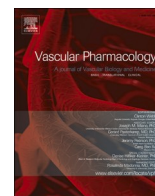Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

In silico Nigellidine (*N. sativa*) bind to viral spike/active-sites of ACE1/2, AT1/2 to prevent COVID-19 induced vaso-tumult/vascular-damage/comorbidity

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A B S T R A C T

COVID-19, a global-pandemic binds human-lung-ACE2. ACE2 causes vasodilatation. ACE2 works in balance with ACE1. The vaso-status maintains blood-pressure/vascular-health which is demolished in Covid-19 manifesting aldosterone/salt-deregulations/inflammations/endothelial-dysfunctions/hyper-hypo-tension, sepsis/hypovolemic-shock and vessel-thrombosis/coagulations. Here, nigellidine, an indazole-alkaloid was analyzed by molecular-docking for binding to different Angiotensin-binding-proteins (enzymes, ACE1(6en5)/ACE2 (4aph)/receptors, AT1(6os1)/AT2(5xjm)) and COVID-19 spike-glycoprotein(6vsb). Nigellidine strongly binds to the spike-protein at the hinge-region/active-site-opening which may hamper proper-binding of nCoV2-ACE2 surface. Nigellidine effectively binds in the Angiotensin- II binding-site/entry-pocket (−7.54 kcal/mol, −211.76, Atomic-Contact-Energy; ACE-value) of ACE2 (Ki 8.68 and 8.3 μmol) in comparison to known-binder EGCG (−4.53) and Theaflavin-di-gallate (−2.85). Nigellidine showed strong-binding (Ki, 50.93 μmol/binding-energy −5.48 kcal/mol) to mono/multi-meric ACE1. Moreover, it binds Angiotensin-receptors, AT1/AT2 (Ki, 42.79/14.22 μmol, binding-energy, −5.96/−6.61 kcal/mol) at active-sites, respectively. This article reports the novel binding of nigellidine and subsequent blockage of angiotensin-binding proteins. The ACEs-blocking could restore Angiotensin-level, restrict vaso-turbulence in Covid patients and receptor-blocking might stop inflammatory/vascular impairment. Nigellidine may slowdown the vaso-fluctuations due to Angiotensin-deregulations in Covid patients. Angiotensin II-ACE2 binding (*ACE*-value −294.81) is more favorable than nigellidine-ACE2. Conversely, nigellidine-ACE1 binding-energy/Ki is lower than nigellidine-ACE2 values indicating a balanced-state between constriction-dilatation. Moreover, nigellidine binds to the viral-spike, closer-proximity to its ACE2 binding-domain. Taken together, Covid patients/elderly-patients, comorbid-patients (with hypertensive/diabetic/cardiac/renal-impairment, counting >80% of non-survivors) could be greatly benefited.

1. Introduction

At the initial phase of infection of SARS CoV2, ACE2 is occupied by the peripheral spike glycoprotein of the virus which is then internalized. In this situation angiotensin II level become high so the blood pressure increases and in the hypertensive individuals this situation becomes even more detrimental for the drastic pressure increase. In elderly persons it may results in cerebral, cardiac and renal vascular failure and death [1,2].But, in the post infection stage and after few cycles of viral generations (considering its extreme high rate of propagation) SARS CoV2 stimulatesACE2 expression on different tissues including the vast beds of arterial endothelial cells and even in several tissue surfaces other than lung [3,4]. It makes the system more vulnerable to viral infection. At the same time extra ACE2 utilizes more angiotensin II to form angiotensin 1–7 peptides which may results in sudden vasodilatation (creating vasogenic or vasoplegic shock,) and hypovolemic shock in association with cardiogenic shock (3). In addition, in advanced viral infection state sepsis shock in response to cytokine storm makes the situation worsen. So, the persons already having an infection/inflammation history with hypertensive/vascular disorder both or alone become extremely vulnerable and morbid in nature [5]. The present status of death versus infection of country-wise/global ratios and elderly

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(having more possibility several disorders/aging processes) persons decisively suggests the comorbidity situation is the strongest determinant of the disease severity and death.

As explained in Li, et al. [6], low infection, moderate infection and severe infection can be represented by no co-morbidity, less number of co-morbidity and more number of co-morbidity situations respectively. Renin-angiotensin system, aldosterone and other steroid regulations are the major and direct determinant of blood pressure and vascular health in an individual. Disorder in the peripheral vasculature may impair three organ-associated vascular systems; namely cardio-vascular, reno-vascular and cerebro-vascular systems. These are also the main target of the age and possible gender associated anomalies [7,8]. Age related impairment of the immune system has been reported [1,9]. Moreover, it has been recently demonstrated that SARS-CoV-2 can infect podocytes and tubular epithelial cells of the kidney [10]. Taken together, co-morbidity is the main factor and possibly can explain the gap between numbers of deaths versus infection in different geographical locations.

Angiotensin II being the main factor of pressure regulation and ACE2 being the specific target of spike glycoprotein of SARS CoV-2, the upstream and downstream regulations of angiotensin II metabolism and functions could best explain about the co-morbidity situations and eventually the therapeutic targeting of this regulatory pathway may protect from the severity of the co-morbidity situation. In addition to the binding affinity between viral spikes to ACE2, only ACE2 expression can be a disease severity factor. So targeting the spike protein is also great therapeutic approach. Earlier finding suggests that tea flavonoids like Epigallocatechin and theaflavin gallate may block the central channel of the spike protein [11]. In the current study we showed that nigellidine can bind to the closer extremities of the Receptor Binding Domain (RBD) of the spike attachment locations and may have some viral attachment inhibition. Further studies are necessary in this regard.

