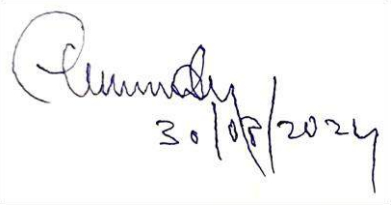
Novel Therapeutic Strategies in Treating Acute Respiratory Distress Syndrome (ARDS) and Pulmonary Fibrosis

Acute Respiratory Distress Syndrome (ARDS)

1. Nimbolide protects against endotoxin-induced ARDS by inhibiting TNF-α mediated NF-κB and HDAC-3 nuclear translocation

Nimbolide is a chemical constituent of *Azadirachta indica*, having multiple biological properties, while its role in ARDS is elusive. Nimbolide could be a potential lead molecule for treating various inflammatory diseases. Herein, we have investigated the protective effects of nimbolide in abrogating the complications associated with ARDS. We showed that nimbolide markedly suppressed the nitrosative-oxidative stress (DCFDA &mitosox), inflammatory cytokines, and chemokines expression by suppressing iNOS, myeloperoxidase, and nitrotyrosine expression in cell line (RAW264.7 & THP-1) **(Fig. 1A)**. Moreover, nimbolide mitigated the migration of neutrophils and mast cells whilst normalizing the LPS-induced hypothermia **(Fig. 1B)**. Also, nimbolide modulated the proinflammatory cytokines and chemokines (IL-6, IL-12 (p40), MIP-1α, MIP-1β, and TNF-α) and anti-inflammatory cytokines (IL-4, IL-10, and IL-13) expression **(Fig. 1C)**. Further, expression of epigenetic regulators with multiple HDAC inhibitory activity by suppressing the nuclear translocation of NF-κB and HDAC-3 **(Fig. 1D)**.

One of the earliest abnormalities seen in lung injury is the elevated levels of inflammatory cytokines, among them, the soluble tumor necrosis factor (TNF-α) has a key role, which exerts cytotoxicity in epithelial and endothelial cells and thus exacerbates edema. Therefore, we extended our studies using molecular docking studies, which demonstrated a strong interaction between nimbolide and TNF-α. Docking model of nimbolide in the active site of TNF-α (PDB ID: 2AZ5) and its ligand-protein interactions in the binding site of TNF-α. The dark pink dashed lines represent hydrogen bonds between heteroatoms of ligand and amino acid residues as follows: Ser60 (3.4Å) and Leu120 (3.2Å). The red line indicates arene-arene interaction with Tyr59 **(Fig. 1E).** Additionally, we observed that treatment with nimbolide increased GSH, Nrf-2, SOD-1, and HO-1 protein expression; concomitantly abrogated the LPS-triggered TNF-α, p38 MAPK, mTOR, and GSK-3β protein expression. Collectively, these results indicate that TNF-α-regulated NF-κB and HDAC-3 crosstalk was ameliorated by nimbolide with promising anti-nitrosative, antioxidant, and anti-inflammatory properties in LPS-induced ARDS **(Fig. 1E).** Our data provided a potential mechanistic link between the TNF-α or LPS-induced nuclear translocation of NF-κB and HDAC-3. This study emphasized



the potential role of nimbolide in inhibiting NF-κB and HDAC-3 translocation, thereby reducing inflammatory cytokines and maintaining redox balance, thus alleviating the inflammatory symptoms associated with ARDS conditions. This work was published in the journal **"Cell Death & Disease", 2019 (I.F.-8.1), 120 Citations**(1).

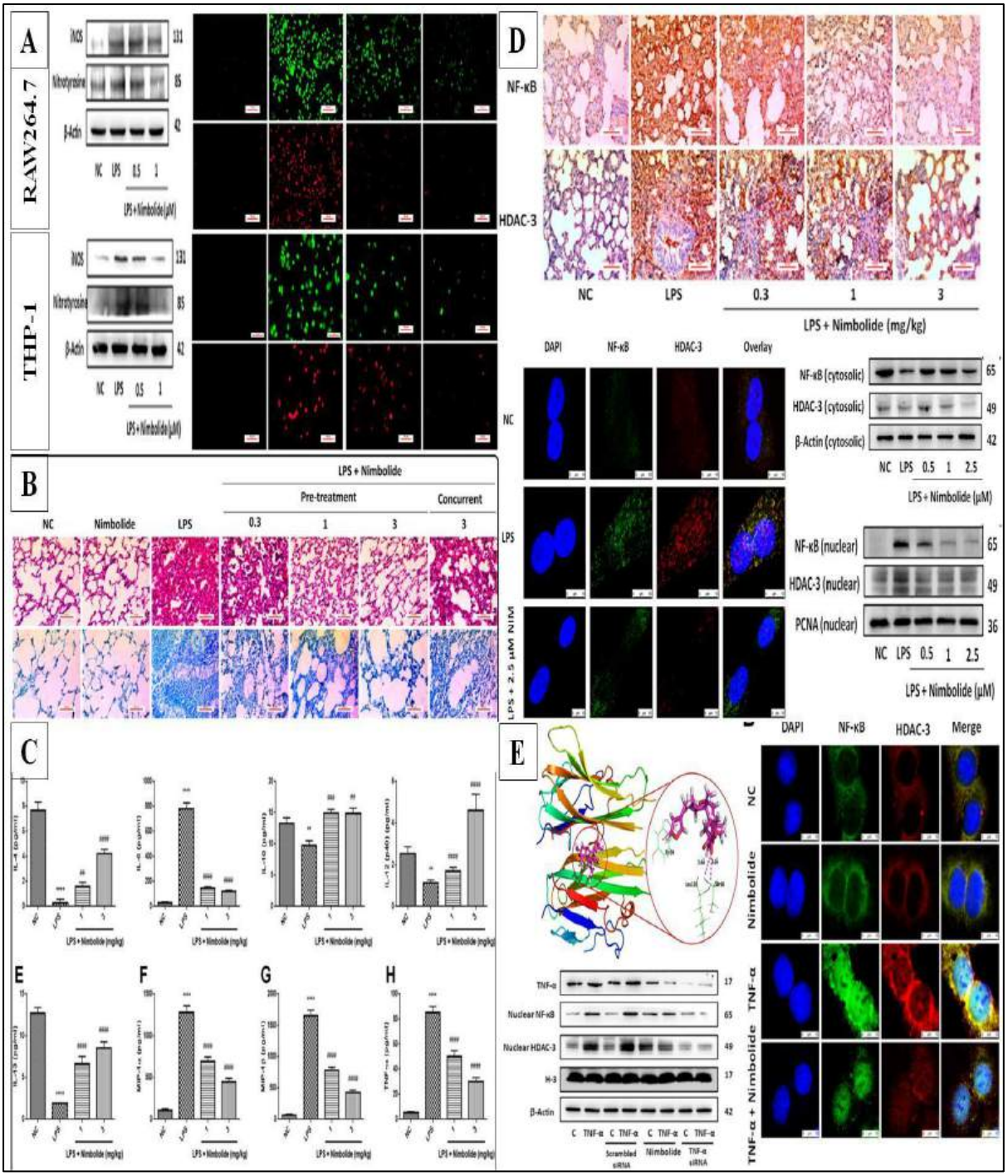


Figure 1: *Nimbolide protects against endotoxin-induced ARDS by inhibiting TNF-α mediated NF-κBandHDAC-3 nuclear translocation.*

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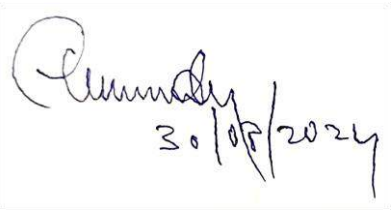
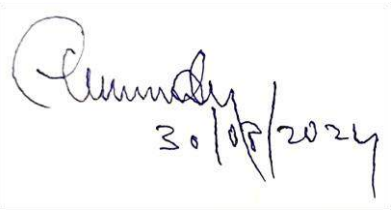
2. iRGD conjugated nimbolide liposomes protect against endotoxin-induced acute respiratory distress syndrome

iRGD is a cyclic peptide that preferentially inhibits ligand binding to integrins with an RGD recognition specificity and suppresses their function by selectively targeting the inflammation sites. For efficient lung targeting and treating ARDS, we designed NIM-encapsulated liposomes conjugated with iRGD peptide. The homing is achieved through a mechanism of selective binding of iRGD to integrins, which are overexpressed in inflammatory conditions. In the present study, we found that iRGDLip, NIMLip, and iRGD-NIMLip exhibited nanosize were negative in charge, and are spherical with clear liposomal compartments **(Fig 2A)**. To improve the therapeutic response with less deleterious effects, the formulation was opted to be delivered through the oropharyngeal route to improve the effectiveness at low doses with minimal accumulation in other organs.

LPS challenge leads to the influx of protein-rich edematous fluids into the lungs, accompanied by the recruitment of inflammatory cells. In agreement with the previous reports, we found a significant loss in the integrity of the lungs with LPS instillation. Remarkably, we observed that treatment counter-regulated the LPS-mediated inflammatory response by suppressing the BAL (Broncho alveolar lavage) total cells, neutrophils, platelets, procalcitonin (PCT), absolute lymphocytes, basophils, monocytes, eosinophils, and white blood cells (WBC) **(Fig. 2D).** H&E staining images inferred that iRGD has normalised the LPS induced thickening of the alveolar wall and accumulation of inflammatory cells **(Fig. 2D).** LPS stimulation in the later phases induces TRAF-6 mediated excessive TNF-α activation through an accompanying switch in mechanisms such as NF-κB, MAPK, and Akt signaling, which was significantly reduced by iRGDNIMLip in macrophages and lung tissue **(Fig. 2B, 2F)**. Accumulating evidences suggest that; the other inflammatory mediators such as COX-2 &iNOS and HIF-1α that enhances the production of pro-inflammatory cytokines especially IL-6 and TNF-α were remarkably suppressed by this novel formulation **(Fig. 2B, 2F)**. Additionally, PI3K-Akt-mTOR signaling that participates in the activation of various inflammatory genes was reversed by iRGD-NIMLip in BEAS-2B cells **(Fig. 2C),** which leads to downregulation of the LPS-induced inflammatory cascade. Furthermore, it reversed the phosphorylation of MAPK and GSK-3β signaling, inhibited STAT3 expression, and disrupted this complex with DNMT1, a beneficial effect in dampening inflammatory cascade responses **(Fig. 2F)**.iRGD-NIMLip abrogated the LPS induced p65 NF-κB, Akt, MAPK, Integrin β3 and β5, STAT3, and DNMT1 expression.



It is noteworthy that iRGD-NIMLip potently boosted the efficacy with remarkable disruption of this reciprocal crosstalk and inhibited a spectrum of cytokines secretions as compared to f-NIM and could be a promising therapeutic option to combat the inflammation associated with ARDS. Generally, the majority of ARDS and COVID-19-associated cases of pneumonia share common pathophysiology like pulmonary edema and oxidative stress, whereas our novel drug delivery system iRGD-NIMLip may protect COVID-19-associated ARDS by suppressing the cytokine storm. Future studies in suitable animal models to simulate the SARS-CoV-2 induced ARDS may provide further evidence of possible clinical translation of our approach to protect patients from COVID-19-associated respiratory complications. This research was published in "**Nanomedicine journal", 2021 (I.F.-4.2), 17 Citations**(2).



