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Azvudine alleviates SARS-CoV-2-induced inflammation by targeting myeloperoxidase in NETosis

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ABSTRACT

Neutrophil extracellular traps (NETs) formation (NETosis), is a crucial immune system mechanism mediated by neutrophils, measuring the capacity to induce NETosis is proposed as a clinical biomarker indicating the severity of COVID-19 and long COVID. Azvudine (FNC), has shown efficacy in treating SARS-CoV-2 infection and potential for alleviating inflammation. However, the molecular mechanism underlying its anti-inflammatory effects has not been extensively investigated. Therefore, a series of experiments were conducted on SARS-CoV-2 infected rhesus macaques (RMs) to investigate the anti-inflammatory effects of FNC. The experiments involved HE staining, mass spectrometry-based proteomics, validation experiments conducted *in vivo* using RMs tissues and *in vitro* differentiation of HL-60 cells. Additionally, interaction investigations were carried out utilizing LiP-MS, CETSA, Co-IP along with molecular docking. The results demonstrated that FNC treatment effectively alleviated neutrophil infiltration and attenuated inflammatory injury following infection. In addition to exhibiting antiviral effects, FNC treatment exhibited a reduction in inflammation-associated proteins and pathways such as myeloperoxidase (MPO) and the formation of NETs, respectively. Validation experiments confirmed the impact of FNC on regulating NETs formation, interaction experiments suggested that MPO may serves as a therapeutic target. The multifaceted properties of FNC, including its antiviral and anti-inflammatory characteristics, highlight the therapeutic potential in diseases associated with NETosis, particularly those involving concurrent SARS-CoV-2 infection, providing insights for drug development targeting MPO and NETosis-associated diseases.

Neutrophil extracellular traps (NETs) formation, also known as NETosis, represents an inflammatory form of neutrophil-specific cell death characterized by non-covalent interactions between DNA and proteins associated with NETs, such as myeloperoxidase (MPO) and citrullinated histone H3 (CitH3) [1,2]. NETosis is a crucial mechanism of the innate immune system, however, accumulating evidence suggests that excessive formation of NETs contributes to chronic inflammation and tissue destruction, leading to detrimental consequences in various disease such as infectious diseases, atherosclerosis, and cancer [3,4]. NETosis is a promising target for therapy, with several drugs already developed or in clinical trials, especially for treating SARS-CoV-2 infection [5,6]. Neutrophil activation and the formation of NETs are crucial risk factors that contribute to multi-organ damage and mortality associated with SARS-CoV-2 infection [7–9]. Infection with SARS-CoV-2 is associated with an increase in neutrophil counts, and the virus induces dose-dependent production of NETs [10]. Accumulation of NETs stimulates the release of inflammatory cytokines, leading to necrotic inflammation, cytokine storm occurrence, thrombotic inflammation, and multi-organ failure. Patients infected with SARS-CoV-2 exhibit higher levels of NETs in blood circulation and tissues (including lungs and livers) compared to healthy controls [4,11,12]. Additionally, the excessive production of NETs has been linked to long COVID [13,14]. The persistent release or impaired clearance of NETs after recovering from COVID-19 may contribute to the development of long COVID [15]. Measuring the capacity for inducing NETosis is proposed as a clinical biomarker reflecting the severity of COVID-19 and long COVID [16].

Targeting NETosis represents a promising therapeutic strategy for SARS-CoV-2 infection, however, the lack of specificity and limited ability to effectively inhibit viral replication at its source restrict the therapeutic efficacy of existing drugs such as Disulfi-

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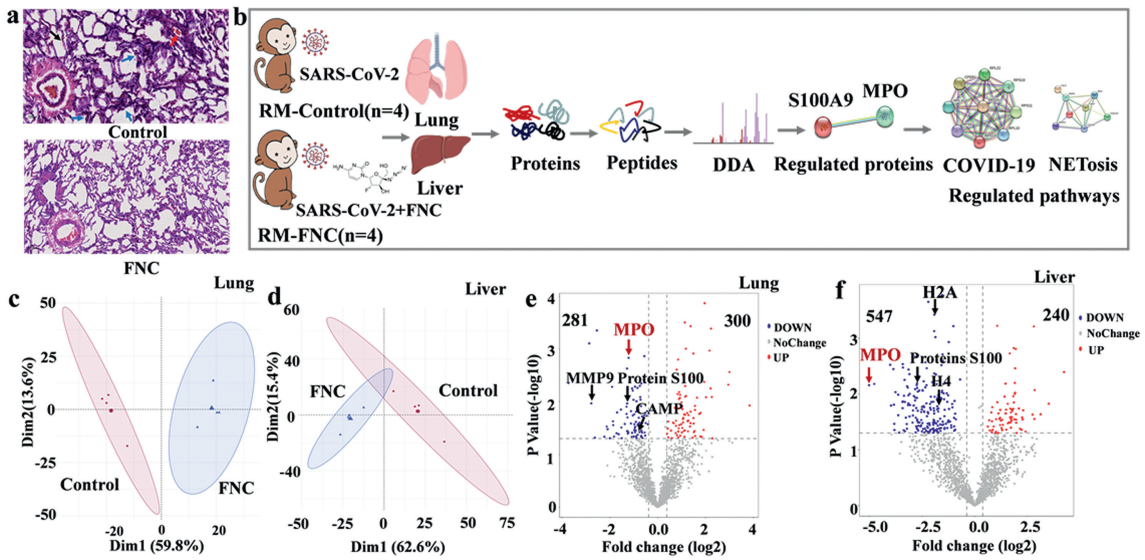

