Furosemide as a possible therapeutic for COVID-19 (#47843)

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Furosemide as a possible therapeutic for COVID-19

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The novel coronavirus SARS-CoV-2 has become a global health concern. The morbidity and mortality of the potentially lethal infection caused by this virus arise from the initial viral infection and the subsequent host inflammatory response. The latter may lead to excessive release of pro-inflammatory cytokines, IL-6 and IL-8, as well as TNF-α ultimately culminating in hypercytokinemia (“cytokine storm”). To address this immuno-inflammatory pathogenesis, multiple clinical trials have been proposed to evaluate anti-inflammatory biologic therapies targeting specific cytokines. However, despite the obvious clinical utility of such biologics, their specific applicability to COVID-19 has multiple drawbacks, including they target only one of the multiple cytokines involved in COVID-19’s immunopathy. Therefore, we set out to identify a small molecule with broad-spectrum anti-inflammatory mechanism of action targeting multiple cytokines of innate immunity. In this study, a library of small molecules endogenous to the human body was assembled, subjected to *in silico* molecular docking simulations and a focused *in vitro* screen to identify anti-pro-inflammatory activity via interleukin inhibition. This has enabled us to identify the loop diuretic furosemide as a candidate molecule. To pre-clinically evaluate furosemide as a putative COVID-19 therapeutic, we studied its anti-inflammatory activity on RAW264.7, THP-1 and SIM-A9 cell lines stimulated by lipopolysaccharide (LPS). Upon treatment with furosemide, LPS-induced production of pro-inflammatory cytokines was reduced, indicating that furosemide suppresses the M1 polarization, including IL-6, TNF-α and IL-1β release. In addition, we found that furosemide promotes the production of anti-inflammatory cytokine products (IL-1RA, arginase), indicating M2 polarization. Accordingly, we conclude that furosemide is a reasonably potent inhibitor of IL-6 and TNF-α that is also safe, inexpensive and well-studied. Our pre-clinical data suggest that it may be a candidate for repurposing as an inhaled therapy against COVID-19.

Furosemide as a Possible Therapeutic for COVID-19

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Abstract

The novel coronavirus SARS-CoV-2 has become a global health concern. The morbidity and mortality of the potentially lethal infection caused by this virus arise from the initial viral infection and the subsequent host inflammatory response. The latter may lead to excessive release of pro-inflammatory cytokines, IL-6 and IL-8, as well as TNF-α ultimately culminating in hypercytokinemia ("cytokine storm"). To address this immuno-inflammatory pathogenesis, multiple clinical trials have been proposed to evaluate anti-inflammatory biologic therapies targeting specific cytokines. However, despite the obvious clinical utility of such biologics, their specific applicability to COVID-19 has multiple drawbacks, including they target only one of the multiple cytokines involved in COVID-19's immunopathy. Therefore, we set out to identify a small molecule with broad-spectrum anti-inflammatory mechanism of action targeting multiple cytokines of innate immunity. In this study, a library of small molecules endogenous to the human body was assembled, subjected to *in silico* molecular docking simulations and a focused *in vitro* screen to identify anti-pro-inflammatory activity via interleukin inhibition. This has enabled us to identify the loop diuretic furosemide as a candidate molecule. To pre-clinically evaluate furosemide as a putative COVID-19 therapeutic, we studied its anti-inflammatory activity on RAW264.7, THP-1 and SIM-A9 cell lines stimulated by lipopolysaccharide (LPS). Upon treatment with furosemide, LPS-induced production of pro-inflammatory cytokines was reduced, indicating that furosemide suppresses the M1 polarization, including IL-6, TNF-α and IL-1β release. In addition, we found that furosemide promotes the production of anti-inflammatory cytokine products (IL-1RA, arginase), indicating M2 polarization. Accordingly, we conclude that furosemide is a reasonably potent inhibitor of IL-6 and TNF-α that is also safe, inexpensive and well-studied. Our pre-clinical data suggest that it may be a candidate for repurposing as an inhaled therapy against COVID-19.

Introduction

COVID-19, a potentially lethal infection caused by the SARS-CoV-2 virus, has emerged as a public health crisis of global concern. Currently, there are no effective curative treatments for COVID-19, affording little patient recourse beyond supportive care (Cortegiani *et al*. 2020). The morbidity and mortality of COVID-19 arise from two competing pathological processes, the initial viral infection and the subsequent host inflammatory response, the latter of which may lead to excessive release of pro-inflammatory cytokines (interleukins [*e.g.* IL-6, IL-8] or non-interleukins [*e.g.* TNF-α]), and may culminate in hypercytokinemia ("cytokine storm"), a self-targeting inflammatory response syndrome (Conti *et al*. 2020; Mehta *et al*. 2020; Qin *et al*. 2020; Huang *et al*. 2020). Reflecting these dichotomous disease processes, current therapeutic development strategies may be categorized into two broad groups: anti-viral and anti-inflammatory. Arguably, truly effective treatments may require both agents, since an antiviral will fail to suppress uncontrolled pro-inflammatory cytokine release once the process has been triggered.

To address the COVID-19 immuno-inflammatory pathogenesis, multiple clinical trials have been proposed to evaluate anti-inflammatory biologic therapies targeting specific cytokines. Agents under consideration include tocilizumab (Peking University First Hospital 2020; Tongji Hospital *et al*. 2020; National Cancer Institute N 2020) and sarilumab (Pharmaceuticals R and Sanofi 2020), both monoclonal antibodies that target the IL-6 pathway (Sebba 2008; Boyce *et al*. 2018), as well as adalimumab, which binds with specificity to TNF-α (Furst *et al*. 2003). Preliminary data from a Chinese study in which tocilizumab was given to 21 patients with severe COVID-19 reported that the patients "improved remarkably" with 19 patients being discharged 13.5 days following treatment, and the remainder "recovering well" (Xu X *et al*. 2020).

However, despite the obvious clinical utility of such biologics for many disorders, their specific applicability to COVID-19 is hampered by various issues: they target only one of the multiple cytokines implicated in COVID-19's immunopathy; if administered systemically, they can predispose patients to secondary infections or other toxicities, such as hepatoxicity; and they may be expensive to mass produce and distribute. Therefore, they are of limited utility in the context of a global pandemic.

Accordingly, we set out to identify a small molecule with the following properties: broad-spectrum anti-inflammatory mechanism of action targeting cytokines of innate immunity; low toxicity and excellent safety profile; chemically stable; easily stored and administered; able to be rapidly adopted in clinical settings worldwide; and, widespread availability with inexpensive and efficient means of production. As described herein, a systemic series of *in silico* and *in vitro* studies have enabled us to identify furosemide as a candidate molecule.

Materials & Methods

In Silico Studies

Molecular docking simulations: Molecular docking simulations on the endogenous molecule (1136) and known drug datasets (1768) against Il-6 and TNF-α were carried out in two steps. The first step involved placement of the endogenous or ligand molecule in an identified binding pocket (docking) of IL-6 and TNF-α. In second step, the calculation of the binding energies of the docked molecules (scoring) was completed.

Preparation of TNF-α and IL-6 3D protein structures: The TNF-α structure (PDB code: 2AZ5) and IL-6 structure (PDB code: 1ALU) were loaded into Molecular Operating Environment (MOE 2019.0101) software (MOE, 2019). The loaded crystal structure was

prepared employing the 'QuickPrep Panel' in MOE, which contains the 'structure preparation' feature. The 'Protonate3D' function was used to optimize ionization states of the added hydrogen atoms. Water molecules, which were present 4.5 Å away from ligand or receptor, were deleted. The MFF94x force field, with RMS gradient of 0.05 kcal/Å, was used for energy minimization (Panigrahi & Desiraju 2007). A Ramachandran plot was used to confirm the geometric and stereochemical qualities of the TNF-α and IL-6 protein structures.