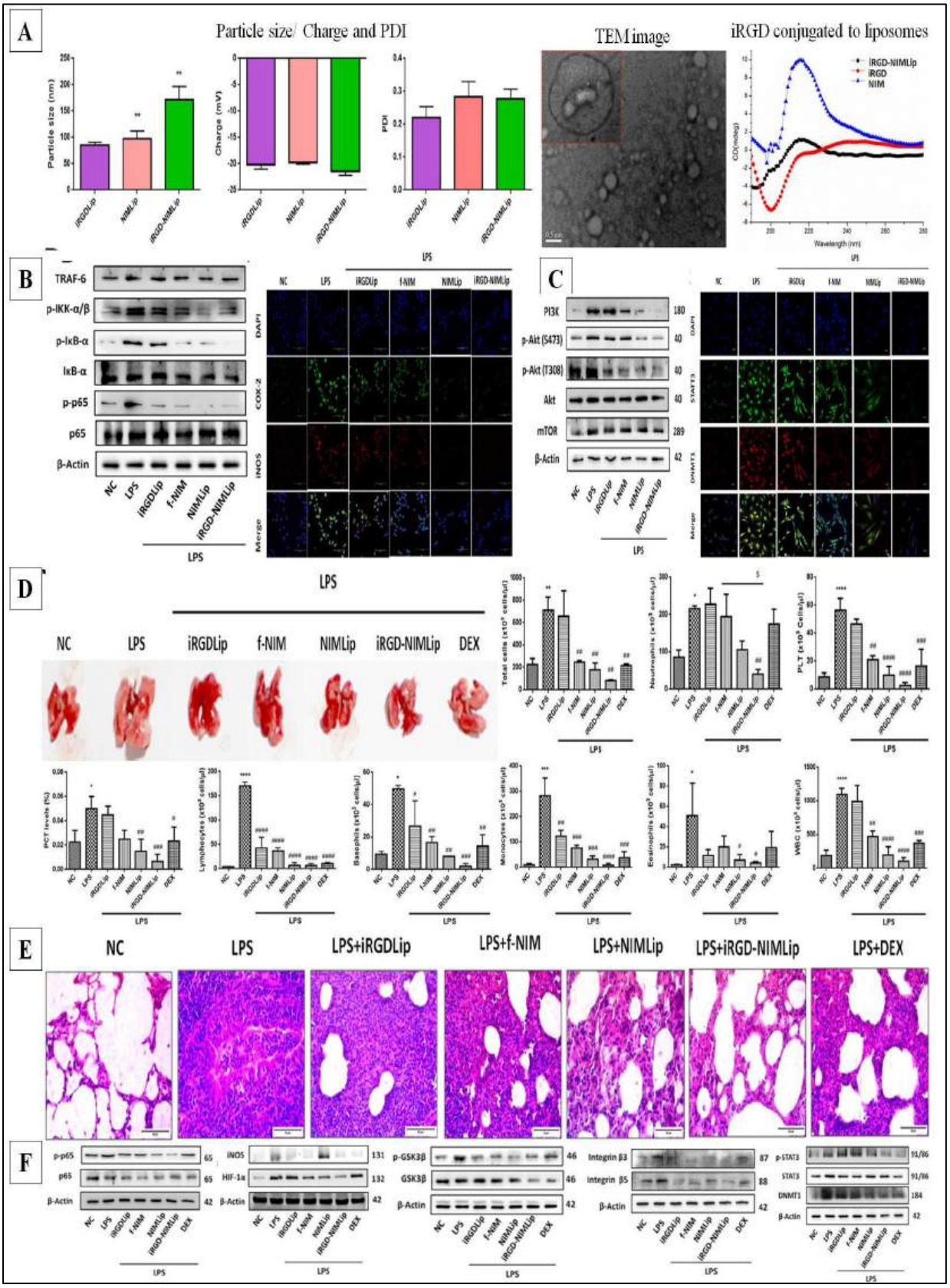


Figure 2: *Demonstration of the protective effect of iRGD-conjugatednimbolide liposomes against endotoxin-induced ARDS.*

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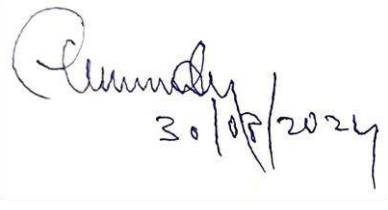
3. BRD4 targeting nanotherapy prevents lipopolysaccharide-induced acute respiratory distress syndrome

BRD4 is implicated in several lung disease conditions such as chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, lung cancer, and, asthma. Moreover, SARS-CoV-2 transmembrane protein E binds with BRD2 and BRD4, thus, targeting BRD4 may inhibit the viral fusion into host cells as well as respiratory illness. These findings raise an intriguing possibility that BRD4 might be involved in ARDS conditions. In the present study, we demonstrated for the first time that BRD4 has a predominant role in exacerbating inflammatory conditions and hence promoting ARDS. Furthermore, in *in vitro* and preclinical ARDS models were used to study and explain BRD4 suppression with the pharmacological inhibitor PFI-1 and BRD4 targeted nanotherapy (siRNA). Where we have developed the BRD4 siRNA lipoplexes (BRD4-siRNA-LP) by thin-film hydration method and characterization showed nano size and exhibited positive charge with acceptable PDI **(Fig. 3B)**. Further, these siRNA lipoplexes were evaluated in LPS (Lipopolysaccharide)induced *in vitro*and*in vivo*modelconsidering PFI-1 as a standard BRD4 inhibitor. Apart from LPS-stimulated macrophages, over-expression of BRD4 was observed by plasmid (pcDNA5-Flag-BRD-4-WT) in THP-1 macrophages which was significantly down-regulated by PFI-1 **(Fig. 3C)**. Consistent with this expectation, after LPS oropharyngeal instillation in mice, BRD4-siRNA-LP suppressed the LPS induced total cells, WBC, neutrophils, monocytes, and basophils counts, and protected the epithelial barrier dysfunction **(Fig. 3I)**. BRD4-siRNA-LP reversed the LPS induced BRD4 expression, morphological alterations and cytokine storm in lungs by significantly suppressing pro-inflammatory cytokine levels (IL1β, IL-6, IL-17A, IL22, and TNF) and protected the lung from immune cell-mediated cytokine storm **(Fig. 3F-H)**. SARS-CoV-2 activates p65 NF-κBsignalingsimilar to SARS-CoV and MERS-CoV, which is critical for pulmonary inflammation and associated series of events. In our experimentation, we observed that LPS induced the phosphorylation of IKK-α/β, IκB-α, and p65, whereas this effect was significantly inhibited by BRD4-siRNA-LP **(Fig. 3E)**. We also noticed that BRD4 inhibition suppressed the STAT3 phosphorylation and BRD4 lipoplexes significantly inhibited p65 and STAT3 and further reduced the activation of inflammatory proteins expression **(Fig. 3A, E,J)**.

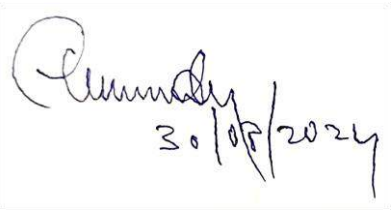
Inhibition of BRD4 by BRD4-siRNA-LP might play an important role in treating clinical COVID-19-ARDS patients potentially through anti-inflammatory and anticytokine storm properties by controlling p65 and STAT3 nuclear translocation. This could be a promising novel target with the potentially advantageous delivery systems, which may be considered as a plausible therapeutic option for treating COVID-19 induced ARDS. Further, analogues of

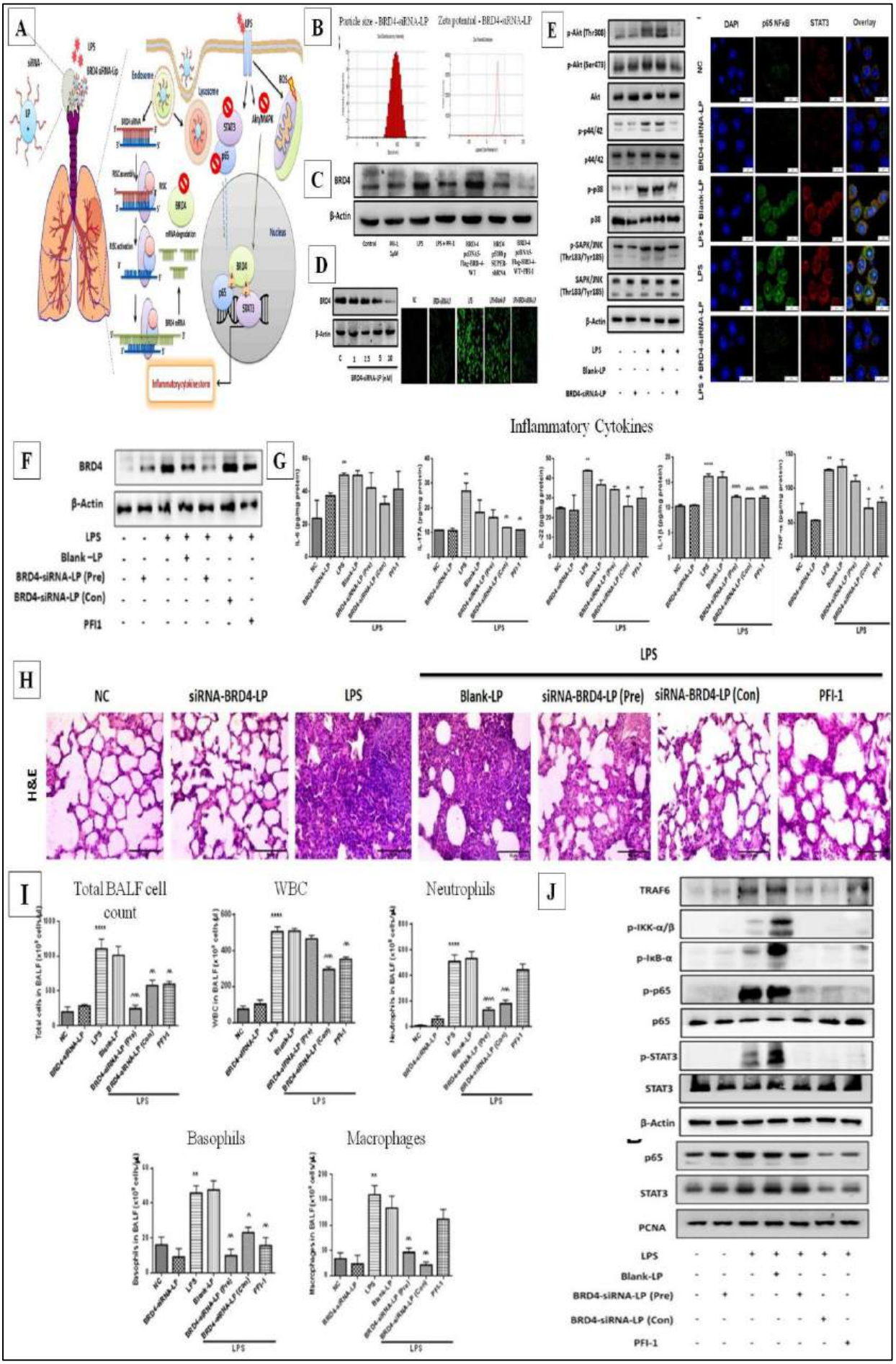


ZL-0420, PFI-1, and IBET-762 potent BRD4 inhibitors with nanomolar binding affinities to bromodomains have been synthesized in collaboration with the medicinal chemistry department. This research outcome has been published in the "**International Journal of Pharmaceutics", 2021 (I.F.-5.3), 20 Citations**(3).

Role of BRD4 in the tumor microenvironment:

Chronic inflammatory disease enhances cancer cell proliferation, progression, and invasion. Effect of acute lung inflammation on the activation and enhancement of lung metastasis in LPS-induced *in vitro* and *in vivo* models. Respiratory illness is mainly caused by cytokine storm, which further influences oxidative and nitrosative stress. The LPS-induced inflammatory cytokines made the conditions suitable for the tumor microenvironment in the lungs. In this study, we observed that LPS induced the cytokine storm and promoted lung inflammation via BRD4, which further caused the nuclear translocation of p65 NF-κB and STAT3 **(Fig. 3L)**. The transcriptional activation additionally triggered the tumor microenvironment and lung metastasis. Thus, BRD4-regulated p65 and STAT3 transcriptional activity in ARDS enhanced lung tumor metastasis. Moreover, LPS-induced ARDS might promote the tumor microenvironment and increase cancer metastasis into the lungs. Collectively, BRD4 plays a vital role in inflammation-mediated tumor metastasis and is found to be a diagnostic and molecular target in inflammation-associated cancers. This work was published in the Journal of **"International Immunopharmacology"**, **2023 (I.F.-4.8), 5 Citations**(4).

