= 100 μ m); Lung tissue; Rat Vehicle group (5 ml NS/kg/day, V); Naftidrofuryl group (50 mg NAFT/kg/day, NAFT); Acute lung injury group (5 ml NS/kg/day, ALI); Dexamethasone-treated group (5 mg/kg/day, DEXA); NAFT 25-treated group (25 mg/kg/day, NAFT 25); NAFT 50-treated group (50 mg/kg/day, NAFT 50); NAFT 25+DEXA group (25 mg NAFT and 5 mg DEXA with 2 hours in-between); and NAFT 50+DEXA group (50 mg NAFT and 5 mg DEXA). All treatments were injected I.P. once daily for 28 days. Lung Sections from V, NAFT, ALI, DEXA, NAFT 25, NAFT 25+DEXA, NAFT 50, NAFT 50+DEXA groups highlighted the pathological changes between groups along bronchioles thickness (rectangle), bronchiole lining epithelium (arrow), alveolar epithelium (arrowhead), inflammatory cells (arch arrow), interstitial hemorrhage (wave arrow), congested blood vessels (star), and collagen fibers (arrow with tail). (A) Bronchial alveolar epithelium; (B) Alveolar wall thickness; (C) Number of inflammatory cells; (D) Amount of collagen fibers; and (E) Number of congested capillaries, n =4, six fields examined. Values were expressed as means $\pm$ SD. Significant difference vs. a V, b NAFT, c ALI, d DEXA, e NAFT 25, f NAFT 50, or g NAFT 25+DEXA groups, each at p<0.05; using One-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc ALI, Acute lung injury; DEXA, Dexamethasone; NAFT, Naftidrofuryl; NS, Normal saline; and V, Vehicle

or with DEXA, had significantly lower lung wet/dry weight ratio, MPO activity, and WBC count than the untreated ALI group, ultimately affirming the efficacy of NAFT against ALI. Because the precise mechanisms by which NAFT may exert its therapeutic effect on ALI, especially when combined with DEXA, remain uncertain, network analysis, molecular docking, and experimental validation were crucial for elucidating the regulatory targets and pathways involved.

Network study revealed 237 shared targets between NAFT and ALI, and 7880 shared targets between DEXA and ALI, with a total of 176 targets common to NAFT-ALI and DEXA-ALI. Interestingly, STAT3 was recorded as the primary target among the top 10 hub targets, with JAK2 in the 14th position. Additionally, KEGG enrichment analysis disclosed JAK-STAT signaling as an important molecular interaction between NAFT and ALI in the three principal

pathways— “Pathways in cancer, EGFR tyrosine kinase inhibitor resistance, and Chemokine signaling pathway”.

Lately, the JAK2/STAT3 pathway has been highlighted as relevant to ALI pathogenesis and potential treatment strategies. For instance, it has been demonstrated that Qingdai, a traditional Chinese medicine, mitigates LPS-induced ALI by decreasing JAK2/STAT3 signaling (38). Similarly, fibroblast growth factor 21 (FGF21) exhibited protective effects against LPS-induced ALI by suppressing JAK2/STAT3 signaling (39). Meanwhile, research has clarified that JAK2/STAT3 may be one of several pathways activated by serotonin 5-HT $_2$ A receptors to induce cellular damage and inflammation (40). These findings suggest that targeting the JAK2/STAT3 pathway, by blocking 5-HT $_2$ A, may efficiently modulate ALI-related inflammation and concomitant pulmonary damage.

In the present research, all NAFT-treated rats, either alone or alongside DEXA, showed a marked decline in JAK2 and STAT3 gene expression as compared to ALI animals. The data agreed with the findings of Wu *et al.* (2019), who reported that ulinastatin protected against lung injury in sepsis-induced ALI by suppressing JAK2/STAT3 activation and reducing inflammatory mediators (41).