Endogenous molecules and known drugs dataset curation: The datasets of endogenous molecules (1136) and drugs (1768) were prepared using MOE software. Two functions 'wash at dominant pH of 7.4' and 'energy minimize' were used to obtain an energy minimized molecular form of molecules present at pH 7.4.

Binding site selection and docking simulations: The active site of IL-6 and TNF-alpha was identified using the 'MOE-site Finder' module. The 'Triangle Matcher' placement method and 'London dG' scoring functions were used for docking simulations. The poses from ligand conformations were generated in the Placement phase. After placement of poses, the refinement was done with the 'GBVI/WSA dG' scoring method using 'induced fit' option. Induced fit refinement allows both the ligand molecule and protein active site to move freely to facilitate residue alignment during docking simulations. Thirty docked poses of placement retention simulation and 10 poses of refinement step were retained. The docking pose corresponding to the highest score for an endogenous or a drug molecule was considered for comparison of binding affinity within datasets. Further details of descriptor calculation and docking simulations are provided in our recent publications (Gupta *et al.* 2019; Gupta *et al.* 2020) and in supporting information.

In Vitro Studies

Inflammation activation on RAW264.7 macrophage: RAW264.7 cells were purchased from ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS) at a final concentration of 10%. RAW264.7 cells were seeded in 12-well plates at 0.25 x 10^6 cells/well seeding density, one day before experiments. To activate the cells, cell culture medium was changed to a lipopolysaccharide (LPS) and Interferon γ (IFNγ) containing medium with dimethyl sulfoxide (DMSO) or furosemide at required concentrations, followed by 24 h incubation at 37 °C; conditioned media and lysates were harvested for analysis.

Inflammation activation on THP-1 monocytic cells: THP-1 cells (ATCC) were maintained in RPMI 1640 medium supplemented with 2-mercaptoethanol at a final concentration of 0.05 mM and FBS at a final concentration of 10%. THP-1 cells were seeded in each well of a 12-well tissue culture plate at a density 0.5 x 10^6 cells/mL, one day before experiments. THP-1 monocytes were differentiated by 150 nM PMA (phorbol 12-myristate 13-acetate) for 24 h. Cells were then treated with LPS+IFNγ with DMSO or furosemide at the required concentration, followed by 48 h incubation; conditioned media and lysates were collected for analysis.

Nitric oxide (NO) production by Griess assay: Nitric oxide production from the conditioned media of RAW264.7 and SIM-A9 cell cultures was examined by a Griess assay. Conditioned medium and sulfanilamide were mixed in a microwell plate to form a transient diazonium salt. Then, N-naphtyl-ethylenediamine was added to all wells to form a stable azo compound by incubating for 5-10 min in a dark at room temperature. The absorbance was measured between 520 nm and 550 nm. The concentration of NO production was quantified by being plotted against a standard curve.

Enzyme-linked immunosorbent assay (ELISA): Cytokines were quantified using ELISA kits following the manufacturer's instructions. Briefly, the high-binding plates were

coated at 100 µL/well with diluted capture antibodies (1:250) at 4ºC overnight. The coated plates were then blocked with the diluent for 1 h before assay. Each sample was diluted accordingly and added to the plates for a 2 h incubation period at room temperature. Plates were then washed with 250 µL/well PBS with 0.05% Tween-20 and incubated with detection antibodies (1:250 in assay diluent) for 1 h at room temperature. After another washing step, 1:250 diluted avidin-HRP (horseradish peroxidase) was added and incubated for 30 min. Next, 100 µL TMB-substrate (3,3',5,5'-tetramethylbenzidine) was added and the plate was incubated in dark until the signal was sufficiently developed. The reaction was stopped with 50 µL of 2 N sulfuric acid. Absorbance was measured at 450 nm with a correction of 570 nm using a plate reader.

Western blotting: Cells were washed twice with ice-cold PBS and harvested in RIPA buffer supplemented with a protease inhibitor cocktail. The whole-cell extracts were then centrifuged at 22,000 xg for 20 min at 4 °C to remove cell debris. Protein concentrations were quantified using a Micro BCA protein assay kit. The absorbance was measured at 595 nm using a microplate reader. Equal amounts of cellular protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes at 100 V for 90 min. The membranes were blocked for 1 h in Tris-buffered saline (TBS), pH 7.4, with 0.1% Tween-20 (TBS-T) containing 10% skim milk. The membrane blot was then incubated overnight at 4 °C with primary antibodies against iNOS (1:1000), IL-1β (1:1000), actin (1:5000) and GAPDH (1:5000) in TBS-T containing 5% skim milk. The membrane was washed with TBS-T 3 x 10 mins and incubated with goat anti-rabbit IgG-horseradish peroxidase (1:5,000) for 1 hour. After the washing step, the immunoblotting was visualized by chemiluminescence HRP-substrate.

Flow cytometry: Cells were harvested and re-suspended with staining buffer. Cells were stained with antibody (1:100) by incubating at 4 °C for 30 min in the dark. Stained cells were centrifuged, and the supernatant was discarded. The cell pellets were then re-suspended in cell flow buffer, transferred to FACS tubes and analyzed by flow cytometry within 48 h. To all cells in the experiment, Fc blocker was added.

Inflammation activation on SIM-A9 cells: SIM-A9 (ATCC) cells were maintained in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM-F12) with 10% fetal bovine serum, 5% horse-serum and antibiotic-antimycotic. SIM-A9 cells were seeded 24 h before the experiment. Culturing medium was replaced with DMEM-F12 medium containing 5% FBS + 2.5% horse serum with required LPS concentration (final volume is 1 mL/well). The conditioned media and lysates were harvested for cytokine and cell marker examination.

Statistical analysis: Data are presented as mean ± SD or ±SEM. Statistical analysis was performed with GraphPad Prism software version 6.01c, applying a two-tailed unpaired t-test. A *p*-value of >0.05 was considered significant.

Results

Identifying an Initial Hit

A screening strategy was devised for identifying an initial compound with potential broad-spectrum anti-inflammatory activity targeting relevant cytokines of the pulmonary innate immune system. Since immunologically-mediated inflammatory responses in the human body are subject to tight homeostatic regulation, there exist multiple endogenous compounds capable of either up- or down-regulation of innate immune processes. Accordingly, we sought to identify an endogenous compound as an initial molecular platform around which to devise a therapeutic. A library of 1,136 small molecules endogenous to the human body was assembled, subjected to

in silico molecular docking simulations and a focused *in vitro* screen to identify anti-pro-inflammatory activity via interleukin inhibition.

Multiple compounds within the tryptophan metabolic pathway were identified, both indoleamine and anthranilate metabolites. In particular, 3-hydroxyanthranilic acid (3-HAA) was found to demonstrate significant anti-inflammatory potential (in accord with previous studies by others, such as Lee *et al.* (2013), who have shown significant activity of 3-HAA in inhibiting IL-6 and TNF-α). Regrettably, 3-HAA is a small polar molecule with poor drug-like properties and is not an approved therapeutic for human use.

Converting Hit to Drug-Like Anthranilate Compound

The task of converting 3-HAA to a drug or drug-like compound can be addressed via two approaches: 1) synthesis of new chemical entities with drug-like properties based on the 3-HAA scaffold, or 2) identification of known drugs with structural properties similar to the 3-HAA scaffold with the goal of repurposing. Because of the urgency imposed by the unfolding pandemic, approach 2 was selected to provide a short-term solution.