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BRD4
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p-SAPK/JNK (Thr183/Tyr185)
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Blank-LP
BRD4-siRNA-LP
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p65 NFκB
STAT3
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LPS + BRD4-siRNA-LP
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Macrophages
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TRAF6
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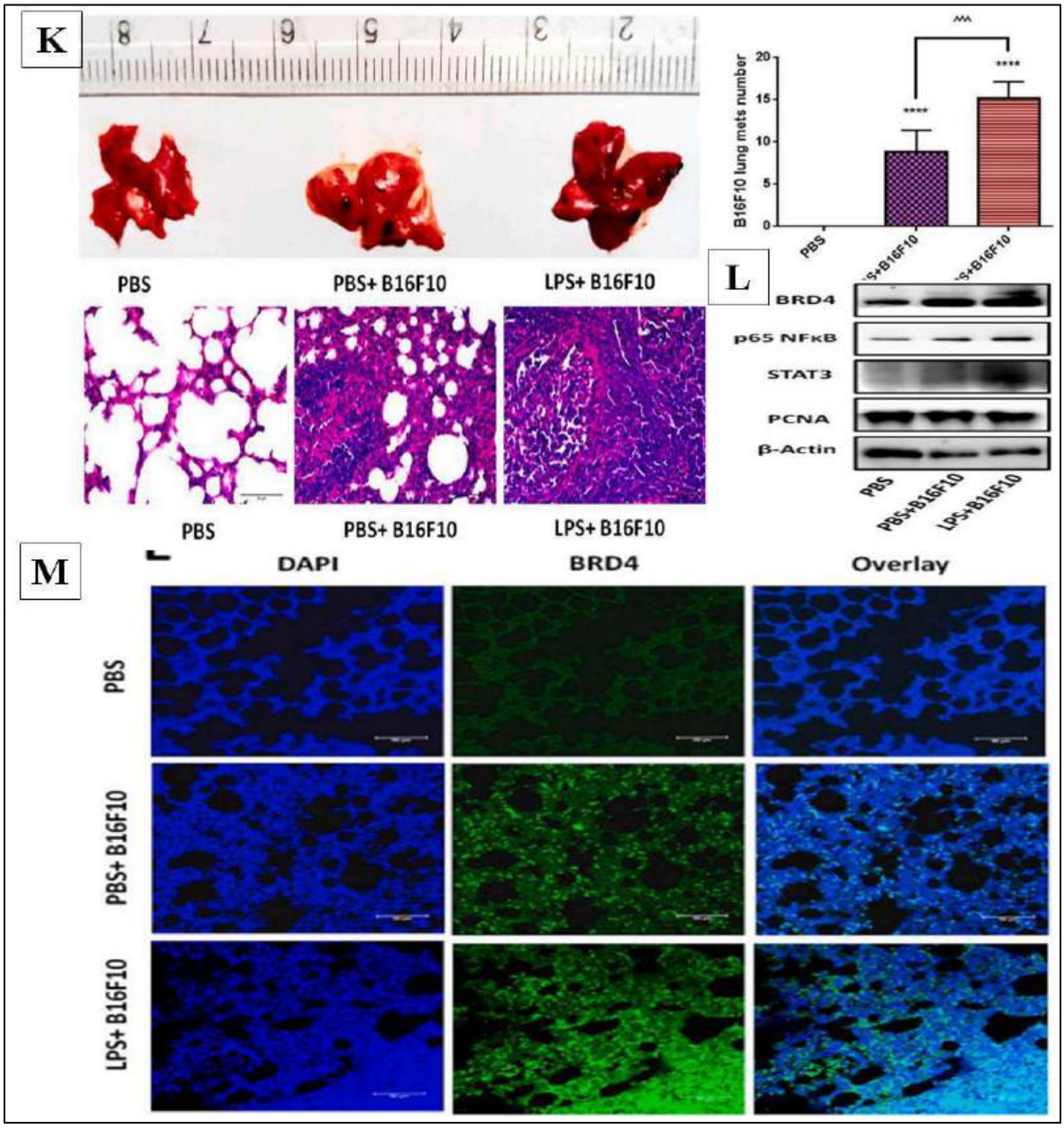

Figure 3: *BRD4 targeting nanotherapy prevents LPS-induced ARDS and* BRD4 plays a vital role in inflammation-mediated tumor metastasis.

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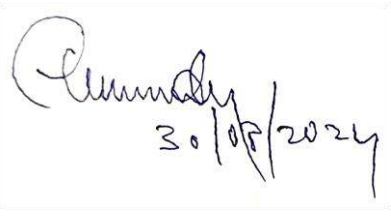
4. Development of Novel Therapeutic Molecules Targeting PAD-4 for the Management of ARDS

NETosis, one of the critically conserved processes for anti-microbial defense that is released by activated neutrophils to trap, immobilize and kill the invading pathogen. While NETs play a crucial role in host defense and pathogen clearance during infection; dysregulation of NETs can lead to autoimmune and inflammatory disorders. The onset of ARDS is triggered by the formation of neutrophil extracellular traps (NETs) released by activated neutrophils due to the enzymatic activity of peptidyl arginine deiminase-4 (PAD-4) in the lungs.

In this study, we evaluated the anti-NETotic and anti-inflammatory effects of novel chemical entities (NCEs) against PAD-4 *in-vitro* (HL-60 & Beas-2B) and *in-vivo*. From 23 synthesized molecules, four (35a, 36a, 36e, 36j) were selected based on cytotoxicity and kit-based anti-PAD-4 activity, with 36e showing the best IC_{50} for enzyme inhibition **(Fig. 4B)**. These molecules reduced NETs release, myeloperoxidase (MPO) and NETotic markers in PMA-induced dHL-60 cells **(Fig. 4C)**. Inflammation was induced in BEAS-2B lung epithelial cells using NETs isolated from PMA-treated and PMA-NCE-treated dHL-60 cells, with NETs from NCE-treated cells reducing oxidative stress and inflammatory markers, and 36e being the most effective **(Fig. 4D)**. Further, we evaluated the anti-inflammatory effects of 36e in a lipopolysaccharide (LPS)-induced ARDS mouse model. Pre-treatment with 36e (oral and IP) followed by LPS-induced inflammation showed improved lung architecture and lung function assessment revealed significant normalization of critical parameters, demonstrating the inhibitor's ability to restore respiratory functions **(Fig. 4F)**. Moreover, lung morphology, showed remarkable improvements, solidifying the inhibitor's potential to ameliorate the disease condition and lower expression of inflammatory markers. Notably, the 36e inhibitor's potency was underscored by analyzing MPO levels and expression of NETosis marker in lung tissue, revealing a remarkable reduction in treated animals, reaffirming its efficacy in combating ARDS with oral administration showing better anti-NETotic activity **(Fig. 4G-I),** where it corroborated better activity through oral dose. These compelling findings offer hope for both patients and medical communities, as our novel PAD4 inhibitor holds immense promise and could potentially revolutionize the management of this life-threatening condition.



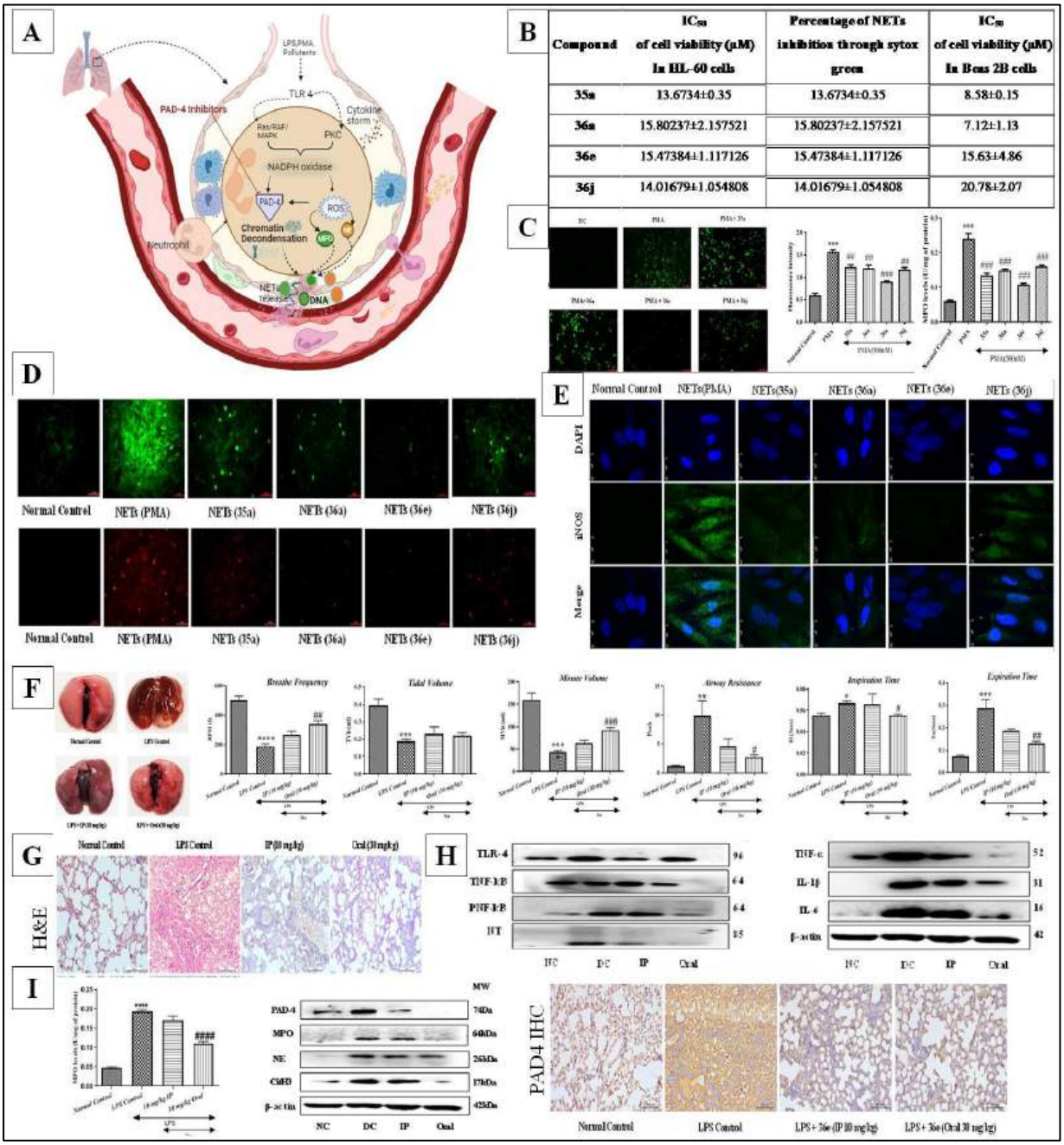


Figure 4: Representation of *novel therapeutic molecules targeting PAD-4 for the treatment of ARDS.*

5. Development of novel nanoceria decorated DNase nanoparticle targeting NETs for the management of ARDS

Neutrophils exert their anti-microbial activity by virtue of neutrophil extracellular traps (NETs) release known as NETosis which traps and kills pathogens. Dysregulation in the NETs activity lead to several inflammatory disorders. Control of NETs is becoming a target for therapeutics in the management of various inflammatory and auto-immune diseases. NETs are web-like structures released by activated neutrophils consisting of decondensed chromatin with an aim to neutralise and terminate invading pathogens. To open the possibility of exploring agents that target NETs, we have prepared a smart nanomedicine

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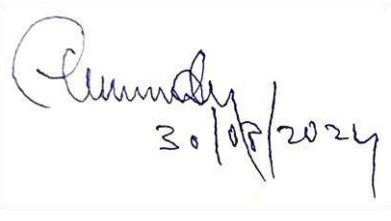
‘nanoceria-coated with polydopamine and DNase-1 (NPD)’ by using excellent bio-adhesive property of mussel-like polydopamine. We have decorated the PDA coated nanoceria with a layer of DNase-1 enzyme which is an endogenous nuclease effectively degrading the extruded NETs which are nothing but DNA in the form of decondensed chromatin. This nanoformulations has a holistic way of approach which targets not only one but multiple pathway in the inflammatory cascade of reactions.