As previously mentioned, there was an association between JAK2/STAT3 activation and PPAR α down-regulation (42). PPAR α could attenuate LPS-induced ALI by reducing leukocyte infiltration, protein permeability, apoptosis, and pro-inflammatory mediator release. Moreover, activating PPAR α regulates lung fibrosis by suppressing TGF- β 1 levels (43). In parallel, our network analysis identified PPAR α and TGFBR1 as common targets of NAFT and ALI at the 29th and 43rd positions, respectively, thereby affirming their potential therapeutic roles against ALI. Similarly, the *in vivo* study revealed that rats administered NAFT, alone or with DEXA, had higher PPAR α levels with lower TGF- β 1 protein expression than ALI rats. These findings coincide with the data recently presented by Oruqaj *et al.* (2023), who investigated that PPAR α attenuates inflammation and the fibrotic process in IPF by inhibiting TGF- β 1 signaling (44).

Simultaneously, an investigation indicated that the anti-inflammatory and antifibrotic properties of PPAR α depend on sirtuin release, notably SIRT1 (45). SIRT1 is highlighted for its role in lung protection by inhibiting the HMGB1 pathway, thereby blocking caspase-3 expression and apoptosis (46). Taken together, HMGB1 and liver kinase B1 (LKB1) may exacerbate inflammatory responses in LPS-induced ALI by preventing AMPK activation. AMPK can notably regulate cellular energy balance, substantially diminishing oxidative stress and inflammation in ALI (47). Additionally, AMPK can activate ATF3, which is deemed essential for pulmonary endothelial cell regeneration after acute injury (48).

From prior research, it has been clearly shown that SIRT1, HMGB1, AMPK, and ATF3 exert distinct roles in ALI pathogenesis; however, our network analysis did not locate them as NAFT molecular targets or as intersecting targets between NAFT and ALI. Plus, none were observed within the KEGG pathways. Consequently, it was imperative to ascertain NAFT affinity towards the appointed targets using molecular docking.

The docking results demonstrated significant selectivity of NAFT for SIRT1, HMGB1, AMPK, and ATF3. This finding aligns with our *in vivo* study, which reported a significant

increase in SIRT1 levels and a decrease in HMGB1 and caspase-3 expression, along with fewer apoptotic lung tissues across all NAFT-treated groups, whether alone or with DEXA, compared with the ALI group. A previous study reported similar outcomes, suggesting that artesunate may mitigate sepsis-induced ALI via a SIRT1-dependent mechanism (49). A notable increase in AMPK expression and ATF3 levels was also observed, consistent with multiple studies elucidating the role of the AMPK-Nrf2 pathway in protecting against inflammation and oxidative stress in ALI models (50).

Additionally, activation of the AMPK-ATF3 axis by naringenin has been assigned to protect mice from endotoxemia by negatively regulating the LPS/TLR4 cascade (51). Following TLR4 activation by LPS, MyD88 is recruited to stimulate the “interleukin-1 receptor-associated kinases (IRAKs)”, thereby activating “tumor necrosis factor receptor-associated factor 6 (TRAF6)” (52). This activates the I κ B kinase (IKK) complex, leading to I κ B phosphorylation and degradation, thereby allowing NF- κ B dimers (mostly p65/p50) to enter the nucleus. In the nucleus, NF- κ B attaches to DNA sequences to facilitate the transcription of numerous pro-inflammatory cytokines (e.g., TNF- α , IL-6, and IL-1 β) (53).

In light of the aforementioned association, it was essential to consider the roles of caspase-3, Nrf-2, HO-1, IL-6, TNF- α , and MYD88 in the current work, particularly after distinguishing them among the targets included in the detected KEGG pathways. Further, the PPI was designed to confirm their association with the ten identified hub targets. This ensured that the therapeutic strategies of NAFT for ALI focused on targeting caspase-3 as an apoptotic indicator, Nrf-2 and HO-1 as antioxidant mediators, and IL-6, TNF- α , and MYD88 as inflammatory markers.