Accordingly, 1,768 known drugs were computationally evaluated for anthranilate structural components. This screen identified mefenamic acid (*N*-(2,3-xylyl)-anthranilate) and furosemide (4-chloro-5-sulfamoyl-*N*-furfuryl-anthranilate) as the two strongest leads. Mefenamic acid is known to provide significant protection against elevated levels of TNF-α and IL-1β in radiation-induced genotoxicity of human lymphocytes (Armagan *et al.* 2012; Hosseinimehr *et al.* 2015); furosemide is known to significantly reduce production of IL-6, IL-8, and TNF-α in bronchial inflammation of asthma (Prandota 2002; Yuengsrigul *et al.* 1999). This screen also identified other loop diuretics structurally related to 3-HAA and furosemide, including

bumetanide, piretanide and azosemide (Fig. 1). Arising from the observation that furosemide can reduce bronchial inflammation, can be administered by inhalation (*vide infra,* Discussion) (Prandota 2002; Inokuchi *et al.* 2014; Waskiw-Ford *et al.* 2018). and is a widely available drug, furosemide was selected to be explored for repurposing in the treatment of COVID-19. Furosemide has been used worldwide for decades as a loop diuretic working via the renal Na^+/K^+-ATP shuttle pathway.

In Vitro Assessment of Lead Anthranilate Compound

To pre-clinically evaluate furosemide as a putative COVID-19 therapeutic, a series of *in vitro* efficacy assessments was performed to investigate its anti-inflammatory properties. First, we investigated if furosemide could reduce the release of pro-inflammatory cytokines induced by LPS in macrophage cell line. RAW264.7 macrophages were stimulated with LPS in the presence or absence of furosemide and the level of TNF-α and NO were measured from the conditioned media using ELISA and Griess assay, respectively. The results in Fig. 2 (A) and (B) show that LPS induces the production of NO and TNF-α, indicating macrophage polarization to an M1 pro-inflammatory phenotype. When cells were treated with LPS in the presence of 25 μM of furosemide, the production of NO and TNF-α significantly decreased. To further investigate the reduction of NO production by furosemide, we determined the expression level of inducible nitric oxide synthase (iNOS) which produces NO in response to inflammatory stimulations. We stimulated RAW264.7 macrophages with LPS and IFNγ. The expression of the iNOS was significantly induced by stimulation with LPS and IFNγ, as shown by the Western blot results in Fig. 2 (C). We found that furosemide was able to suppress the expression of iNOS during LPS

and IFNγ induced stimulation. Densitometry analysis showed that normalized iNOS/GAPDH ratio was reduced from 1 to 0.88 by furosemide.

LPS is recognized by the cell surface pattern-recognition receptors such as the toll-like receptor 4 protein (TLR4) and triggers downstream signaling pathways. LPS induces expression of the pro-inflammatory cytokine IFNγ. IFNγ is known to increase TLR4 expression which may then promote the response to LPS stimulation. We investigated the effect of furosemide on LPS-induced TLR4 expression in RAW264.7 macrophage cells using flow cytometry. As shown in Fig. 3, LPS increased the TLR4+ cell population significantly. Whereas IL-4, an anti-inflammatory cytokine, barely induced TLR expression from the cells. Interestingly, furosemide completely blocked LPS-induced TLR4 expression, suggesting the possible involvement of furosemide in the LPS- or IFNγ-induced inflammation.

As the next stage of macrophage activation, we studied the activity of furosemide on the expression and secretion of IL-1β which plays a key role in modulating inflammatory response, as a downstream pro-inflammatory marker of the TLR4 signalling pathway by using differentiated THP-1 monocytes. We analyzed the expression of IL-1β precursor protein (pro-IL-1β) and the secretion of mature IL-1β from THP-1 macrophage cells by using Western blot analysis and ELISA, respectively. Furosemide, as shown in Fig. 4, significantly decreases the expression of pro-IL-1β in differentiated THP-1 cells as identified by Western blot analysis. As the secreted form, we measured soluble IL-1β from the conditioned medium of cell culture via ELISA. The differentiated THP-1 macrophages secrete IL-1β upon LPS stimulation and the amount of secreted IL-1β slightly decreased with co-treatment of LPS with furosemide, demonstrating yet another inflammatory cytokine targeted by furosemide.

To further explore the effect of furosemide on different macrophages, we next tested it on SIM-A9 cells. We stimulated SIM-A9 macrophages with LPS and analyzed the release of pro-inflammatory markers such as NO, IL-6 and TNF-α. The results in Fig. 5 show that LPS induced the production of NO, IL-6 and TNF-α from SIM-A9 cells. Similar to RAW264.7 cell results, furosemide significantly reduced the production of all these pro-inflammatory markers from SIM-A9 cells as well.

We have tested three different macrophage cell lines for the effects of furosemide on pro-inflammatory markers. Furosemide consistently reduced pro-inflammatory markers such as NO production, secretion of IL-6, TNF-α and IL-1β from different macrophage cell lines, implying that furosemide has broad inhibitory activity against pro-inflammatory cytokines.

Then, we evaluated if furosemide exhibits any effects on anti-inflammatory cytokines. We measured the levels of anti-inflammatory cytokines from the conditioned medium of differentiated THP-1 cells after 48 h of stimulation with LPS and IFNγ by Multiplex assay using flow cytometry. Furosemide induces the expression of IL-4, interleukin-1 receptor antagonist (IL-1RA) and arginase, which are anti-inflammatory markers, suggesting the polarization of THP-1 macrophage to an M2 phenotype (Fig. 6).

Together with the experiments discussed above, these results show that furosemide inhibits the expression of M1 pro-inflammatory markers and promotes the expression of M2 anti-inflammatory markers. Thus, furosemide is a broad-spectrum anti-inflammatory drug candidate targeting multiple cytokines.

In Silico Mechanism of Action Simulations

The molecular mechanism of action whereby 3-HAA and furosemide inhibit IL-6 and TNF-α activity is unknown at this time and lies outside the scope of the present study. Interestingly, in an *in silico* screen of possible binding sites for 3-HAA and related drug anthranilates, we noted favourable interactions with both the IL-6 and TNF-α proteins. Whilst it is improbable that such interactions are completely responsible for the observed anti-inflammatory effects, this notable observation is worthy of future experimental evaluation. These computational simulations are briefly outlined below.

The binding of 3-HAA in IL-6 and TNF-α active sites is presented in Figs. 7 and 8. The docking score (S) of minimum energy pose of 3-HAA in IL-6 is -4.620, and in TNF-α is -4.487. In Figs. 7 (A) and 8 (A), the ligand 3-HAA is shown in yellow and amino acid residues of binding pocket have been labelled. Figures 7 (B) and 8 (B) represent the ligand interaction diagrams of 3-HAA with active site residues of TNF-α and IL-6.

Figures 9 and 10 present docking simulations of furosemide with TNF-α and IL-6, respectively. In Figs. 9 (A) and 10 (A), furosemide is shown in yellow and the amino acid residues of the binding site are labelled. The TNF-α protein complex (PDB: 2AZ5) contains four identical chains having 148 amino acids each and two bound ligands. The A and B chains were retained (coloured orange and purple) and co-crystalized; the ligand was removed while preparing the protein structure for docking.

Figure 9 (A) shows the minimum energy docking pose of furosemide in the TNF-α active site. The amino acid residues Leu B57, Tyr B59, Gly B121, Tyr A151, Tyr A119 and Leu A120 are involved in forming hydrogen bonding and π-π interactions (arene-arene, arene-H, arene-cation) with furosemide in minimum energy docked poses. The docking score of the minimum

energy pose is -6.0854, which indicating that furosemide fits well into the active site of TNF-α and inhibits its activity.