In lung functional tests using whole-body plethysmography, LPS-induced alterations in breath frequency, tidal volume, minute volume, airway resistance, inspiration, and expiration time were effectively normalized by nanoformulations treatment, particularly with NPD **(Fig. 5A)**. Oro-pharyngeal LPS administration resulted in significant weight loss, increased lung weight, and size, which were markedly restored to normal levels by pre-treatment with NPD, indicating its superior efficacy **(Fig. 5B)**. NPD pre-treatment significantly decreased total cell count and lymphocyte/neutrophil percentages in BALF, while also mitigating LPS-induced albumin increase, a marker of lung injury, more effectively than other pre-treatment groups **(Fig. 5C-F)**. Neutrophil NETotic ability, increased in the LPS group, was majorly mitigated by all pre-treatment groups, particularly NPD and DNase. Pro-inflammatory cytokines (TNF-α, IL- 1β, IL-6) increased 2-4 folds in LPS-treated animals, effectively ameliorated and normalized by NP pre-treatment, with NPD showing better activity **(Fig. 5G-I)**. Histologically, LPS control group lungs exhibited distorted morphology with inflammation and damage to alveoli septa. NP pre-treatment resulted in better lung histology, resisting LPS-induced changes **(Fig. 5J)**. Immunohistochemical studies revealed increased expression of TNF-α and NF-κB in LPS-treated animals, reduced by NP pre-treatment, particularly NPD **(Fig. 5K-L)**. Immunofluorescence studies on NETotic markers showed a 3-4 fold increase in LPS-treated lungs, significantly reduced by NP pre-treatment, with NPD exhibiting the most reduction **(Fig. 5N)**. Blot analysis reflected increase in pro-inflammatory cytokines in LPS-treated animals, effectively normalized by NP pre-treatment, with NPD showing maximum inhibition **(Fig. 5M)**. These comprehensive findings underscore the potent anti-inflammatory and protective effects of NPD, positioning it as a promising therapeutic candidate for mitigating lung injury and inflammation induced by LPS. These encouraging results suggest that NPD may hold promise as a potential therapeutic approach to counter acute inflammatory conditions like ARDS which is commonly observed in severe respiratory failures in conditions like COVID-19 and sepsis.



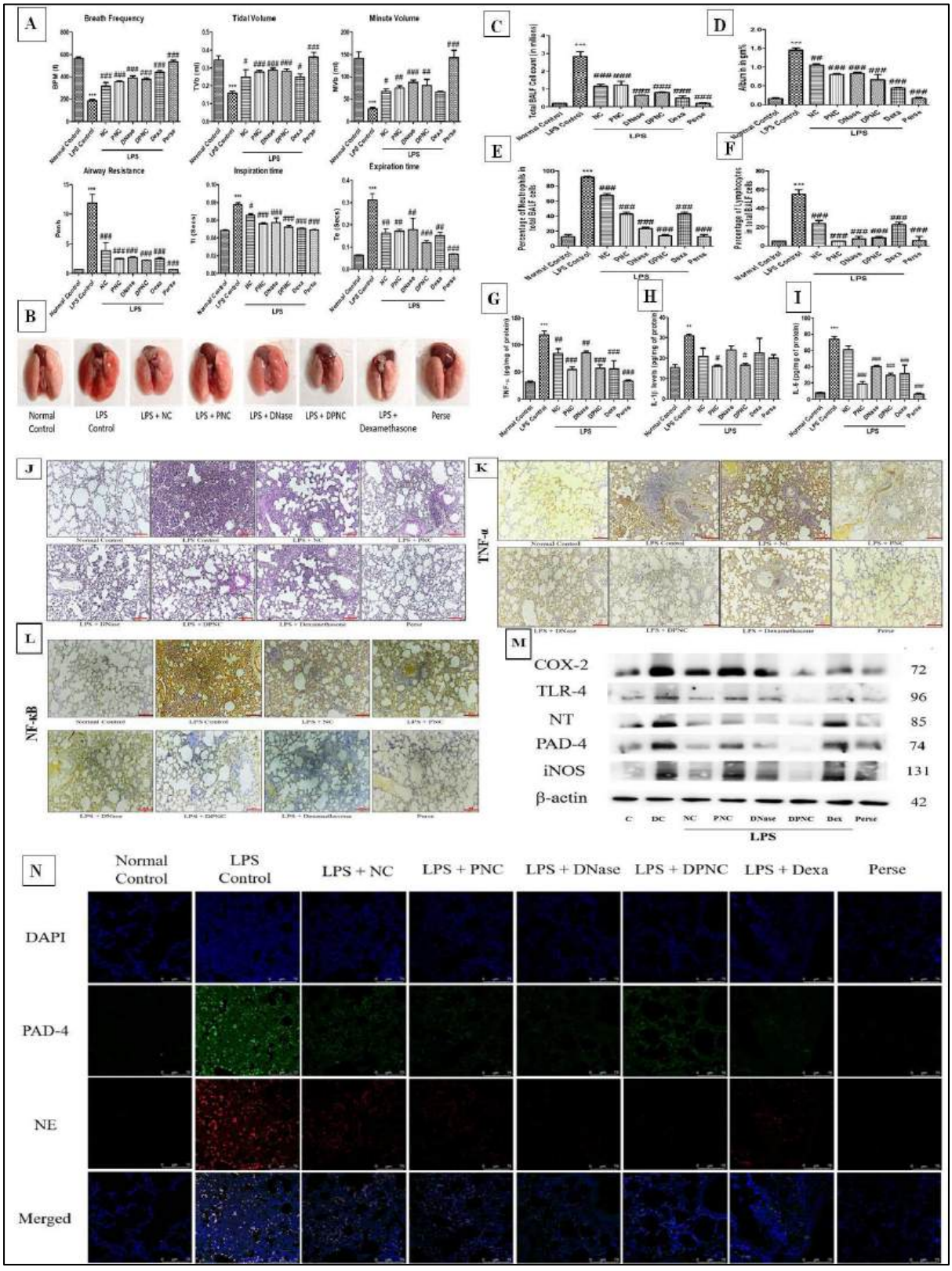


Figure 5: *Schematic representation of novel nanoceria decorated DNase nanoparticle targeting NETs for the management of ARDS.*

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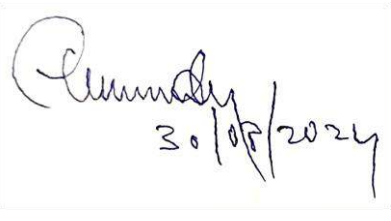
Pulmonary Fibrosis

6. An adaptogen: withaferin A ameliorates *in vitro* and *in vivo* pulmonary fibrosis by modulating the interplay of fibrotic, matricellular proteins, and cytokines

Withaferin A (WFA) is a natural immunomodulator and a pre-dominant bioactive constituent obtained from *Withania somnifera* (Ashwagandha) exhibiting an array of potential biological activities including anti-inflammatory, anti-invasive, pro-apoptotic, and anti-fibrotic effects and is remarkably safe. Thus, the present study is aimed at demonstrating the role of WFA in mitigating pulmonary fibrosis (PF), a chronic lung disease with only two Food and Drug Administration (FDA) approved clinically available drugs, with limited safety profiles. Inadequate therapy motivated us to explore the effect of vimentin inhibitor Withaferin A, as an anti-fibrotic agent against TGF-β1-induced *in vitro* fibrotic events and bleomycin-induced *in vivo* fibrosis with an emphasis on epithelial to mesenchymal transition (EMT), extracellular matrix deposition (ECM), inflammation, and angiogenesis. In vitro EMT and fibrotic events were induced by TGF-β1 in alveolar epithelial cells and human fetal lung fibroblasts followed by treatment with Withaferin A (0.25, 0.5, and 1 µM concentrations) to explore its anti-fibrotic effects **(Fig. 6A)**. *In vivo* potential of Withaferin A (2 and 4 mg/kg) was assessed in a murine model of bleomycin-induced PF. All the parameters and molecular studies related to PF were performed at the end of the treatment period. WFA exerts anti-fibrotic effects by modulating histological changes, we performed Hematoxylin and Eosin (H&E) staining to observe key morphological alveolar changes, while Masson's trichrome and picrosirius red stains and hydroxyproline assay were performed to evaluate collagen deposition **(Fig. 6B)** . WFA ameliorated the expression of inflammatory cytokines including NF-κB p65, IL-1β and TNF-α, as well as attenuated the expression of pro-fibrotic proteins including CTGF, collagen 1A2, collagen 3A1, and fibronectin **(Fig. 6C-D)**. Expression of angiogenic factors like VEGF, FAK, p38 MAPK, and PLC-γ1 was also inhibited by Withaferin A **(Fig. 6D)**. Phosphorylation of Smad 2/3 which was induced by TGF-β1 and bleomycin were significantly inhibited **(Fig. 6D)**. Taken together, the present study throws light on the significance of interplay of various pro-fibrotic and cellular matrix proteins, pro-inflammatory cytokines, and angiogenic factors in pathogenesis of PF and emphasizes on targeting such integral cellular processes for heading towards the development of effective therapeutic interventions. WFA could fit into the role of mitigating PF by inhibiting EMT and ECM progression, inflammation, and angiogenesis. However, further decisive studies are required to establish WFA as a promising therapeutic intervention for treating PF. This



research has been published in **"Frontiers in Pharmacology", 2018 (I.F.-4.4), 69 Citations**(5).

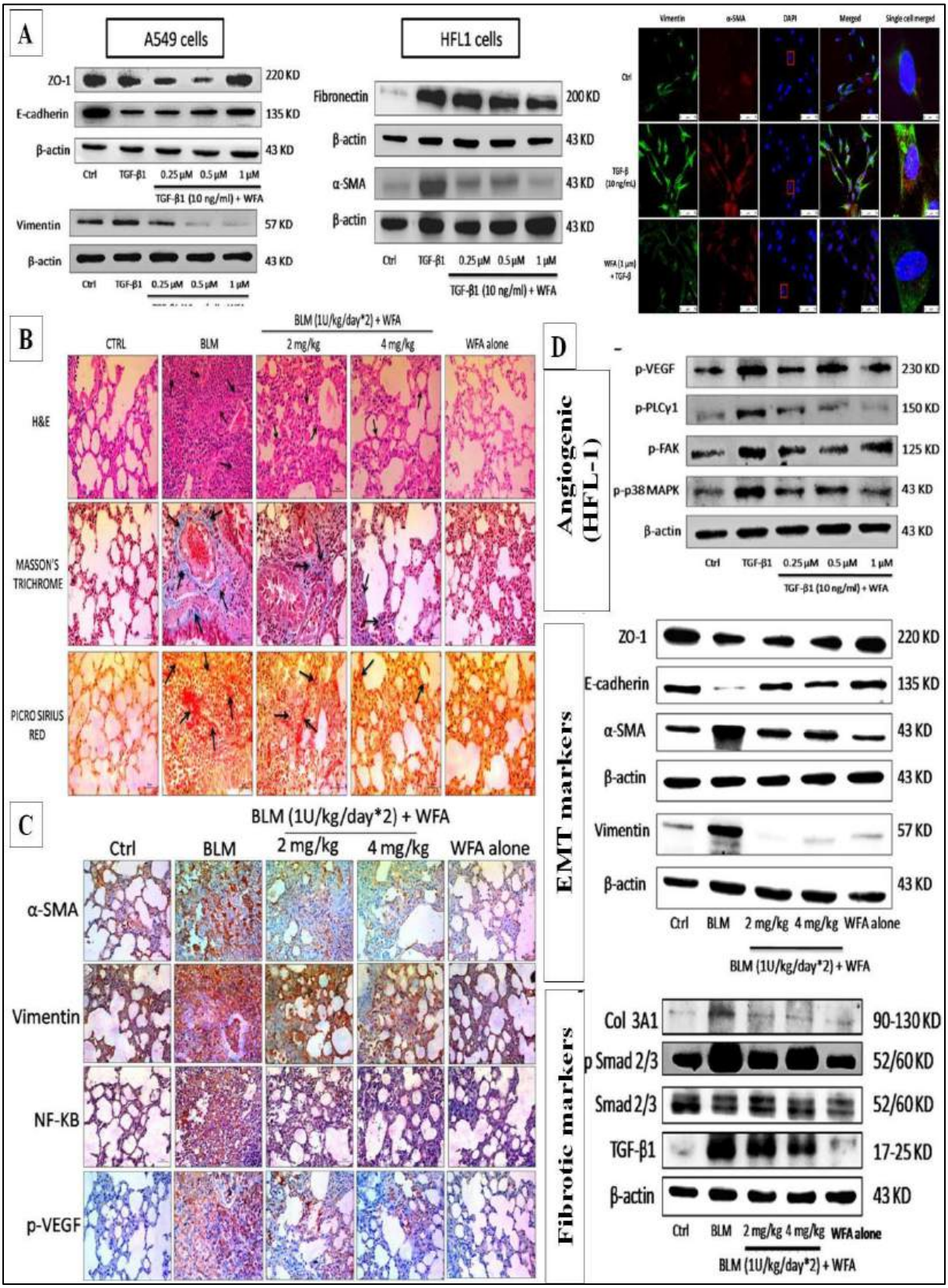


Figure 6: *WFA ameliorates in vitro and in vivo pulmonary fibrosis by modulating the interplay of fibrotic, matricellular proteins, and cytokines.*