Fig. 1. FNC treatment suppressed virus replication and NETs formation in RMs infected with SARS-CoV-2. (a) Representative HE-stained sections of lung tissues from SARS-CoV-2-infected RMs. The blue arrow showed predominantly neutrophils, indicating inflammatory cells. the black arrow indicated compensatory pulmonary emphysema, the red arrow pointed to congestion and edema, scale bar = 100 μm. (b) General workflow of proteomics research. (c, e) PCA analysis results and volcano plot of proteins in the lung. (d, f) PCA analysis results and volcano plot of proteins in the liver.

ram, Colchicine, and Baricitinib [4,6]. Therefore, it is imperative to develop drugs with dual functionality, inhibiting viral replication while targeting NETosis. Azvudine (FNC), a nucleoside analog, exerts its antiviral activity by targeting the RNA-dependent RNA polymerase [17]. FNC treatment effectively reduced viral load, inflammation, and organ damage in SARS-CoV-2 infected rhesus monkeys (RMs) [7]. Clinical trials have shown that FNC treatment shorten the time of nucleic acid negativity conversion, reduced the in-hospital mortality of overall COVID-19 patients, provided benefits for subgroups with moderate, severe and critical conditions as well as with pre-existing cardiovascular disease, and exhibited comparable effectiveness on in-hospital all-cause mortality when compared to Paxlovid [18–22]. In addition to its antiviral properties, clinical research has demonstrated the potential efficacy of FNC in mitigating inflammation by down-regulating IL-6 and C-reactive protein levels among individuals with normal SARS-CoV-2 infection and patients co-infected with hemodialysis [23,24]. The dysregulation of the immune system and excessive inflammatory response are identified as the primary causes of physiological deterioration and mortality in COVID-19 infection, necessitating the use of anti-inflammatory medication [25–27]. FNC exhibits both antiviral and anti-inflammatory properties, potentially obviating the necessity for separate anti-inflammatory treatments. This has the potential to reduce treatment costs, unnecessary medication usage, and offer significant implications in terms of cost-effectiveness and streamlined therapeutic approaches. However, there remains an incomplete understanding of the molecular mechanisms underlying the regulatory phenomenon of FNC on inflammation. Therefore, this study aims to investigate and elucidate the anti-inflammatory mechanism of FNC.

Initially, lung tissues from SARS-CoV-2 infected RMs were examined using hematoxylin-eosin (HE) staining to investigate the pathological impact of FNC. The RMs experiment was conducted following the methodology as described in an earlier publication [7], which was approved by the Institutional Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Science (Ethics number: DWSP202006001). In the control group (with vehicle administration, Fig. 1a), a significant infiltration of inflammatory cells, predominantly neutrophils, was observed. Additionally, compensatory pulmonary emphysema, congestion, and edema were evident. However, following FNC treatment (Fig. 1a), a distinct reduction in inflammatory exudate was apparent, indicating its beneficial effect in alleviating neutrophil infiltration. Neutrophils play a crucial role in initiating immune responses against pathogens and their expression level increases upon infection, positively correlating with disease severity [28]. The previous study has demonstrated a significant decrease in neutrophil count following FNC treatment compared to the control group [7]. The reduction in neutrophil infiltration and neutrophil count suggested that FNC may modulate this immune response and suppress excessive inflammation-induced damage.