Figure 10 shows the minimum energy docking pose of furosemide in the protein structure of human IL-6 (PDB: 1ALU), having 186 amino acid residues and a co-crystallized ligand (tartaric acid). The co-crystallized ligand had been removed during the structure preparation step of the IL-6 protein. Furosemide interacts with Arg 182, Gln 175, Leu 33 and Arg 179 in most of the minimum energy poses. The docking score (S) of the minimum energy pose of furosemide with IL-6 is -5.1343, which computationally supports the ability of furosemide to inhibit IL-6 activity.

Discussion

The pathogenic mechanisms of COVID-19 morbidity and mortality are diverse, though immuno-inflammatory contributions are likely a central player. It is now appreciated that COVID-19 afflicted individuals with major respiratory symptoms have pathologically elevated levels of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α (Conti *et al.* 2020; Mehta *et al.* 2020; Qin *et al.* 2020; Huang *et al.* 2020). A logical therapeutic approach to the management of COVID-19 thus includes a need to modulate immunotoxicity.

Tryptophan and its metabolites, particularly via the indoleamine-2,3-dioxygenase initiated pathway, have a previously described role as endogenous modulators of innate immunity. Of these various metabolites, 3-HAA has been identified to exhibit a significant anti-inflammatory ability to suppress inflammation mediated via multiple pro-inflammatory interleukin cytokines, including IL-1β, IL-6, IL-8 and TNF-α (Lee *et al.* 2013). This motivated our search for an anthranilate-based 3-HAA-like agent from a library of known drugs; furosemide emerged as a possible candidate from this search.

As part of its pre-clinical evaluation, we studied furosemide's anti-inflammatory activity on multiple macrophage cell lines involved in innate immunity. Broadly conceptualized stimulated macrophages can be polarized to either an M1 pro-inflammatory phenotypes or an M2 anti-inflammatory phenotype. We investigated if furosemide inhibits the production of pro-inflammatory cytokines (M1) or promotes the secretion of anti-inflammatory cytokines (M2) involved in COVID-19 disease progression, using RAW264.7, THP-1 and SIM-A9 cell lines. Upon stimulation, these cell lines initiate an immune response by producing cellular stress signals and pro-inflammatory cytokines. Upon treatment with furosemide, these pro-inflammatory markers were reduced, indicating that furosemide suppresses the M1 polarization, including NO, IL-6, TNF-α and IL-1β. More importantly, our Multiplex ELISA results demonstrate furosemide promotes the production of anti-inflammatory cytokine products (IL-1RA, arginase), indicating M2 polarization.

Furosemide is a small molecule with a molecular weight of 330.75 g/mol and relatively low lipophilicity (logP = 2.03) (Hardman *et al.* 2001). Although the drug has low water solubility at pH 7, furosemide can be formulated in weakly basic buffer solution (pH 8) to achieve 10 mg/mL solutions suitable for intravenous administration. Due to the presence a primary sulfonamide and carboxylic acid group, furosemide is highly bound to albumin with a human plasma protein binding value of 98.6 ± 0.4%. The drug has a very low volume of distribution (V_D = 0.13 ± 0.06 L/kg) and a relatively short half-life of 1.3 ± 0.8 h (Hardman *et al.* 2001). These pharmacokinetic parameters along with high plasma protein binding equates to low tissue distribution with furosemide being retained with the blood. These intrinsic molecular properties are ideal for targeting macrophages contained within the blood stream prior to their tissue distribution. Furosemide exhibits a large therapeutic window.

Furosemide is listed on the WHO's List of "Essential Medicines"; it is readily available worldwide, is easily manufactured, and has a long record of safety and efficacy when given orally or intravenously. More importantly, furosemide may also be administered safely by inhalation. More than 20 years ago, the concept of inhaled furosemide was explored as an approach to reduce dyspnoea, primarily based on the rationale that edematous airway mast cells would be reduced in size following diuresis (Prandota 2002). However, further investigations established that the mechanism of action was not related to local diuretic effect or engagement of the Na^+/K^+-ATP shuttle. More recent studies have reported reduction in pulmonary IL-6, IL-8, and TNF-α levels upon administering inhaled furosemide to patients with conditions including tachypnoea (Armed Forces Hospital Pakistan 2016; University of Cologne 2012), bronchopulmonary dysplasia (University of North Carolina *et al*. 2015), and chronic lung disease (Center BIDM and Research NIoN 2014; McGill University 2016; Oxford Brookes University 2015). A 2018 double-blind, placebo-controlled trial by Grogono *et al.* (2018) evaluated inhaled nebulized furosemide (40 mg furosemide in 4 mL 0.9% saline), demonstrating improved efficacy after multiple dosing per day with no untoward effects. Therefore, accumulated data indicate that furosemide is a cytokine-targeting anti-inflammatory, which may be safely administered by inhalation multiple times per day.

Our *in silico* screening identified other loop diuretics with structural similarities to furosemide. Bumetanide exhibits anti-inflammatory properties in LPS stimulated RAW264.7 cells, reduces LPS-induced production of cytokines following direct pulmonary administration, and lowers levels of TNF-α production in lung-injured mice (Hung *et al.* 2018). Bumetanide however failed to inhibit sodium metabisulfite induced bronchoconstriction in asthmatic subjects (O'Connor *et al*. 1991). Piretanide and azosemide have also been variously studied in models of

cytokine-mediated inflammation and bronchoconstriction (Yeo *et al*. 1992). Since none of these agents are in widespread clinical use, we have elected to pursue the development of furosemide in COVID-19 because of its worldwide availability in the time of a global pandemic.

The potential use of furosemide in the anti-inflammatory treatment of COVID-19 has strengths and weaknesses. Furosemide is inexpensive and available in every country in the world; it is safe and has profound anti-inflammatory cytokine activity, particularly against IL-6 and TNF-α. If administered orally, furosemide can produce a profound diuresis which would be a clinical detriment in a febrile and potentially dehydrated person. When administered orally, the pharmacokinetics of furosemide indicate that it would have primarily intra-vascular distribution, suggesting greater utility early in the course of the disease, but less so in later-stage ventilator-supported individuals in whom macrophage migration from bloodstream to pulmonary tissue has occurred. As the disease progresses, direct administration of furosemide to the lungs by nebulized delivery adequately addresses the need to have furosemide reach intra-alveolar macrophages. Care must be taken to prevent viral contamination and bystander exposure during the aerosolized administration of the drug, but this can be achieved with appropriate cautions in place. We are currently pursuing a clinical study of inhaled furosemide in people with COVID-19.

Conclusions

COVID-19 is a pandemic threatening global health. The need to identify innovative therapeutics which may be deployed rapidly and efficiently is a pharmacological priority. Furosemide is a safe, inexpensive, well-studied small molecule which is a reasonably potent inhibitor of IL-6 and TNF-α and may be administered locally to the lungs; pre-clinical data from *in silico* and *in vitro*

experiments suggest that it may be a candidate for repurposing as an inhaled therapy against the immunopathologies of COVID-19.

Supporting Information: A list of physiochemical descriptors used as initial screening test of the dataset; a list of promising drug candidates along with corresponding physiochemical descriptors; Docking Score 'S' of promising drug candidates with IL-6 and TNF-α; Figures of Mefenamic acid, Bumetanide, Piretanide and Azosemide drugs docking into binding site of TNF-α and IL-6 and Ligand interaction diagram of Mefenamic acid Mefenamic acid, Bumetanide, Piretanide and Azosemide drugs in active site of TNF-α and IL-6. This information is available free of charge on *PeerJ*.