This explains why, in NAFT groups, whether independently used or with DEXA, there was an elevation in Nrf2 expression and HO-1 levels, confirming their antioxidant properties. The same was revealed by Jia *et al.*, 2024, who reported that activating Nrf2 mitigated ferroptosis and oxidative stress in sepsis-induced ALI (54). The impact was also corroborated in our study by diminished MDA levels alongside elevated TAC. As well, the effectiveness of NAFT as an anti-inflammatory agent in the present investigation is evidenced by an apparent reduction in TLR4 levels, expression of MyD88 and NF- κ B, along with the levels of TNF- α , IL-6, and IL-1 β relative to the ALI group.

In tandem, combining NAFT with DEXA during treatment may confer additional benefit for patients with ALI, since DEXA, as a glucocorticoid (GC), may exhibit resistance and poor treatment response (55). Research suggests that PPAR- α may increase the anti-inflammatory effects of GCs like DEXA in spinal cord injury (SCI) (56). Jointly, a prior investigation indicated that GC and PPAR α agonists reduced LPS-induced inflammation via inhibiting NF- κ B-mediated expression in A549 hepatocytes, hence fostering a sustained additive anti-inflammatory effect (57).

Concurrently, evidence suggests that obstructing pathways associated with resistance, such as “transcriptional activator protein-1 (AP-1)” and “macrophage migration inhibitory factor (MIF)”, may improve therapeutic outcomes in GC resistance (9). Thereby, DEXA resistance can be mitigated by activating SIRT1, which inhibits macrophage

AP-1 activity (58). Also, stimulating ATF3 may modulate DEXA resistance by facilitating macrophage migration and M1/M2 polarization, hence counteracting MIF effects (59). Thus, by activating the PPAR α /SIRT1/ATF3 pathway in this study, the ALI rats administered NAFT 50+DEXA could achieve the most favorable results in ameliorating lung injury.

Given the preceding, it is reasonable to anticipate that the rats in the NAFT 50+DEXA group exhibited the least histopathological alterations in their lung tissues compared to the ALI group. They displayed less bronchiole lining epithelium and alveolar epithelium, with only localized areas of intermediate thickness, few inflammatory cells, slight interstitial hemorrhages, and limited blood vessel congestion.

Conclusion

The regulatory targets and putative mechanisms by which NAFT exerts its therapeutic role against ALI, particularly when co-administered with DEXA, were identified in this study using network analysis, docking, and *in vivo* validation. By targeting the serotonergic system, NAFT may influence the PPAR α /SIRT1/ATF3 axis. The results revealed that NAFT inhibited the JAK2/STAT3 axis, leading to elevated PPAR α levels and decreased TGF β 1-induced fibrosis. PPAR α enhanced SIRT1-mediated HMGB1 deacetylation, reducing caspase-3 expression and apoptosis. Meanwhile, HMGB1 suppression activated AMPK/Nrf2/HO-1 and prevented oxidative stress. ATF3 release by AMPK stopped the TLR4/MyD88 cascade, which in turn inhibited the NF- κ B-dependent release of TNF α , IL-6, and IL-1 β cytokines. In addition, activating PPAR α /SIRT1/ATF3 signaling by NAFT can mitigate inflammation and potentially enhance the efficacy of DEXA treatment. Hence, NAFT represents a promising adjunctive therapy for the management of ALI, particularly in patients resistant to DEXA. Future research should explore the clinical implications of this approach and the mechanisms underlying DEXA resistance in ALI.

In light of recent reports on NAFT-mediated regulation of macrophage polarization, NLRP3/TLR4 signaling, and inflammatory pathways in LPS-induced lung injury (55, 56), the main added value of this work is the proposed and experimentally supported 5-HT2A/PPAR α /SIRT1/ATF3-centered mechanism and the evaluation of NAFT as an adjunct to DEXA.

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Availability of Data

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of

privacy or ethical restrictions.

Authors' Contributions

MM ET, ES H, E R, and SEA R conceived and designed the study. MM ET acquired the data. MM ET, ES H, and SEA R analyzed and/or interpreted the data. MME T, ES H, and SEA R drafted the manuscript. All authors critically revised the manuscript for important intellectual content and approved the version to be published.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

The authors further assert that no AI tools or technologies have been used to prepare this manuscript.

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Corrected Proof