The impact of FNC on inflammation and neutrophils has been demonstrated. To gain a comprehensive understanding of this phenomenon, proteomic research was conducted on lung and liver tissues from SARS-CoV-2 infected RMs. Considering that SARS-CoV-2 infection primarily affects the lungs and approximately half of infected individuals manifest liver injury, hepatic also serves as a site for neutrophil elimination [29–31]. Fig. 1b shows the general workflow of the proteomics study, proteins were identified and differently expressed proteins were screened in the FNC-treated group compared to the control group (Figs. 1c-f and Fig. S1 in Supporting information). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were conducted to investigate the differentially expressed proteins, with a primary focus on those down-regulated proteins. The results of up-regulated proteins can be found in Figs. S2 and S3 (Supporting information). KEGG enrichment results demonstrated a significant down-regulation of the coronavirus-COVID-19 and ribosome pathways in both lung (Fig. 2a) and liver (Fig. 2c) following FNC treatment. Notably, up-regulation of proteins involved in the ribosome pathway has been reported following SARS-CoV-2 infection, indicating active utilization of host machinery for viral replication [26]. However, treatment with FNC effectively inhibited this up-regulation, demonstrating its inhibitory effect on SARS-CoV-2, GO enrichment analysis revealed the similar changes (Figs. 2b and d), and the down-regulated proteins enrichment in the coronavirus-COVID-19 pathway were shown in Fig. 2e. In addition to its antivirus effect, FNC treatment led to the down-regulation of inflammatory-related pathways, including leukocyte trans-endothelial migration, IL-17 signaling pathway, and NETs for-