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Figure 1

Loop diuretics structurally related to 3-HAA.

(A) 3-Hydroxyanthranilic acid, (B) furosemide, (C) bumetanide, (D) piretanide and (E) azosemide.

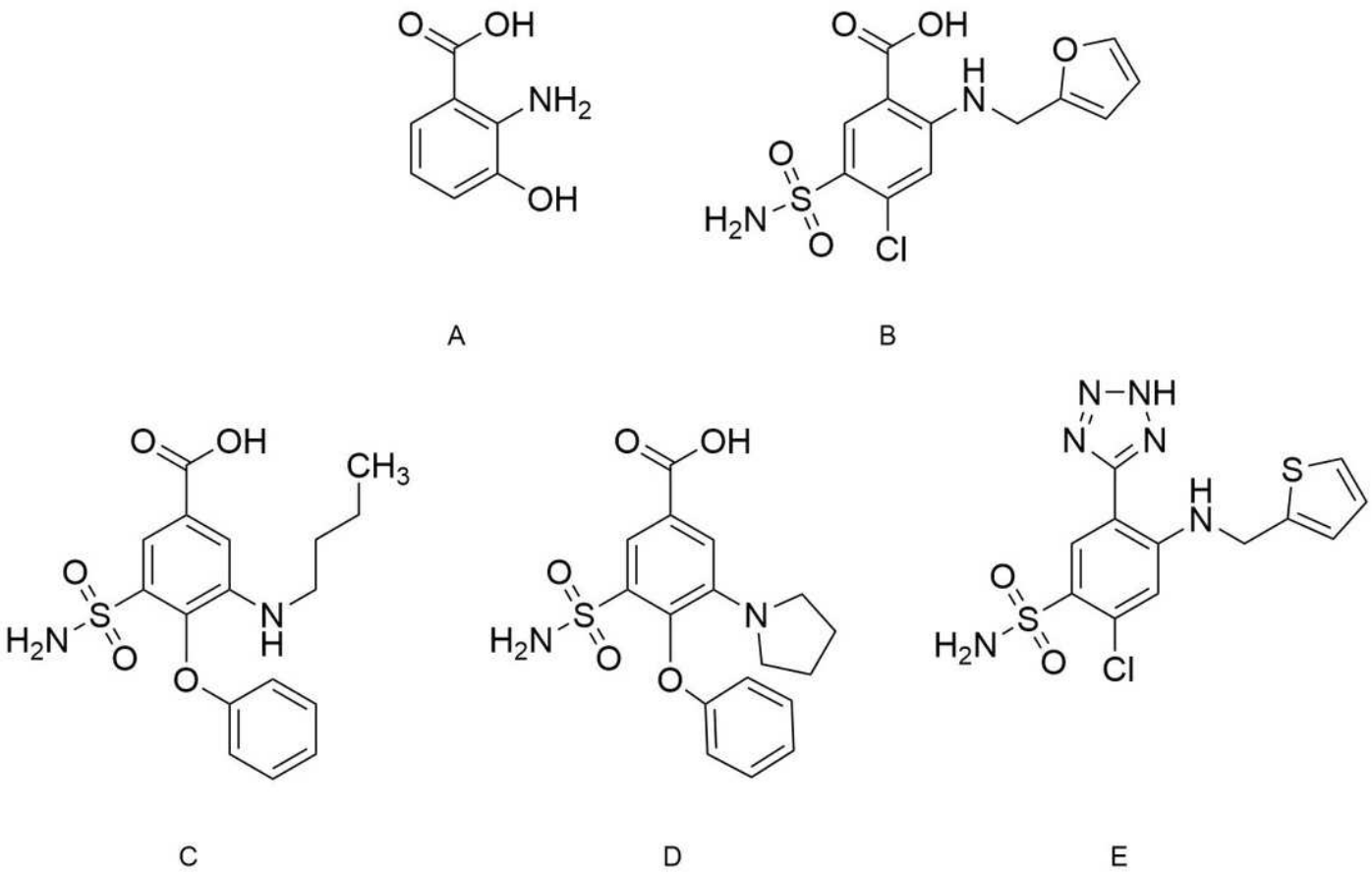

Figure 2

Furosemide decreases the production of NO and TNF-α.

Production of (A) NO and (B) TNF-α from RAW264.7 cells upon LPS induction were determined by Griess assay and ELISA from the conditioned medium. Error bars show SEM, n=3. *, $p < 0.05$. (C) iNOS expression level from RAW264.7 cells was assessed by Western blot analysis. Cells were treated with LPS+IFNγ with DMSO or 25 μM furosemide. After 24h of incubation, cell lysate was harvested for Western blot analysis. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

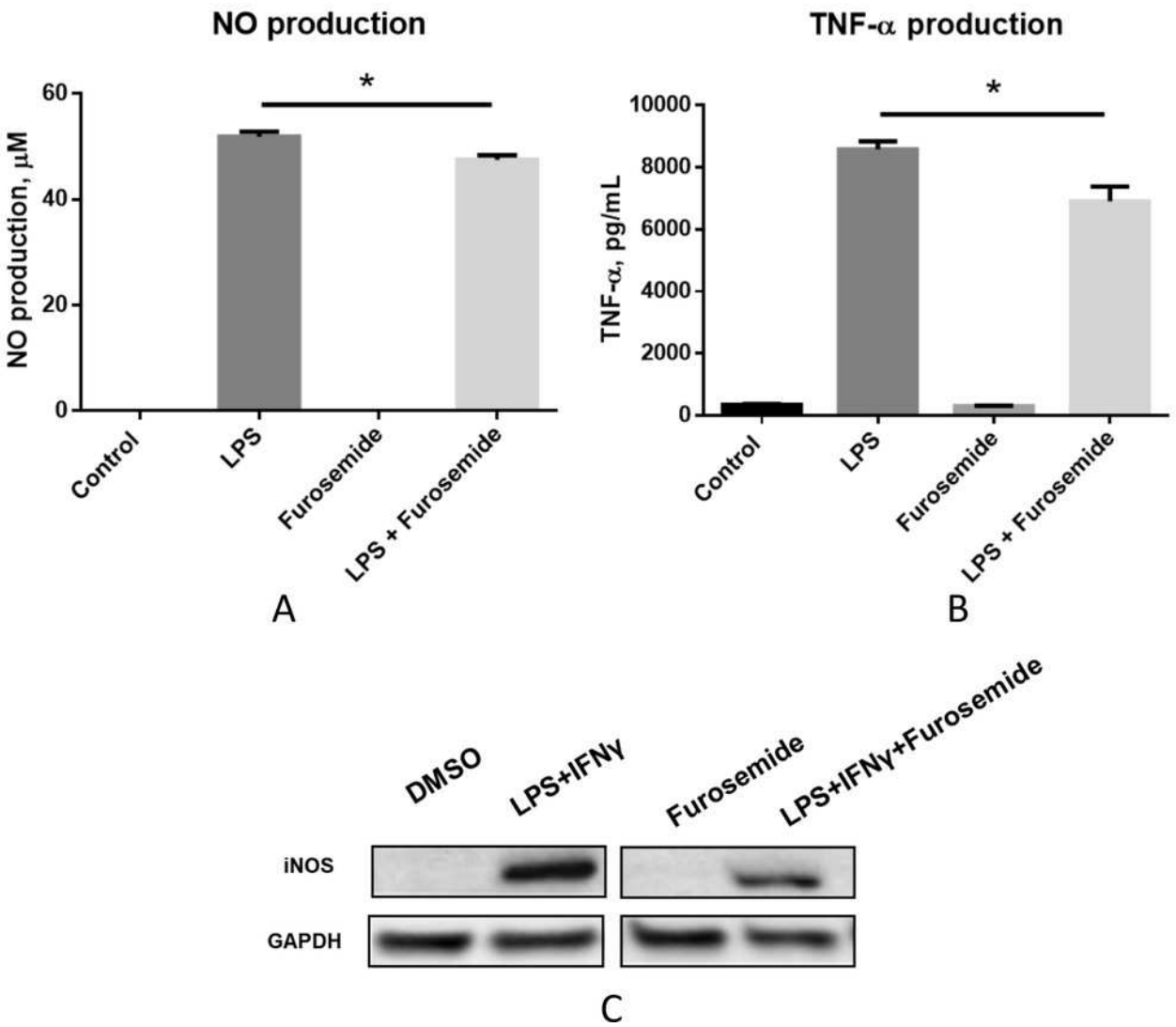

Figure 3

Furosemide significantly decreases TLR4+ cell population.