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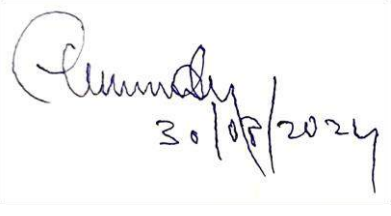
7. Therapeutic effects of Nimbolide, an autophagy regulator, in ameliorating pulmonary fibrosis through attenuation of TGF-β1 driven epithelial-to-mesenchymal transition

Autophagy is a catabolic intracellular pathway that maintains cellular homeostasis, which is involved in many disorders including fibrotic diseases. Our study was oriented on demonstrating the anti-fibrotic effects of NIM with a slight emphasis on the regulation of fibrosis-related autophagy. Nimbolide (NIM) is a bioactive molecule that has garnered significant attention due to its ability to interact with multiple molecular targets such as growth factors, their receptors, transcription factors, protein kinases, and genes that regulate crucial cellular processes like proliferation and apoptosis.

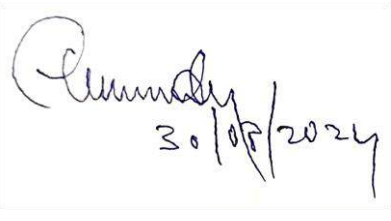
To gain insight into the underlying mechanisms by which NIM attenuated EMT, we investigated EMT-related changes *in vitro* in human type II alveolar epithelial A549 and lung fibroblast HFL1 cells due to predominant EMT occurrence in both cell lines. While whole fibrotic events were explored only in HFL1 cells due to inherent lung matrix homeostasis. NIM suitably modulated the expression of EMT markers (α-SMA, E-cadherin, fibronectin, and ZO-1) by affecting the fibroblast differentiation into myofibroblasts. TGF-β1 stimulation in A549 and HFL 1 cells resulted in the alteration of EMT and fibrotic markers, whereupon NIM treatment restored the levels in A549 cells and HFL1 cells (**Fig. 7A**). This implies that NIM inhibits various stages in the process of EMT. To understand the effect of NIM on TGF-β1 and its downstream fibrotic signaling, we studied NIM in an experimental model of bleomycin (BLM) induced pulmonary fibrosis. As evident from various literature reports, over-expression of TGF-β1-induced pro-fibrotic CTGF expression mediates the upregulation of collagen deposition and dramatic thickening of perivascular regions of the lungs. A prominent decrease in collagen expression was observed upon treatment with NIM that was reflected from *in vivo* immunoblot of Col 3A1 and Smad which was consistent with reduced lung/body weight index by NIM as evident from macroscopic findings and BAL analysis. Microscopic evidence was accumulated by H&E, Masson's trichrome and Sirius red tissue staining. Also, NIM was capable of sharply decreasing the increased levels of hydroxyproline *in vivo***(Fig. 7B)**. These results satisfactorily convinced that abnormal collagen accumulation was blocked by NIM.

Mounting evidence displays role of autophagy in pathogenesis of PF as ambiguous with few sources stating it as a contributor to pathology of PF and certain other reports stating it to be otherwise. In the present study, we determined the role of NIM in regulating autophagy



proteins Beclin 1, LC-3A/B-I/II and p-62 through various protein expression studies including immunohistochemistry of LC-3, p-62 and Beclin 1, Bcl2 via immunoblot in lung tissue **(Fig. 7C)**. Treatment with NIM enhanced autophagy vacuoles in perinuclear regions of TGF-β1 stimulated HFL 1 cells as evident from monodensylcadaverine (MDC). NIM significantly dissipated the expression of p-62 in both TGF-β1 treated cells and also in cells that received a combination of TGF-β1 and chloroquine indicating that autophagy flux was altered by NIM **(Fig. 7D)**. These observations concerning the regulation of autophagy proteins imply that NIM stimulates autophagy.

In conclusion, *in vitro* and *in vivo* assays performed in the present study sufficiently demonstrate NIM as a very promising anti-fibrotic molecule that impedes the EMT pathway, reasonably blocks TGF-β/Smad and its downstream signaling events, thus inhibiting collagen deposition and substantially modulates major proteins involved in the autophagy signaling cascade. Further understanding of the precise mechanisms of NIM is required. Our study prompts research on the use of natural products like NIM with favorable safe properties as a pharmacological intervention for PF. This work was published in the Journal of **"International Immunopharmacology", 2019 (I.F.-4.8), 28 Citations**(6).



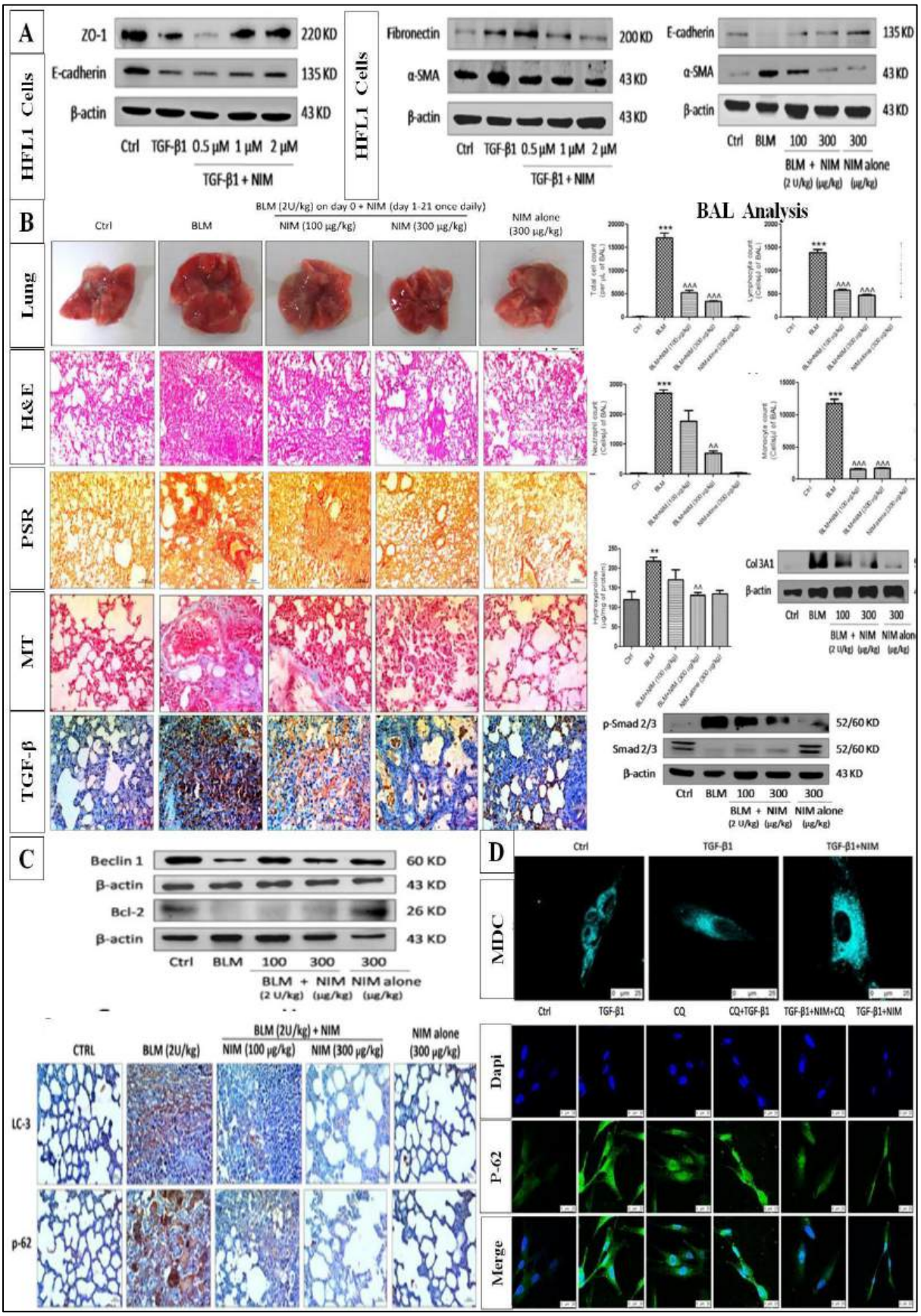


Figure 7: *Data representing the effectofnimbolide in ameliorating pulmonary fibrosisthrough attenuation of TGF-β1 driven EMT and regulating autophagy.*

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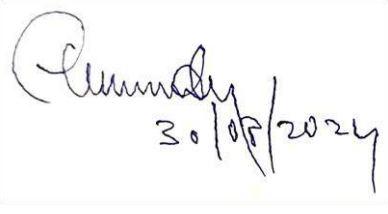
8. Effect of Honokiol (HNK) on IL-6/CD44/STAT3 axis and TGF-β/Smad signalling in both *in vitro* and *in vivo* models of Pulmonary fibrosis

Apart from central mechanisms of TGF-β/Smad signaling and EMT, there also exist other signaling cascades which govern the pathogenesis of pulmonary fibrosis (PF).. CD44/IL-6/STAT3 axis is one such signaling cascade gaining importance as perpetrator in pathogenesis of PF. CD44 is a transmembrane glycoprotein belonging to the family of adhesion molecules known to be multistructural and multifunctional in nature which is implicated in regulation of various adhesion dependent cellular processes like cell proliferation, migration and differentiation. Abridging its role in fibrogenesis, recent research suggests that CD44 keeps the ability to activate latent TGF-β, increases fibroblast proliferation and contribute to the propagation of EMT. Specifically in fibrogenesis, IL-6 and CD44 promote the phosphorylation of STAT3 and its translocation to nucleus for further gene transcription. Additionally, STAT3 controls crosstalk between epithelial cells and fibroblasts which in turn contributes to disease progression. Honokiol (HNK) ((3′,5-di-(2-propenyl)-1,1′-biphenyl-2,2′-diol)), a polyphenol neolignan derived from barks, seed cones and leaves of Magnolia officinalis. HNK is a pleiotropic bioactive lignan with diverse pharmacological effects like anti-tumorogenic, anti-angiogenic, anti-bacterial, anti-diarrhoeal, anti-thrombosis, anti-inflammation, anxiolytic and anti-stress activities. These findings raise an intriguing possibility that HNK might be a novel approach in PF. We for the first time attempted to demonstrate the role of HNK in modulating TGF-β mediated CD44/IL-6/STAT3 axis for exerting anti-fibrotic effects.

This study has been conducted in TGF-β1 induced *in vitro* model and 21 21-day *in vivo* murine model of Bleomycin (BLM) induced PF. The findings of our study suggest that HNK was able to inhibit fundamental pathways of epithelial to mesenchymal transition (EMT) and TGF-β/Smad signaling both *in vitro* and *in vivo***(Fig. 8A-B)**. Additionally, HNK also attenuated collagen deposition and inflammation associated with fibrosis **(Fig. 8C)**. We also hypothesized that HNK interfered with IL6/CD44/STAT3 axis. As hypothesized, HNK significantly mitigated the IL-6/CD44/STAT3 axis both *in vitro* and *in vivo* as evident from outcomes of various protein expression studies like western blotting, immunohistochemistry and ELISA **(Fig. 8D)**. Taken together, it can be concluded that HNK reversed pulmonary fibrotic changes in both in vitro and *in vivo* experimental models of PF and HNK showed promising inhibition of critical pathways involved in pathogenesis of PF such as EMT, TGFβ/Smad signaling and ECM deposition. Additionally, HNK also attenuated CD44/IL-6/STAT3 signaling cascade which also appreciably contributed to the regression of fibrotic



changes. This work was published in the **"Toxicology and Applied Pharmacology Journal", 2020 (I.F.-3.3), 42 Citations**(7).