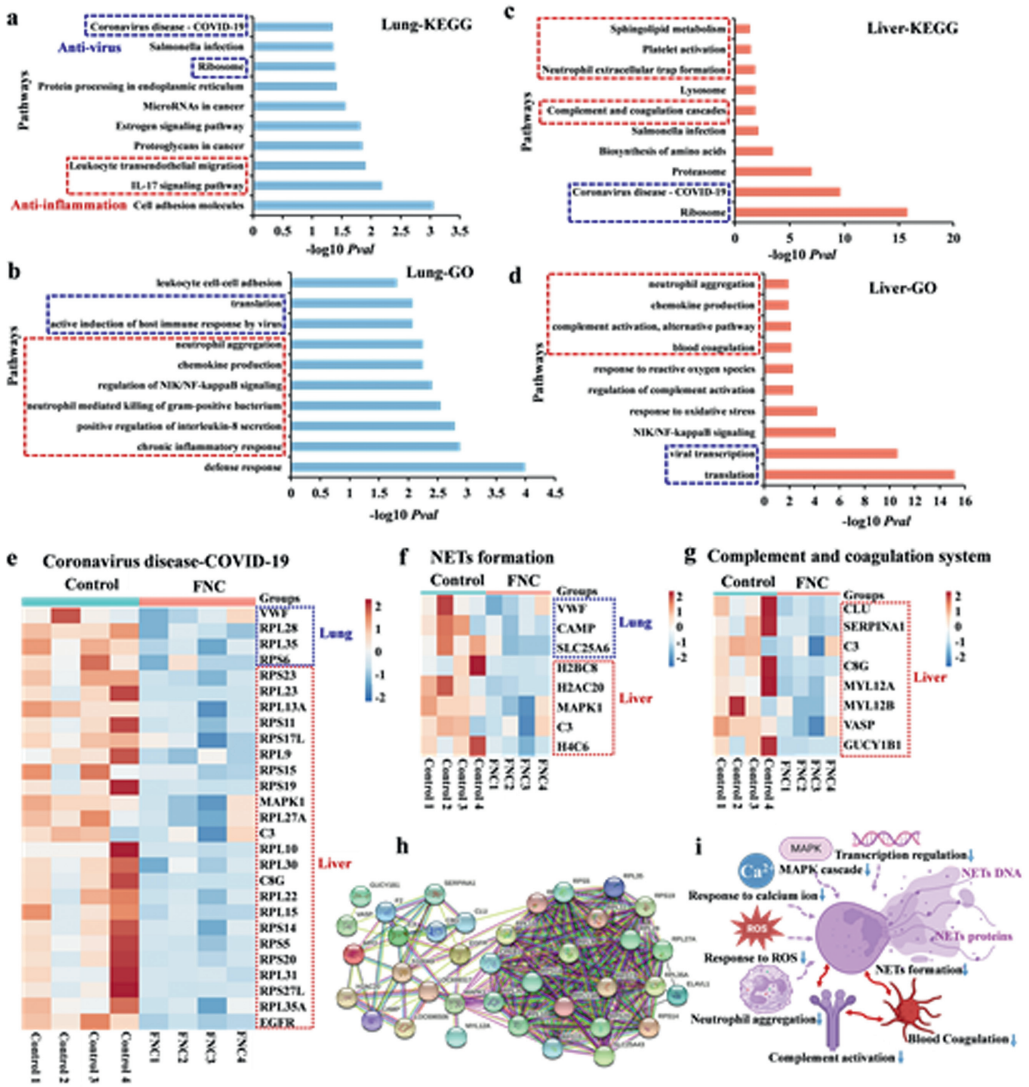

Fig. 2. FNC treatment exhibited both antiviral and anti-inflammatory effects in RMs infected with SARS-CoV-2. (a, c) Selected down-regulated pathways in the lung and liver were identified through KEGG enrichment analysis. (b, d) Selected down-regulated pathways in the lung and liver were identified through GO enrichment analysis. (e–g) Heatmap of down-regulated proteins enrichment in coronavirus diseases-COVID-19, NETs formation and complement and coagulation system pathways. (h) PPI results of differentially expressed proteins. (i) Diagram of altered pathways related to the formation of NETs.

mation pathway, as revealed by KEGG enrichment analysis (Figs. 2a and c). Similarly, GO enrichment analysis yielded consistent findings (Figs. 2b and d). Among the pathways related to inflammation, the formation of NETs, a mechanism employed by activated neutrophils to trap and neutralize pathogens and known as a double-edged sword, has further captured our attention. The proteins enrichment in this pathway was depicted in Fig. 2f, demonstrating the inhibitory effect of FNC on NETs formation. Additionally, a regulatory effect of FNC on the complement and coagulation systems was observed in the liver, as evidenced by both KEGG and GO analyses (Figs. 2c and d) and the proteins enrichment in those pathways were shown in Fig. 2g. It has been reported that the complement and coagulation systems are intricately linked to the formation of NETs, mutually promoting each other's progression [5]. The protein-protein interaction (PPI) analysis of differentially expressed proteins was depicted in Fig. 2h, illustrating the impact of FNC on the network associated with COVID-19, complement and coagulation system, as well as NETosis. Besides, several processes associated with the formation of NETs were observed and documented in Fig. 2i and Table S1 (Supporting information), such as neutrophil aggregation, response to reactive oxygen species (ROS) production [32–34]. All of these findings indicated the potential of FNC in modulating NETosis and its associated immune response and inflammation related to SARS-CoV-2 infection.

Moreover, the above findings were validated through parallel reaction monitoring (PRM). The PRM analysis validated a significant down-regulation of two cytoplasmic proteins, namely protein S100 and MPO, in both lung and liver (Figs. 3a and b). These proteins are predominantly secreted by neutrophils and classified as NETs proteins, which play crucial roles in the process of NETosis [35–37]. The up-regulation of S100 proteins and MPO has been documented in response to COVID-19 infection, indicating the presence of inflammation and NETosis [26]. Treatment with FNC resulted in a significant down-regulation of both aforementioned proteins, suggesting a reduction in both NETosis and inflammatory response. Additionally, the down-regulation of matrix metallopeptidase 9 (MMP9), a constituent of neutrophil granules [6], and cathelicidin antimicrobial peptide (CAMP), released by neutrophils and involved in NETosis [38], was confirmed in the lung (Fig. 3a). Besides, the down-regulation of proteins involved in NETosis such as histone H2A (H2A) and histone H4 (H4) [35]; complement C3 enrichment in complement and coagulation cascades, NETs forma-