RAW264.7 macrophage cells were stimulated with LPS and flow cytometry was used to determine TLR4+ cell population. (A), % of Vis and (B), cell numbers for TLR4+. F = furosemide, FSC-A = front scatter.

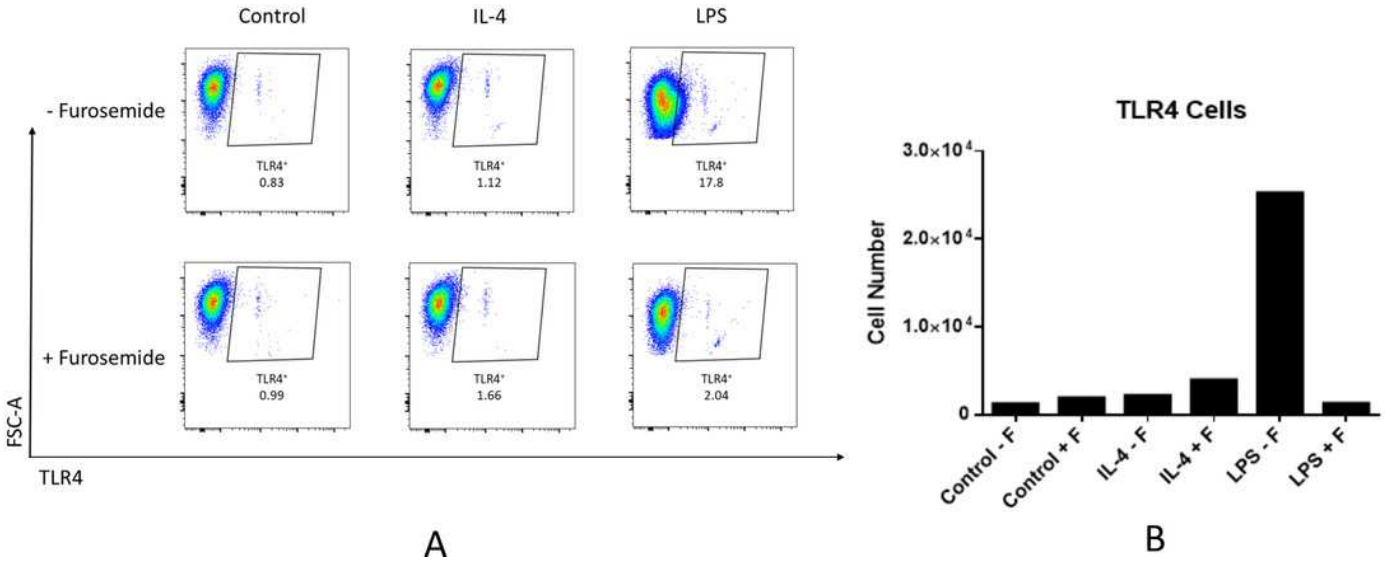

Figure 4

Furosemide significantly decreases both the expression of pro-IL-1β and the secretion of IL-1β from differentiated THP-1 cells.

Effect of furosemide on differentiated THP-1 monocytes: (A) Western blot showing the expression of pro-IL-1β upon treatment with DMSO and furosemide, respectively. Actin was used as a loading control. (B) Concentration of IL-1β was measured by ELISA from the conditioned medium after treating cells with furosemide, LPS or both. Error bars show SD, n=6.

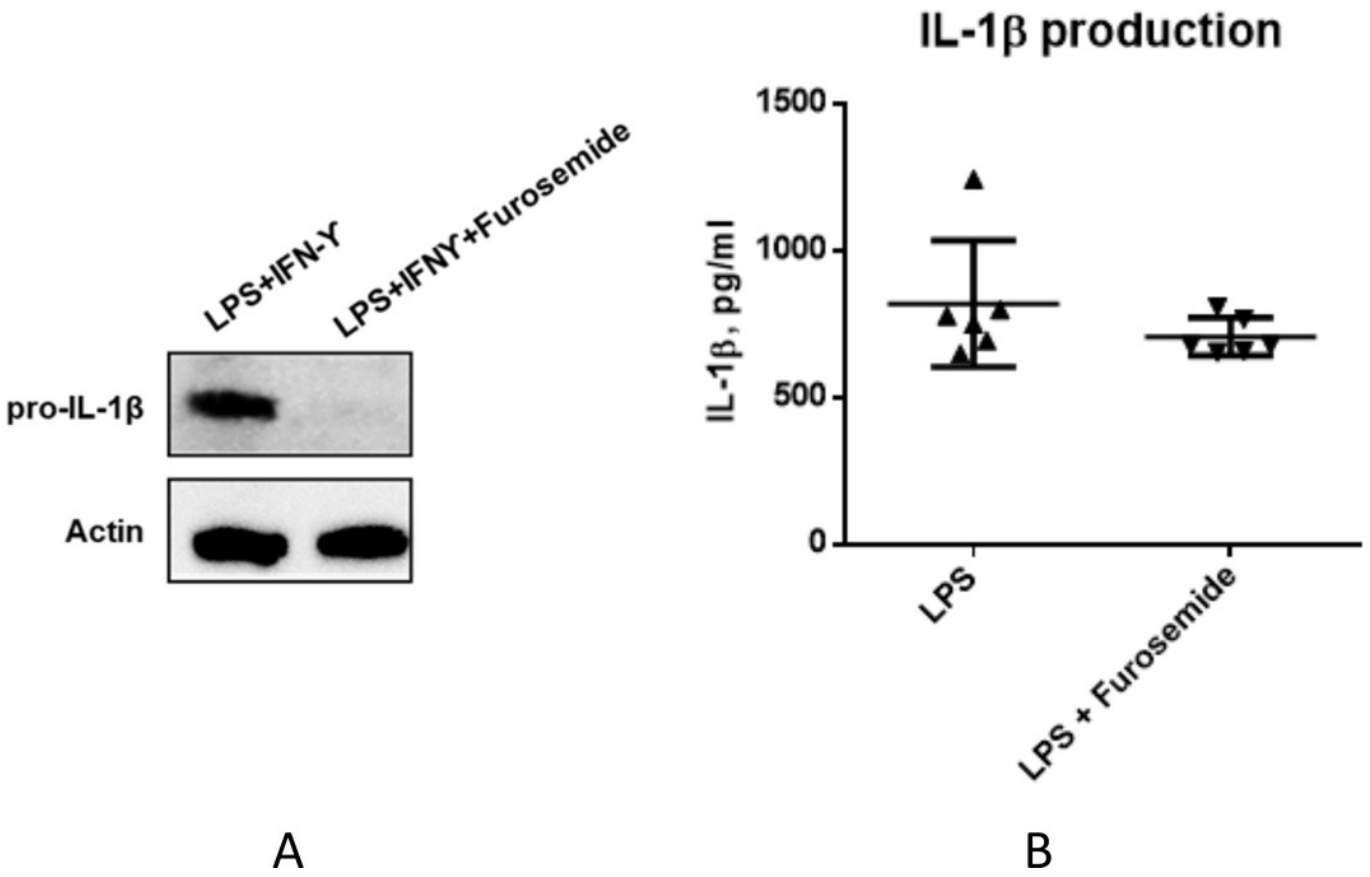

Figure 5

Furosemide decreases production of pro-inflammatory markers from LPS-stimulated SIM-A9 cells.