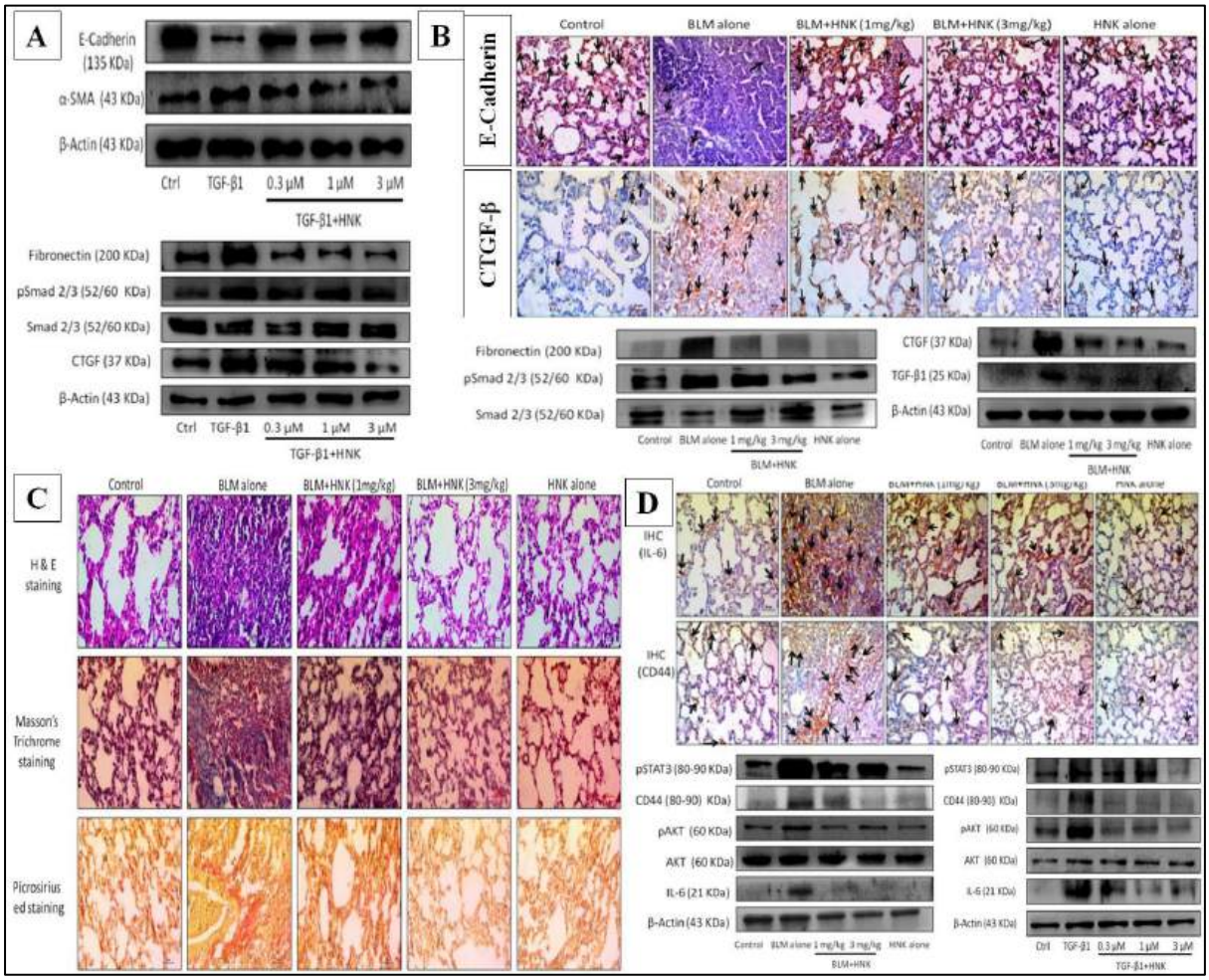


Figure 8: *HonokioltargetingTGF-β/Smad signaling and IL-6/CD44/STAT3 axis in both TGF-β induced in vitro and bleomycin-induced in vivo models ameliorates pulmonary fibrosis.*

9. Niclosamide alleviates pulmonary fibrosis in vitro and in vivo by attenuation of epithelial-to-mesenchymal transition, matrix proteins & Wnt/β-catenin signaling: A drug repurposing study

Evidences from existing literature are strong enough to demonstrate beyond doubt the aberrant activation ofWingless/Int (WNT)/β-catenin signaling associated with downregulation of endogenous WNT antagonists inPF. First report of WNT and it effector β-catenin involvement in fibrogenesis was established several yearsago, since then various studies have proven the pathogenic role of this signaling cascade in development ofpulmonary fibrosis. Additionally, nuclear accumulation of β-catenin is known to induce the process of EMT infibroblasts.

Our current study is aimed at repurposing the old anti-helimenthic drug Niclosamide as an antifibrotic drug against pulmonary fibrosis (PF). PF is most common lethal interstitial lung disease hallmarked by deposition of extracelluar matrix and scarring of lung. Heterogenous nature, untimely diagnosis and lack of appropriate treatment options make PF an inexorable lung disorder. Prevailing void in PF

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treatment and drug repositioning strategy of drugs kindled our interest to demonstrate the anti-fibrotic activity of Niclosamide. Our study is aimed at investigating the anti-fibrotic potential of Niclosamide in TGF-β1 induced in vitro model of pulmonary fibrosis and 21-day model of Bleomycin induced PF in vivo respectively. Our study results showed that Niclosamide holds the potential to exert anti-fibrotic effect by hampering fibroblast migration, attenuating EMT, inhibiting fibrotic signaling and by regulating WNT/βcatenin signaling as evident from protein expression studies. Our study findings can give new directions to development of Niclosamide as an anti-fibrotic agent for treatment of pulmonary fibrosis. Published in "**Life sciences", 2019 (I.F.-5.2), 42 Citations** (8)

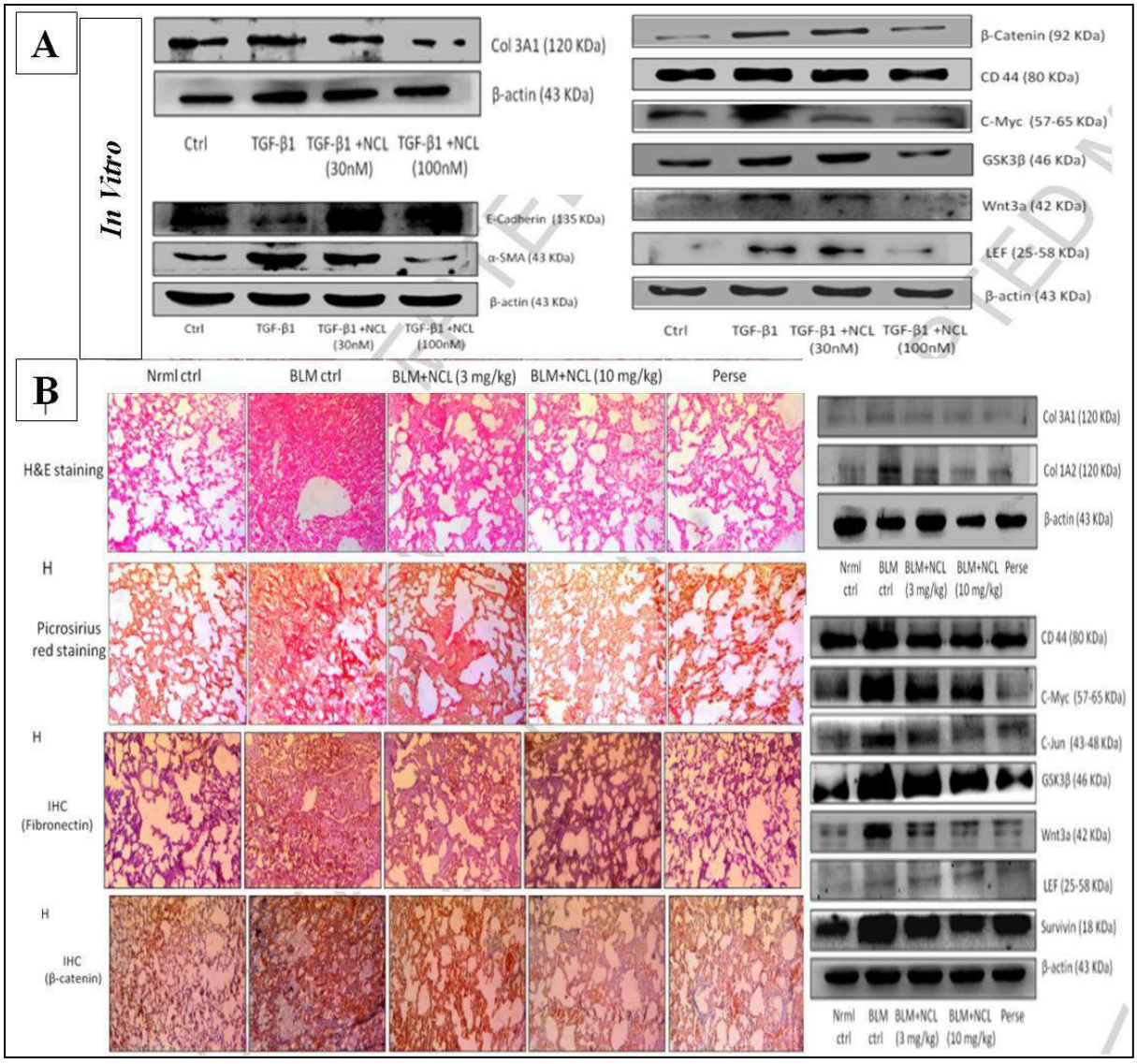

Figure 9: *Data showing niclosamide alleviating pulmonary fibrosis in vitro and in vivo by attenuation of EMT, matrix proteins &.Wnt/β-catenin signalling.*

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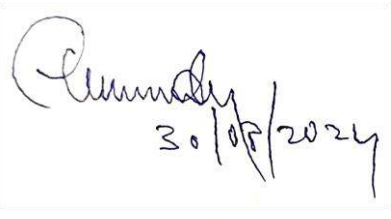
10. Effect of 7-hydroxy-3-(4'-methoxyphenyl) coumarin (C12) SIRT 3 activator in Bleomycin induced pulmonary fibrosis

Silent mating-type information regulation 2 homology 3 (SIRT3) is a member of the sirtuins family expressed in mitochondria performs deacetylation of metabolic enzymes and promotes longevity. SIRT3 is the major mitochondrial protein and functions as a substrate for almost 30 mitochondrial proteins crucial for mitochondrial homeostasis. It is well reported the downregulation of SIRT3 in pulmonary fibrosis. 7-hydroxy-3-(4'-methoxyphenyl) coumarin (C12) is a small molecule first ever known for its direct activation of SIRT3. For the first time, we reported that activation of SIRT3 by C12 attenuated bleomycin (BLM)-induced acute lung injury and pulmonary fibrosis and these results were compared with the known SIRT3 activator honokiol (HNK). C12 prevented the oxidative stress and injury caused by BLM in alveolar epithelial cells (BEAS-2B) and TGF-β-induced reactive oxygen species (ROS) in fibroblasts (MRC-5) as shown in **Fig. 10A&B.** Gross morphology images of lungs in **Fig. 10C** show BLM caused severe damage to the organ with fibrotic lesions and this damage was prevented by C12 treatment and these results were aligned with the HNK treatment group. The development of fibrosis is majorly centred on accumulation of inflammatory cells in lung alveolar spaces. To study the protective effects of C12 in pulmonary fibrosis, %granulocytes were estimated in bronchoalveolar lavage fluid (BALF) collected from mice from various groups. The BALF samples from the disease control (DC) group of animals showed a significant increase in the granulocyte population measured by flow cytometry indicating inflammatory cell accumulation in the lungs and C12 prevented this accumulation **Fig. 10D**. Additionally, the protein expression studies by western blot revealed the activation of SIRT3 by C12 **(Fig. 10E).** *In vivo* mice model alleviated BLM-induced pulmonary fibrosis and downregulated the protein expression of fibrotic markers **(Fig. 10F)** (collagen-1A and 3A, smooth muscle actin (α-SMA), vimentin, fibronectin, N-cadherin and TGF-β). Further histological images of lung tissue sections stained with hematoxylin and eosin (H&E), Masson's trichrome and picrosirius red (PSR) clearly showing that C12 prevented the accumulation of collagen fibres and retained the lung alveolar architecture by preventing the accumulation of extra parenchymal tissue, **(Fig. 10G)** similar to the normal control animals. The observations and findings through this study revealed that C12 can be potential therapeutic option for treating pulmonary fibrosis through activation of SIRT3 and its downstream proteins superoxide dismutase 2 or manganese superoxide dismutase (MnSOD) and 8-Oxoguanine glycosylase (OGG1). This study also providies the novel insights that SIRT3 upregulation could treate several diseases associated with oxidative stress and mitochondrial dysfinction.