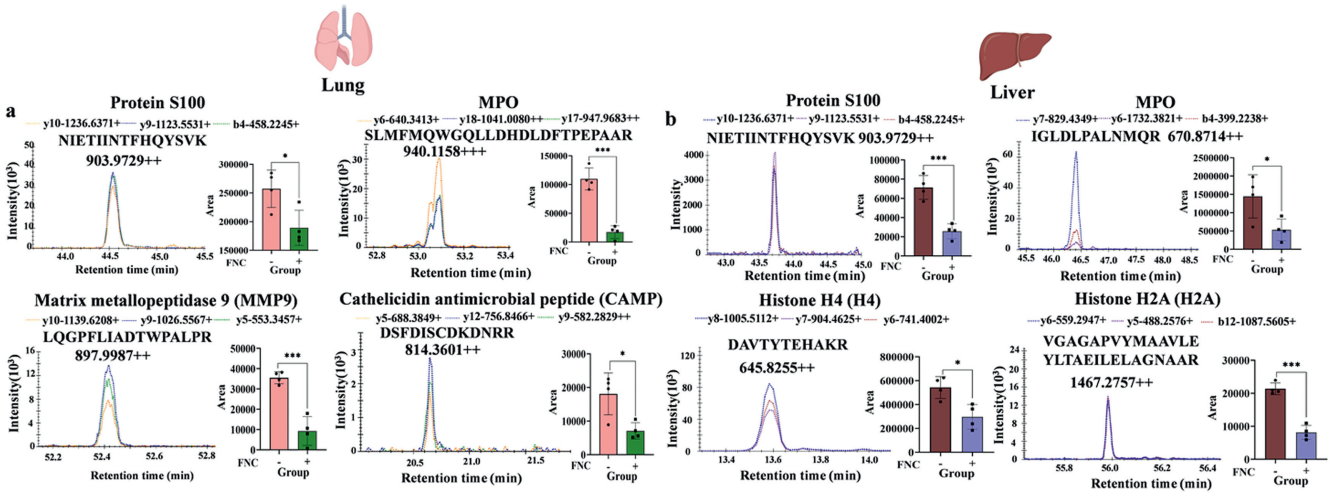

Fig. 3. PRM proteomics validated the effect of FNC on NETs-related proteins. PRM proteomics result of proteins in the (a) lung and (b) liver of RMs infected with SARS-CoV-2 (left: representative chromatogram; right: statistical analysis of the change of protein, $n = 4$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

tion, and coronavirus-COVID-19 pathways; ribosome proteins including 40S ribosomal protein S19 and ubiquitin-40S ribosomal protein S27a; as well as cathepsins B which has been reported to play crucial roles in the entry of SARS-CoV-2 into cells [39], was validated in the liver (Fig. 3b and Table S2 in Supporting information). The PRM results validated FNC's antivirus and anti-inflammatory effects, shedding light on its potential mechanism of action in suppressing NETosis. Additionally, immunofluorescence (IF) analysis conducted on lung tissues from RMs also confirmed the impact of FNC on NETosis by observing a reduction in proteins associated with NETs such as protein S100, MPO, and CitH3, which have been identified in proteomics research and reported in literature (Fig. S4 in Supporting information).

Further investigation was conducted at the cellular level to validate the regulatory effect of FNC on NETosis. Differentiated HL-60 (dHL-60) cells were utilized as a replacement model to overcome the limited lifespan of neutrophils [40,41]. The entry of FNC into cells was verified (Fig. S5 in Supporting information) and the general workflow of *in vitro* validation was depicted in Fig. 4a, wherein both the impact of FNC on DNA and NETs proteins was investigated. The generation of dHL-60 cells resembling neutrophils was assessed, and their viability under varying concentrations of FNC and different treatment durations is illustrated in Figs. 4b, c, and Fig. S6 (Supporting information), respectively. NETosis was induced using Phorbol 12-myristate 13-acetate (PMA) or calcimycin (A23187) [2,6]. To evaluate the extent of NETosis, the levels of double strands DNA (dsDNA) and the fluorescence intensity of SYTOX-Green were quantified in cell culture. Fig. 4d demonstrated the results obtained from both PMA and A23187 stimulation, revealing a significant increase in dsDNA content and fluorescence intensity of SYTOX-Green compared to the control group, indicating successful induction of NETosis by these stimulants. However, treatment with FNC resulted in a noticeable reduction in both dsDNA content and SYTOX-Green fluorescence intensity, suggesting the regulatory effect of FNC on NETosis. In addition to the impact of DNA, the results depicted in Fig. 4e provided evidence for the regulatory effect of FNC on NETs proteins, such as MPO and CitH3, the markers to evaluate the extent of NETosis. The quantification of MPO levels in cell cultures (Fig. 4f) revealed analogous alterations. The reported findings suggest that PMA-induced NETosis primarily relies on ROS [2,42], While the A23187 is known to induce NETs formation in a ROS-independent manner by elevating intracellular Ca^{2+} levels [43]. Investigation was conducted to assess the impact of FNC on ROS induction in PMA-induced NETosis. As illustrated in Fig. 4g, treatment with PMA significantly increased the levels of ROS compared to the control group; however, a noticeable decrease in ROS was observed when treated with FNC, suggesting its effective regulation of PMA-induced NETosis. Additionally, γ-H2AX expression was detected, indicating DNA damage occurrence. As shown in Fig. 4h, γ-H2AX expression was enhanced after stimulation with PMA and A23187 but alleviated after exposure to FNC treatment, demonstrating the protective effect of FNC against DNA damage. Cytokine storm is a hallmark feature of COVID-19 and is closely associated with NETosis. The impact of FNC on pro-inflammatory cytokines was further evaluated. As shown in Fig. 4i, treatment with FNC significantly reduced the levels of IL-1β and IL-6 during PMA and A23187-induced NETosis, as well as TNF-α during PMA-induced NETosis. This observation suggested that FNC possesses anti-inflammatory properties and effectively suppresses the release of these inflammatory mediators during NETosis.