Production of (A) NO, (B) IL-6 and (C) TNFα after stimulation with LPS was measured from the conditioned media by either Griess assay or ELISA. Error bars show n=6. **, $p < 0.01$; ***, $p < 0.001$.

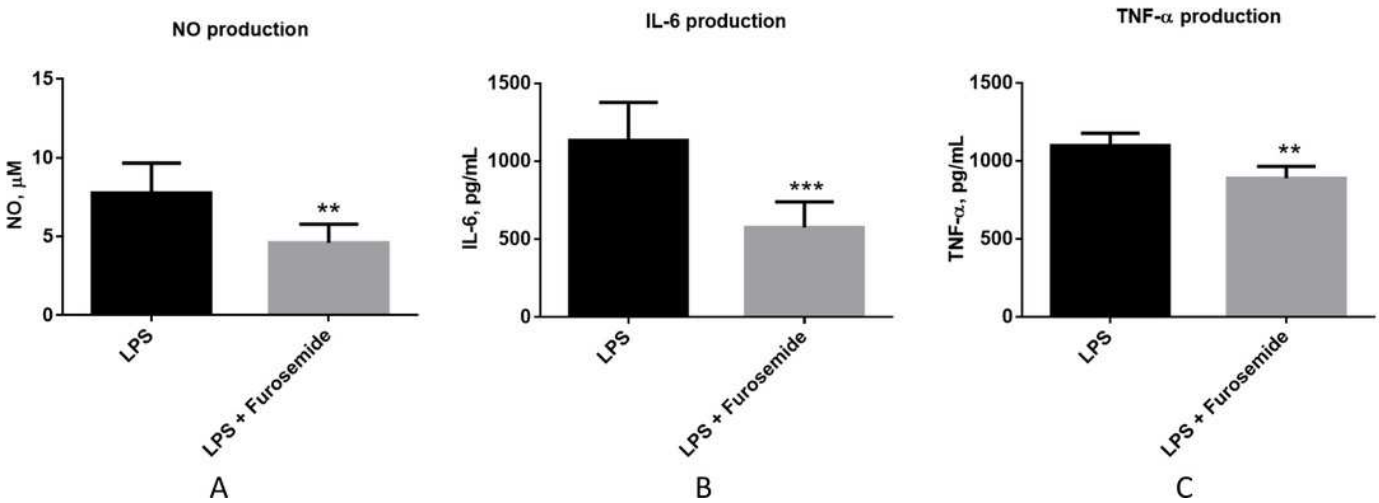

Figure 6

Furosemide induces expression of anti-inflammatory phenotype markers.

Analysis of the anti-inflammatory markers (A) IL-4, (B) IL-1RA and (C) Arginase from differentiated THP-1 macrophage upon treatment with LPS and INFγ with DMSO or 25 µM furosemide. After 48h of incubation, conditioned medium was harvested for cytokine analysis. Error bars show SD, n=2. **, $p < 0.01$.

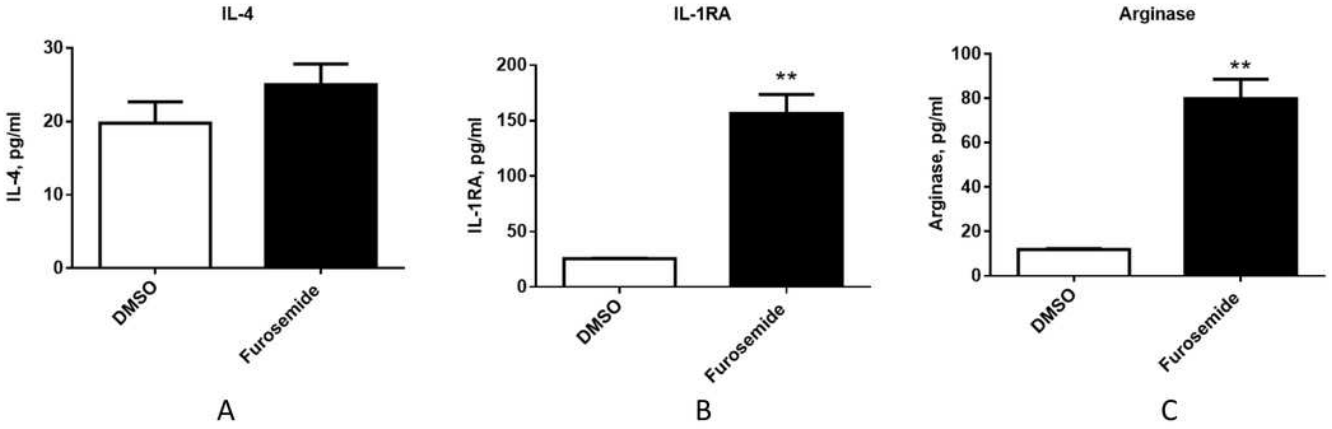

Figure 7

Figure 7 Interaction of 3-HAA with TNF-α active site.

(A) Binding of 3-HAA in the active site of tumor necrosis factor α (TNF-α); (B) Ligand interaction diagram of 3-HAA in binding site of TNF-α.

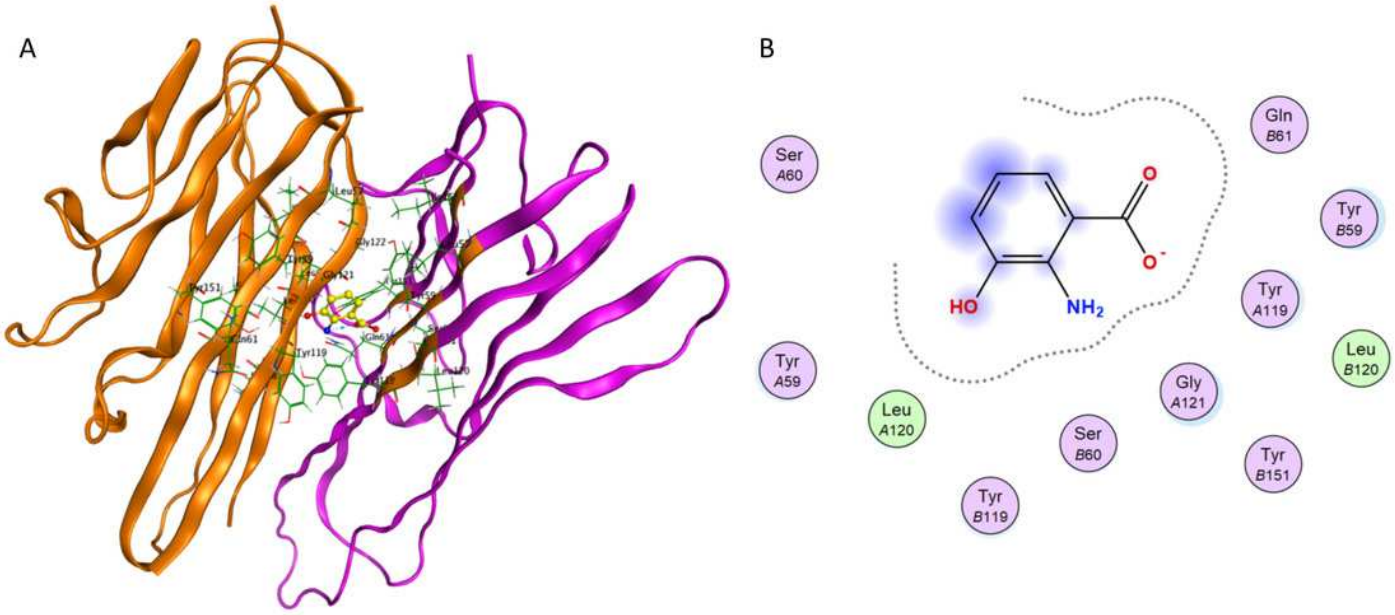

Figure 8

Interaction of 3-HAA with active site of IL-6.