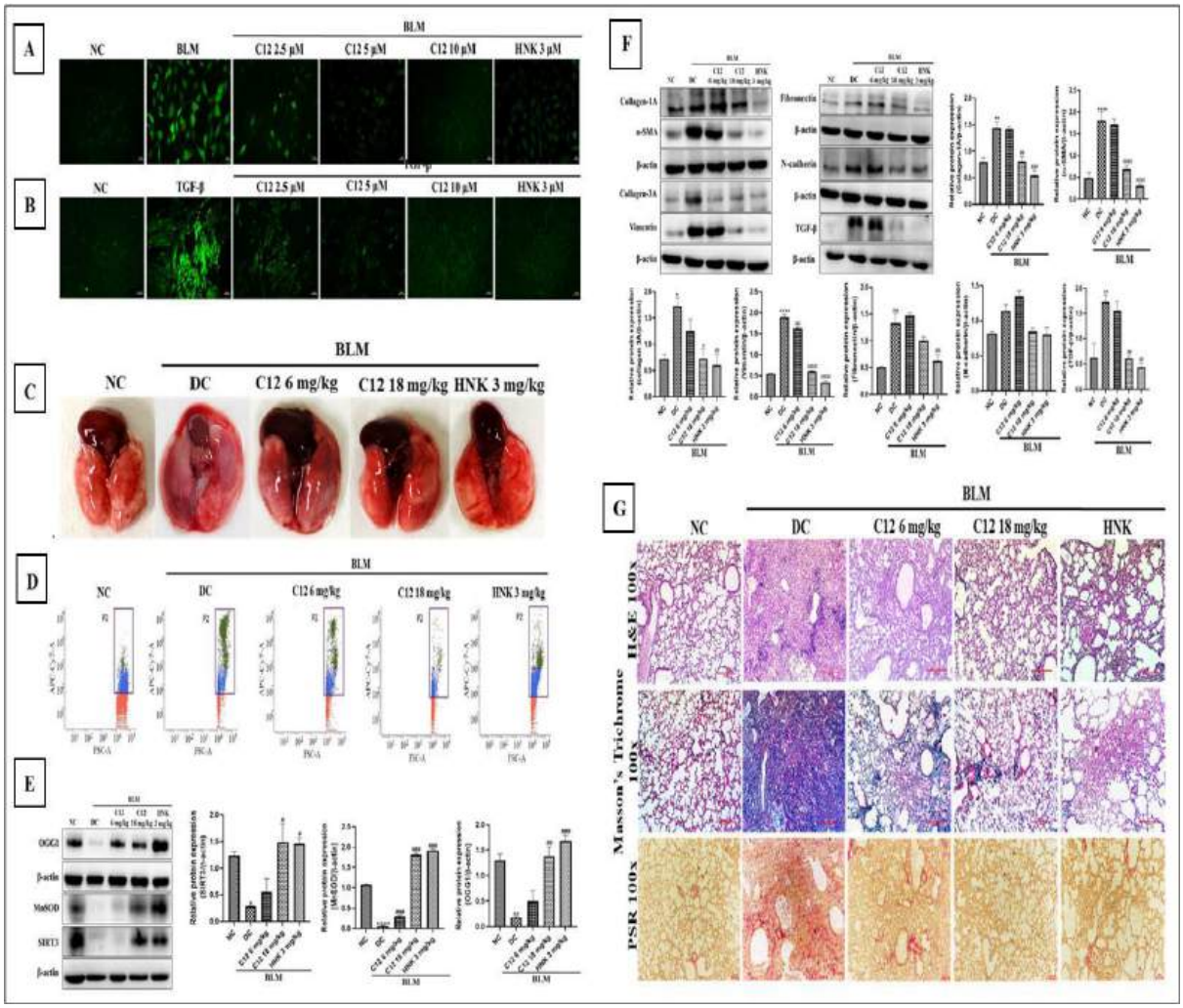


Figure 10: *Datarepresenting the effect ofC12 in Bleomycin-induced pulmonary fibrosis.*

11. Mitochondria-Targeted SIRT3 activator combats bleomycin induced pulmonary fibrosis in mice

Divergent factors often contribute to mitochondrial dysfunction. Oxidative stress is one of the major triggers for the development of pulmonary fibrosis through downregulation of SIRT3. This study aims to enhance the SIRT3 activity at the organelle level by targeted drug delivery approach. C12 is a known molecule as a SIRT3 activator and shown to be protective in pulmonary fibrosis in our previous studies. We designed a mitochondrial-targeted delivery approach by introducing a triphenylphosphonium cation (TPP^+) into the C12 molecule to enhance its specificity and efficacy. The newly designed MitoC12 inhibited the both cellular and mitochondrial oxidative stress in both BEAS-2B and MRC-5 cells**(Fig. 11A-D)**.In vivo studies in mice showing MitoC12 attenuated the BLM-induced lung damage and cellular infiltration **(Fig. 11E&F)**. MitoC12 also protected the lungs from collagen accumulation and restored the tissue architecture as shown in the histological images **(Fig. 11G)**. the protective effect of MitoC12 is through downregulation of fibrotic proteins **(Fig. 11H)** and upregulation of SIRT3 **(Fig. 11I)**and when compared these results with C12 group treatment showing the

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MitoC12 has more promising effects than C12.Overall this study suggests that the MitoC12 could be a potential therapeutic option for pulmonary fibrosis emphasizing TPP^+ conjugated molecules in treating mitochondrial dysfunction-related diseases. This study also has been under patenting and in communication.

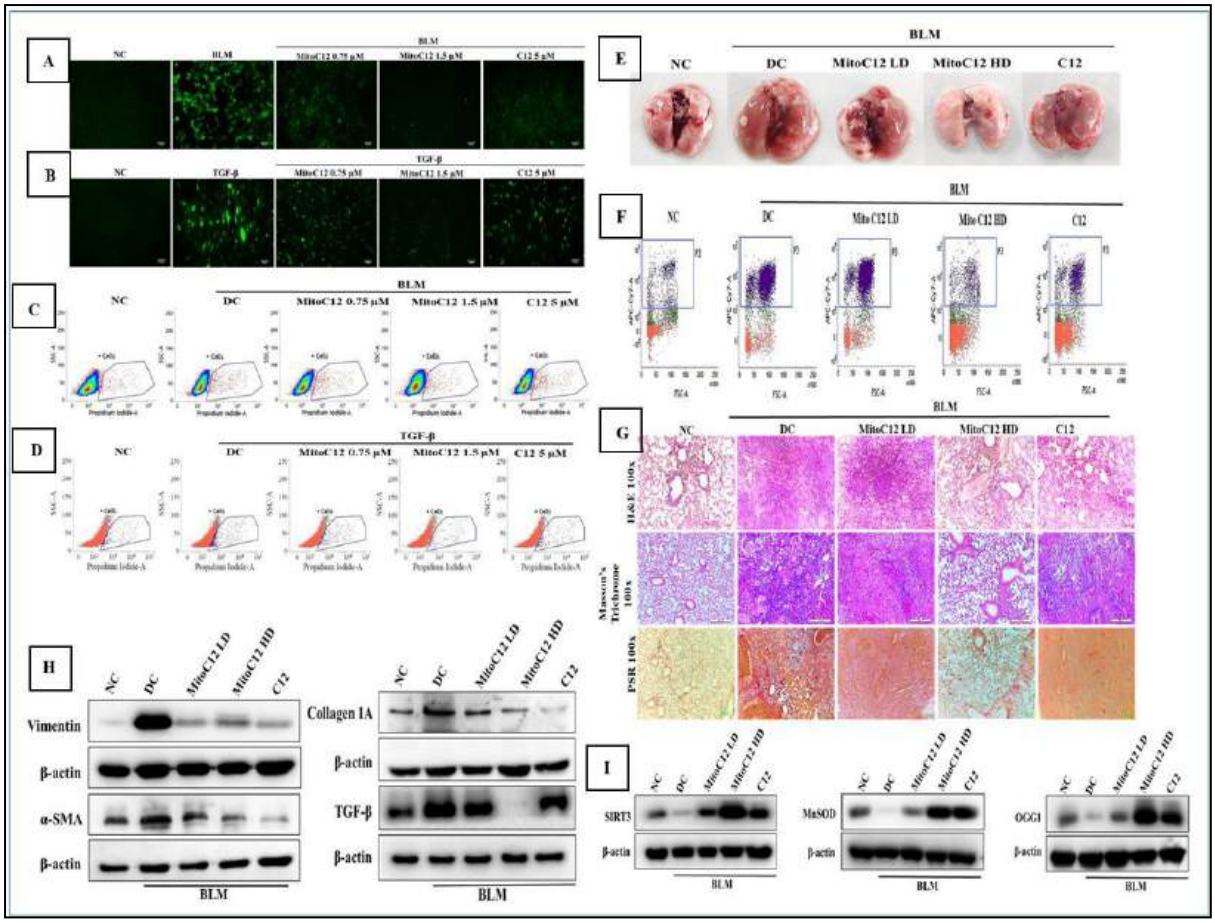

Figure 11: *Mitochondria-targeted C12,combattingbleomycin-induced pulmonary fibrosis in mice.*

12. Peptidyl arginine deiminase-4 inhibitor ameliorates pulmonary fibrosis through positive regulation of developmental endothelial locus-1

Neutrophil extracellular traps (NETs) are released by activated neutrophils to trap, immobilize and kill invading pathogens that are facilitated by Peptidyl arginine deiminase-4 (PAD-4) mediated release of neutrophils trap terminatingthe invading pathogens. However, exaggerated NETs release due to abnormal PAD-4 activation plays a crucial role in activating pro-fibrotic events in PF and inflammatory cascades. Developmental endothelial locus-1 (Del-1), expressed by the endothelial cells of lungs and brain acts as an endogenous inhibitor of inflammation and fibrosis and PAD-4 is thought to inhibit the transcription of Del-1. We have hypothesised that PAD-4 inhibitor exerts anti-inflammatory and anti-fibrotic effects in mice model of PF which is also promoted by activation in transcription of the anti-

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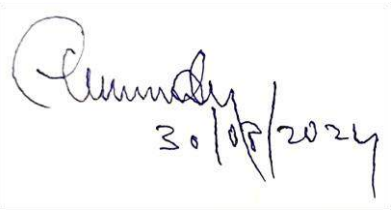
inflammatory mediator Del-1. In a bleomycin (BLM) induced PF mice model, CLA improved lung functional parameters like breathe frequency, tidal volume, minute volume, airway resistance, inspiration time and expiration time **(Fig. 11A)**. Significant increase in the number of total bronchoalveolar lavage fluid (BALF) cells, neutrophils, macrophages and lymphocytes is a marker of fibrotic disease as induced by BLM. However, CLA dose-dependently normalized parameters **(Fig. 12B-I)**. Further, it was observed that the lung histology has degenerated by BLM administration including decrease in alveolar space and increased inflammatory infiltration of cytokines with excess deposition of collagen in the lung tissues. CLA dose-dependently improved lug histology and reduced the collagen deposition **(Fig. 12J-N)**. This is also corroborated by CLA mediated decrease in expression of mesenchymal cell markers like α-smooth muscle actin and N-cadherin and decrease in expression of mesenchymal cell markers like fibronectin, COL1A1, SNAIL and SLUG, thereby attenuated fibrotic events **(Fig. 12O-S)**. Being a PAD-4 inhibitor, CLA also reduced the expression of NETotic markers like neutrophil elastase (NE), myeloperoxidase (MPO) and Citrullinated H3 (CitH3). Furthermore, BLM-induced PF reduced Del-1 expression, which was normalized by CLA treatment dose-dependently **(Fig. 12T-V)**. These findings suggests that inhibition of PAD-4 can prove to be beneficial for PF patients as there is an urgent need to expand the anti-fibrotic regimen of medications due to the availability of only two drugs including pirfenidone and nintedanib. Owing to the significance of this work, we are developing new chemical entities (NCEs) against PAD-4 that will be evaluating for anti-fibrotic effects in the animal model of PF. Published in **"International Immunopharmacology",2024 (I.F-4.8)**(9).