Furthermore, the potential mechanism and target of FNC on NETosis were investigated using limited proteolysis mass spectrometry (Lip-MS) experiment, as shown in Fig. 5a. Protein S100A9, MPO, and neutrophil elastase, among others, which play a crucial role in NETosis, were employed as the reference library for screening various peptides derived from the identified proteins (Table S3 in Supporting information). A total of 38 distinct peptides were obtained, with 17 originating from MPO (Table S4 in Supporting information), suggesting a potential binding interaction between FNC and MPO. Moreover, the cellular thermal shift assay (CETSA) experiment confirmed the molecular-level interaction between FNC and MPO by demonstrating that the degradation of MPO in HL-60 cells increased with temperature but was suppressed when incubated with FNC (Fig. 5b). The original western blot plots of the CETSA experiments were shown in Fig. S7 (Supporting information). Additionally, Co-immunoprecipitation (Co-IP)-MS experiment validated the FNC-MPO interactions (Fig. 5c), demonstrating successful immunoprecipitation of MPO protein and detection of FNC-MPO interaction through MS analysis. The original western blot plots of the Co-IP experiments were shown in Fig. S8 (Supporting information). Docking analyses were then performed to explore the possible binding domain and mode. The results in Fig. 6 revealing that FNC exhibited spatial bonding capabilities around the NIH-binding domain and UE8-binding domain of MPO, with higher CDOCKER_Interaction_Energy values compared to the ligands (Fig. S9 in Supporting information). Additionally, Fig. 6 depicted the specific amino acids through 2D and 3D representations for each domain where FNC binds, respectively. Specifically, FNC primarily