(A) Binding of 3-HAA in the active site of interleukin-6 (IL-6); (B) Ligand interaction diagram of 3-HAA in binding site of IL-6.

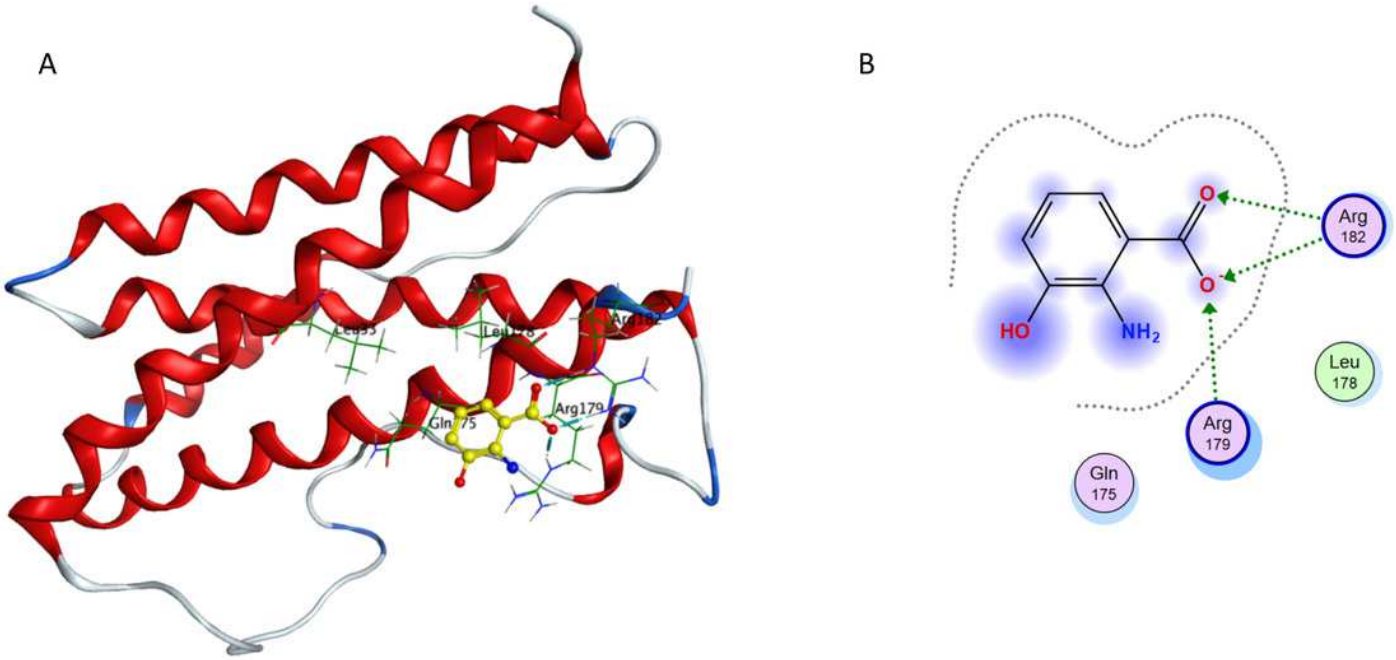

Figure 9

Interaction of furosemide with active site of TNF-α.

(A) Binding of furosemide in the active site of tumor necrosis factor α (TNF-α); (B) Ligand interaction diagram of furosemide in binding site of TNF-α.

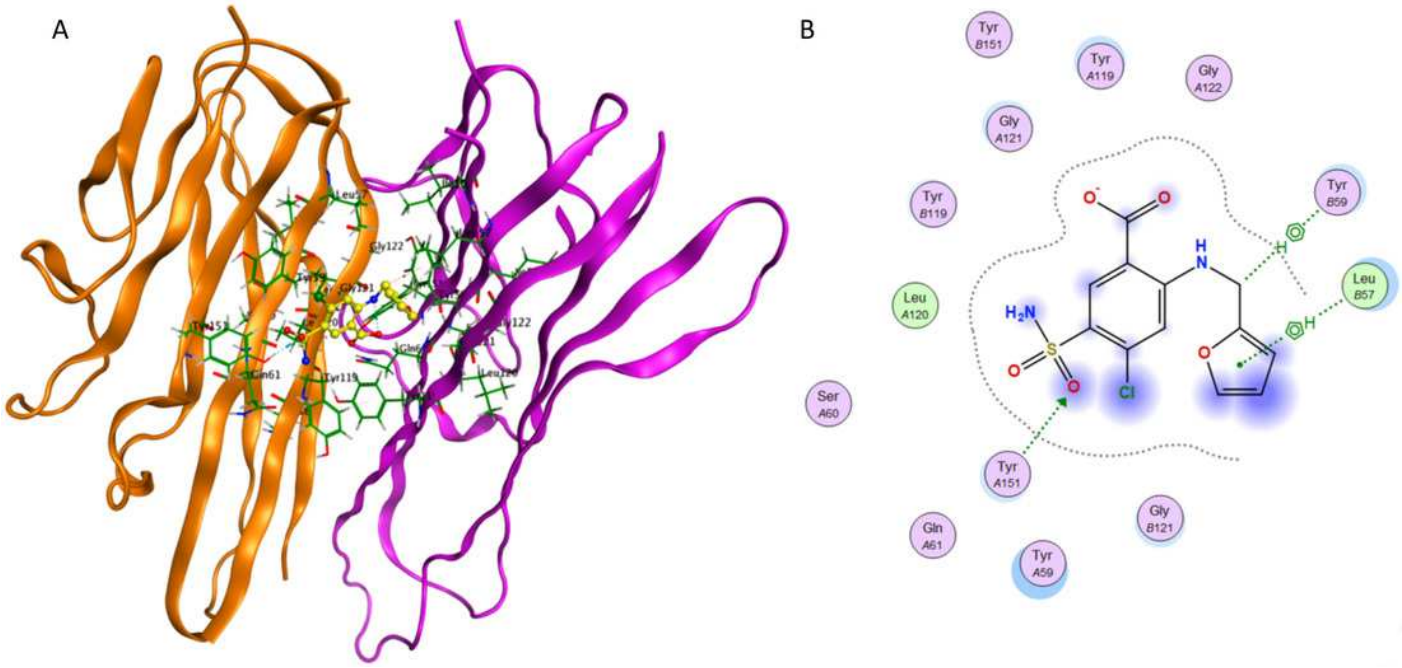

Figure 10

Interaction of furosemide with active site of IL-6.

Binding of furosemide in the active site of interleukin-6 (IL-6); (B) Ligand interaction diagram of furosemide with binding site of IL-6.