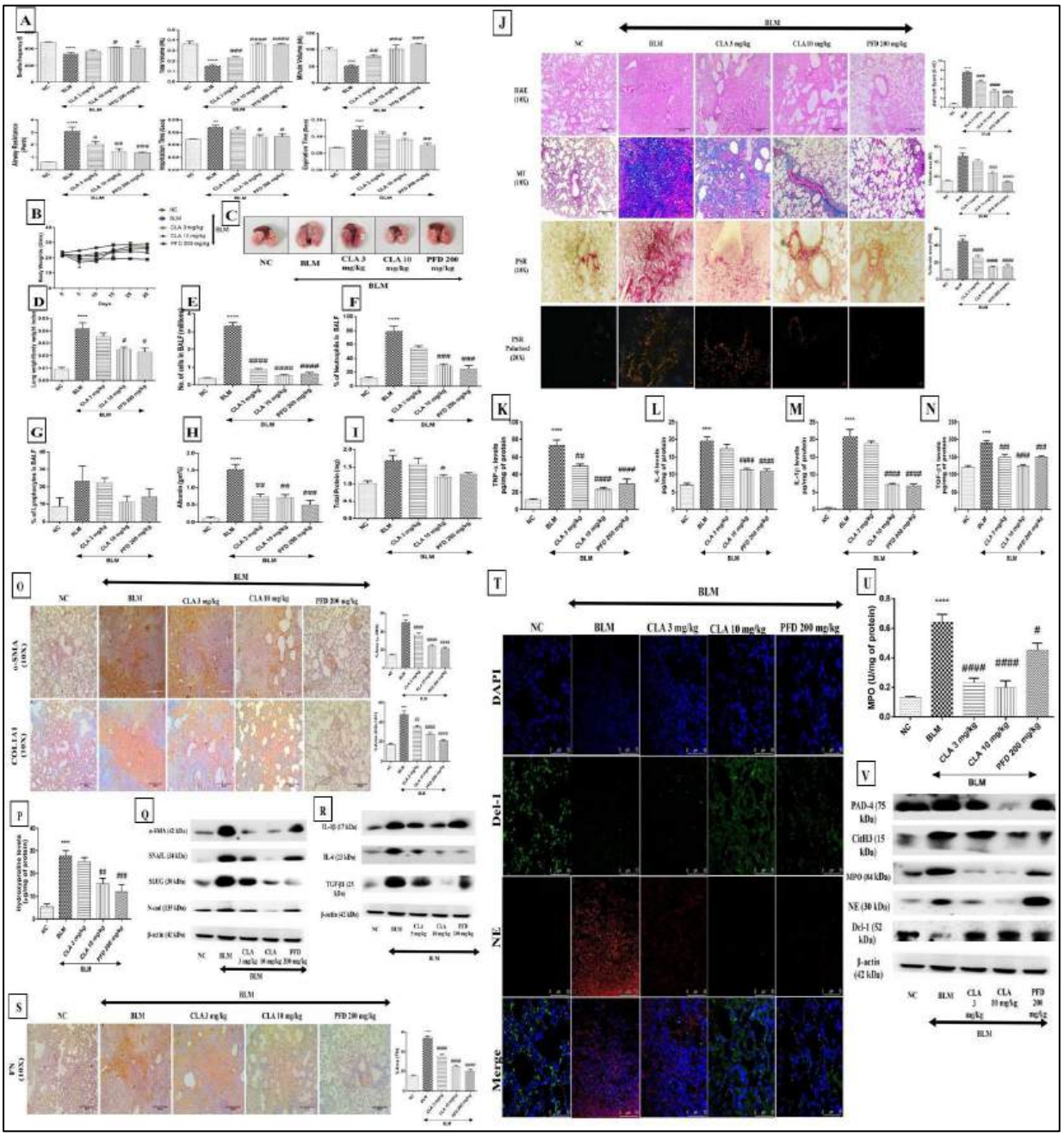


Figure 12: *Data representingPAD-4 inhibitor ameliorating pulmonary fibrosis through positive regulation of del -1.*

13. Designing of Novel Therapeutic New Chemical Entities Targeting PAD-4 for the management of Pulmonary Fibrosis

In the earlier two studies, it was confirmed that PAD-4 inhibition ameliorated NETosis, inflammation and fibrosis in mice models of BLM-induced PF. Here, we have synthesized new chemical entities (NCEs) against PAD-4 and evaluated anti-NETotic and anti-fibrotic effects in *invivo* models of PF. NCEs were synthesized based on the available PAD-4 inhibitors from literature. Following a detailed molecular docking and molecular dynamics simulation, a total of 23 NCEs were synthesised which were indole-pyrazolopyrimidine

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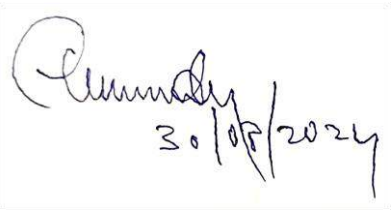
derivatives. After rigourous*in vitro* assays, a single NCE coded 36e was selected for detailed *in-vivo* assay in BLM-induced PF mice model. 36e was administered through IP route and oral routes. BLM-induced PF showed abnormality of lung functional parameters which was significantly ameliorated dose-dependently by 36e **(Fig. 13A)**. This was followed by BALF analysis where it was observed that 36e led to amelioration of aberrant BALF count of total BALF cells, neutrophils, macrophages and increase in inflammatory markers in the BALF such as albumin, LDH and total protein **(Fig. 13E-J)**. Further, it was also confirmed from ELISA studies that 36e led to decrease in BLM-induced increased levels of pro-inflammatory cytokines in the lungs **(Fig. 13K-P)**. Likewise, CLA, 36e also led to dose-dependent normalization of histological markers including increase in alveolar space and clear demarcation of alveolar septa with decreased infiltration. The results of Masson's trichrome and picrosirius red staining suggested that 36e dose-dependently decreased the deposition of collagen in the lung tissues **(Fig. 13Q)**. This is also affirmed by separated IHC studies where it was observed that treatment with 36e resulted in decreased positivity of COL1A1 and fibronectin in the lung tissues **(Fig. 13R-S)**. NETotic inhibition of 36e was higher than that of CLA where it dose-dependently reduced the expression of PAD-4, neutrophil elastase (NE) and myeloperoxidase (MPO) **(Fig. 13T-U)**. We also evaluated the NETs release in the lungs by carrying out the fluorescence staining of Ly6G and sytox green in the lungs. Ly6G is a specific marker for neutrophils and sytox green excellently stains extracellular DNA and is a marker for NETs release. 36e treatment resulted in a dose-dependent decrease in the positivity of Ly6G and sytox green in the treated lung tissues **(Fig. 13V)**. These results confirmed that 36e can prove to be a potent PAD-4 inhibitor. However, optimization of 36e can result in the development of modified NCEs with higher selectivity of PAD-4 and higher anti-fibrotic effects.



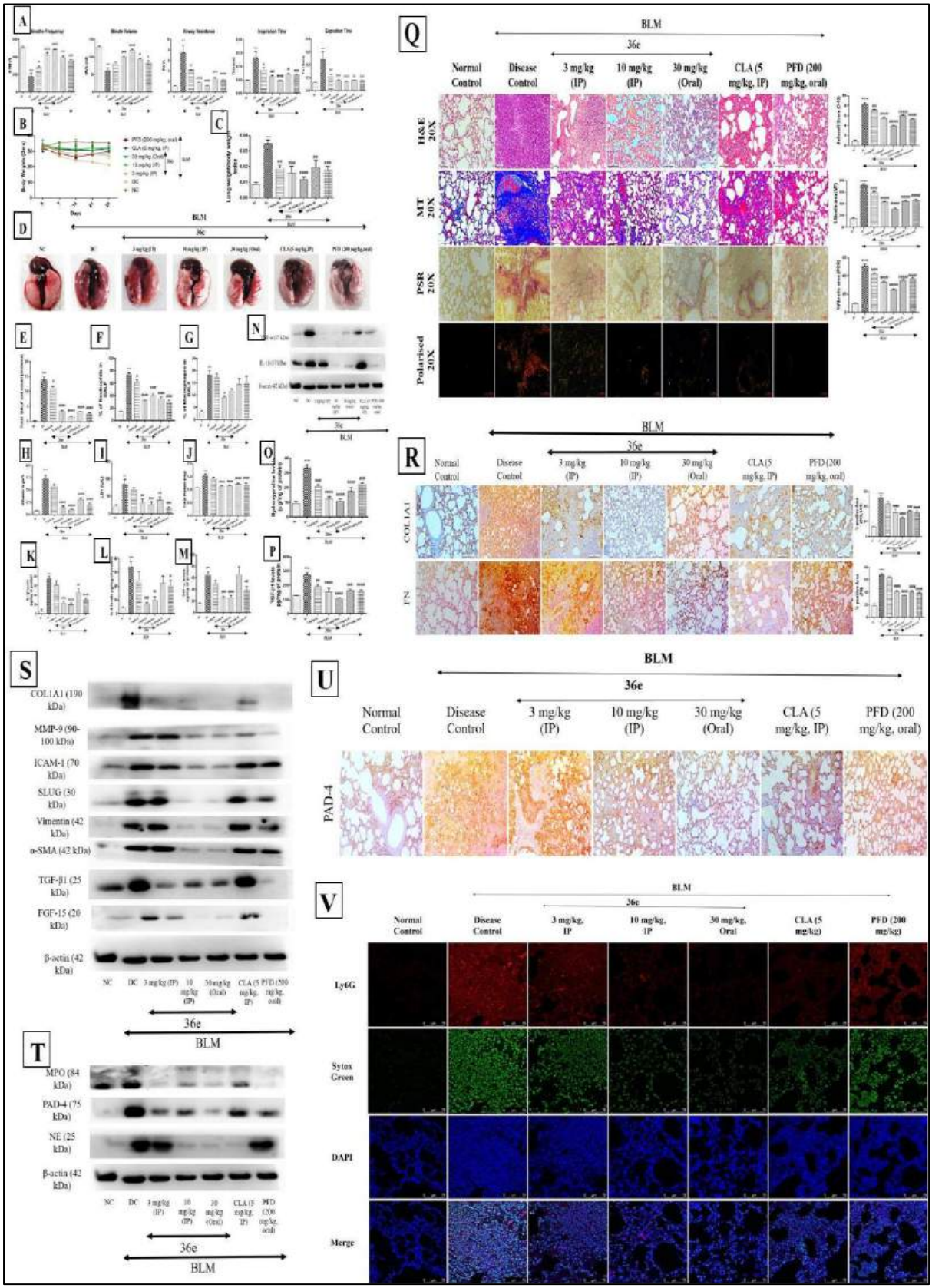


Figure 13: *Representation ofNew Chemical Entities Targeting PAD-4 for the treatment of Pulmonary Fibrosis presenting the inhibition of NETs and fibrotic markers.*

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