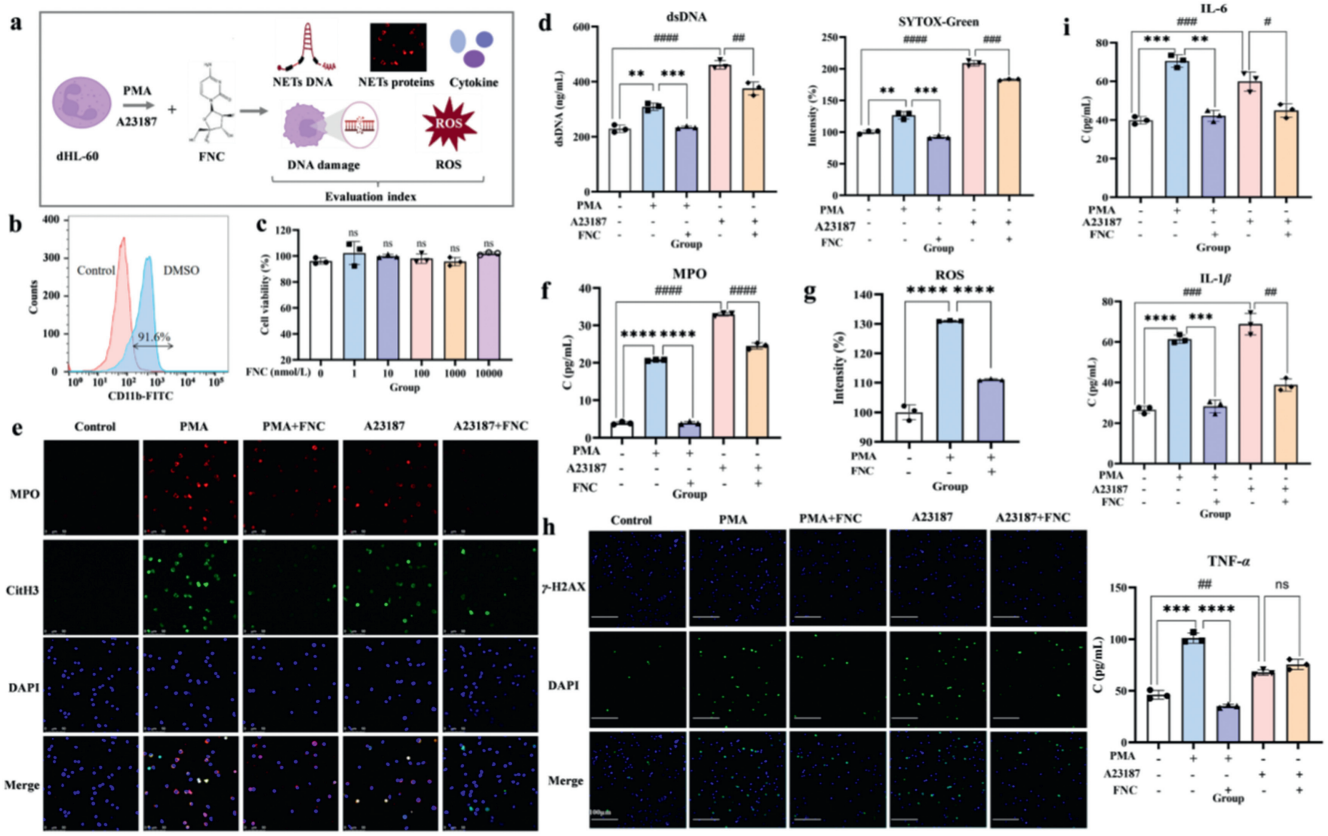

Fig. 4. FNC regulated NETosis in dHL-60 cells. (a) General workflow for *in vitro* cell investigations. (b) The differentiation of HL-60 cells was evaluated by flow cytometry based on the expression of CD11b. (c) Cell viability of dHL-60 cells was assessed using CCK8 assay with varying concentrations of FNC ranging from 0 to 10,000 nmol/L ($n=3$). (d) The levels of dsDNA and the fluorescence intensity of SYTOX-Green were quantified to evaluate the impact of FNC on NETs DNA ($n=3$). (e) Representative confocal microscopy images to evaluate the impact of FNC on NETs proteins, including MPO and CitH3 (scale bar = 50 μm). (f) The impact of FNC on MPO in cell culture was evaluated by quantifying MPO levels ($n=3$). (g) The levels of ROS were measured using flow cytometry to assess the impact of FNC on PMA-induced NETosis ($n=3$). (h) Representative microscopy images to evaluate the impact of FNC on DNA damage based on γ-H2AX expression (scale bar = 100 μm). (i) The levels of IL-6, IL-1β, and TNF-α were quantified in cell culture to evaluate the impact of FNC on inflammatory cytokines ($n=3$). * or $^{\#}$ $P<0.05$, ** or $^{\#\#}$ $P<0.01$, *** or $^{\#\#\#}$ $P<0.001$, **** or $^{\#\#\#\#}$ $P<0.0001$. ns: no significant.

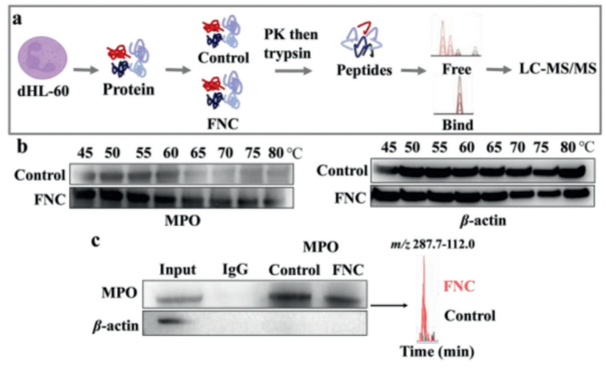

Fig. 5. FNC selectively targets MPO to regulate NETosis. (a) Workflow of Lip-MS experiment. (b) The interaction between FNC and MPO was assessed in the CETSA experiment using Western blot analysis, with β-actin serving as an internal control. (c) Co-IP-MS experiment to assess the interaction of FNC and MPO, β-actin was employed as an internal control.

binds to the MPO protein through hydrogen bonding and attractive charge. It can form hydrogen bonds with amino acid residues 91 GLN, 95 HIS, 98 ASP, 100 THR, 333 ARG, 336 HIS, and 424 ARG of the MPO protein. Additionally, it interacts through attractive charge with amino acid residues 102 GLU, 239 ARG and 242 GLU as well as the ARG residue at position 424. Pi-alkyl forces, van der Waals interactions, halogen (fluorine) bonding and unfavorable negative-negative forces are also observed in the binding process. These various forces contribute to the binding of FNC to the receptor protein MPO. Based on the altered peptides identified through Lip-MS and the amino acids involved in the docking analyses, it can be inferred that the peptides TDQLTPDQER (239–248), NQINALTSFVDAS-MVYGSEEPLAR (328–351) and SSEMPELTSMHTLLLR (406–421) play a crucial role in mediating the interaction between FNC and MPO (Fig. 6 and Table S4).

Since its discovery, research on NETs has emerged as a prominent area of investigation, particularly in immune and inflammatory disorders. The emergence of SARS-CoV-2 in 2019 has further emphasized the importance of NETs, as excessive NETs formation may contribute to cytokine storms and uncontrolled host response, which are considered key factors for mortality [26,44,45]. Requisite prevention and therapeutic strategies are urgently imperative to combat SARS-CoV-2 [45,46]. Existing drugs can target NETs through various mechanisms, however, rare of them possess direct antiviral activity against SARS-CoV-2. The FDA-approved JAK1/JAK2 inhibitor, Baricitinib, has demonstrated effective inhibition of NETosis and cytokine signaling pathways, making it a potential treatment for SARS-CoV-2-induced inflammation [6]. However, its impact on the kinetics of SARS-CoV-2 replication is negligible, necessitating combination therapy with other antiviral drugs such as Remdesivir to enhance therapeutic efficacy at the expense of increased costs. Moreover, being a JAK inhibitor, it may potentially modulate downstream anti-immune activation effects that could impede immune responses and exacerbate infection [6,47].

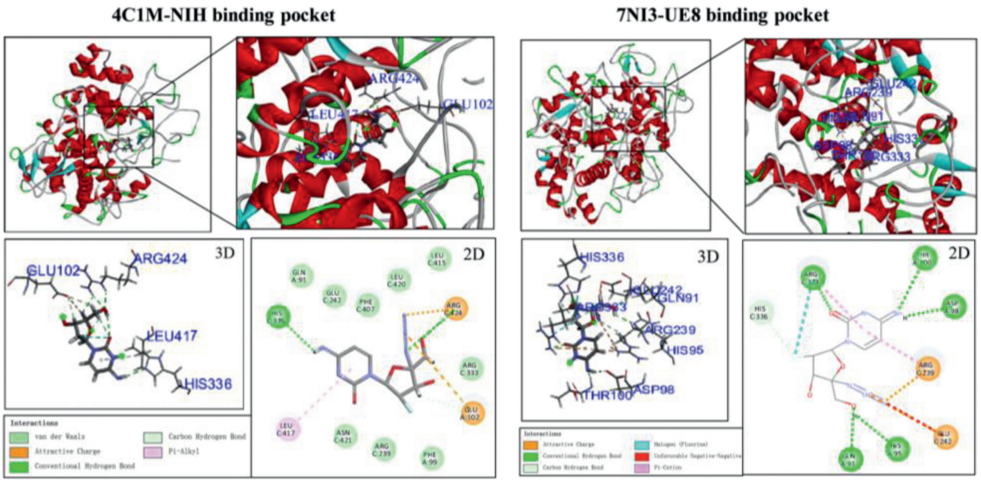

Fig. 6. Docking analysis of FNC spatially bond around NIH and UE8-binding domain of MPO.

The urgent need for the development of drugs with dual antiviral and anti-NETosis properties is evident. The nucleoside-based drug, FNC, received conditional approval from the National Medical Products Administration (NMPA) of China on July 25, 2022 [48]. Several real-world studies have confirmed its safety and effectiveness [20–22]. In addition to its well-established antiviral properties, the supplementary anti-inflammatory effects of FNC, as evidenced by clinical studies, remain relatively unexplored. Based on our study and the existing knowledge of FNC and NETosis, we proposed that the regulatory effect of FNC may be exerted through multifaceted attributes. Firstly, in the context of SARS-CoV-2 infection, FNC demonstrated potent efficacy in inhibiting viral replication and reducing viral load. Additionally, it effectively suppressed complement and coagulation pathways activation, thereby addressing neutrophil activation at its fundamental level and attenuating intracellular interactions involved in NETosis to alleviate inflammation with a dose level almost equivalent to Baricitinib (4 mg/day, with FNC being 5 mg/day) [6,20]. Additionally, in cellular model, in the absence of virus, we observed that FNC exhibited regulatory effects on NETosis by selectively targeting MPO. The MPO enzyme represents a promising therapeutic target for the treatment of chronic inflammatory diseases, atherosclerosis, and acute cardiovascular events [49]. It plays a pivotal role in facilitating the production of reactive oxygen intermediates [50], contributing to chromatin depolymerization and promoting the formation of NETs [51]. The interaction between FNC and MPO highlighted the therapeutic potential of FNC as a promising option for managing MPO and NETosis-associated inflammatory diseases, particularly in cases of concurrent SARS-CoV-2 infection.

Overall, our findings demonstrated that FNC treatment not only effectively suppressed viral replication but also exerted regulatory effects on NETosis through targeted modulation of MPO, thereby alleviating inflammation and tissue damage. These results underscored the therapeutic potential of FNC in diseases associated with NETosis, particularly in cases of SARS-CoV-2 co-infection. This not only facilitated cost reduction and streamlined therapeutic approaches for SARS-CoV-2 infection but also established a robust foundation for its application and drug development targeting MPO and NETosis-associated diseases.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yang Li: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ning Sheng:** Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Kun Wang:** Investigation, Formal analysis. **Yuhuan Li:** Investigation, Formal analysis. **Jiandong Jiang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jinlan Zhang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110238.

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