In classical physiology, it is well-known that angiotensinogen is converted to angiotensin I which is further converted to angiotensin II with the help of ACE. Angiotensin II is a vasoconstriction agent. Now the ACE2 can convert the angiotensin II to angiotensin (1–7) peptides. These peptides are altogether vasodilatation agents. ACE2 performs pivotally counter-balancing to ACE by catalyzing angiotensin II which is the key regulator in the renin-angiotensin-aldosterone system. In case of lower function of ACE2, angiotensin II remain high and performs as the ligand function to AT1 and AT2 surface receptors of several tissues. Binding to AT1 has a strong vasoconstriction function and that is opposite when the binding is on AT2. Other than vaso-status determination, this bunch of peptides and their target enzymes/receptors have other function like inflammatory, gene expressions, signal transductions function. Those states can perform in multi-phasic manner ending up with good or bad outcome. Very often in case of impairment of this tight regulation angiotensin II may act as a double sided sword. So, the up and down stream regulations of Angiotensin II functions which is targeted by SARS CoV-2 has been screened. Here, the focus has been pointed on possible drug targets to this system to forbid the co-morbid situation during this viral infection. Our study suggests that nigellidine an indazole alkaloid strongly resists the SARS Cov-2 associated ACE2 deregulations at its different regulatory steps with most balanced outcome. It is proposed that medications which inhibit the RAS ACE-dependent pathway may be beneficial in treating COVID-19 [12,13]. The basal level of Ang II (basal constriction state) is depleted. It is called post sepsis viral shock, hypotonic/hypovolemic shock. These extra ACE2 may bind with the nigellidine and reduce extra Angiotensin II breakdown. It will maintain normal physiological vaso-state (constriction/dilatation state).

In this background, our objectivity was to screen a suitable molecule which can effectively bind to the angiotensin binding proteins (enzymes ACEs and receptors ATs) and as a whole down-regulates Angiotensin II mediated vaso-functions to restore the normal vaso-state and vascular-inflammatory activities. From a bunch of molecules tested, we found that nigellidine can effectively perform this function which could be helpful in the comorbid and other Covid patients. Moreover, nigellidine

was found to bind in the viral spike glycoprotein that is close proximity of RBD and ACE2. For the first time, we showed that nigellidine can bind to the active binding locations of several angiotensin binding proteins. This finding may have great clinical implications in the present pandemic situation of Covid infection. Further studies are necessary in this regard.

2. Materials and methods

2.1. Protein tertiary structure retrieval

Present study has represented the interaction pattern of nigellidine with different Angiotensin receptors like Angiotensin Converting Enzyme 1 (ACE1), Angiotensin Converting Enzyme 2 (ACE2), AT1 and AT2 and COVID 19 spike glycoprotein. The 3D structures of these receptors were retrieved from Protein Data Bank (PDB) in .pdb file written in flat file format (https://www.rcsb.org/). That PDB ID, used in this study were 6en5, 4aph, 6os1, 5xjm and 6vsb representing ACE1, ACE2, AT1, AT2 and COVID 19 spike glycoprotein respectively.

2.2. Structural modification

The selected PDB structures were found with different molecules like H_2O, NAG, BAM etc. including Angiotensin molecules in Angiotensin receptors. Whereas, COVID 19 spike glycoprotein showed with multiple NAG unit at different position. So, for molecular docking study different attached molecules were removed from the receptor molecules using Pymol molecular visualization software [14]. After removal, receptor molecules were saved in .pdb file for further analysis.

2.3. Ligand structure retrieval and modification

The ligand molecule, nigellidine is a rare indazole-type alkaloid also known as 6,7,8,9-Tetrahydro-1-hydroxy-11-(4-hydroxyphenyl)-3-methylpyridazino[1,2-a]indazol-5-ium inner salt, 9CI. It contains three benzene ring attached through a pentose structure. The molecular formula and weight were $C_{18}H_{18}N_2O_2$ and 294.3 g/mol. The canonical smile was CC1 = CC(=O)C2 = C(N3CCCCN3C2 = C1)C4 = CC=C(C=C4)O. The three dimensional (3D) structure of nigellidine with PubChem ID of 136,828,302 was retrieved from PubChem chemical structure database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format. The retrieved SDF format was saved in PDB format using Pymol.

2.4. Molecular docking with Patchdock

ACE2-Nigellidine binding analysis has been performed through molecular docking analysis. For comparative study, twosoftware were used like Patchdock and Autodock. PatchDock facilitate with molecular docking within two molecules of protein, DNA, peptides or drugs etc. It is based on shape complementarily algorithm [15]. This algorithm works through three different steps; first is recognition of molecular shape of inputs. After recognition the shapes at different surface areas a segmentation algorithm works to find out geometric patches (flat surface, concave and convex). Among them only the 'hot spot' patches are taken for analysis. Second is the surface patch matching algorithm which is the hybrid method of Pose-Clustering matching techniques and Geometric Hashing. In this step, all the preselected geometric patches were cross matched to find out most important patches based on different values. Third is the filtering and scoring matrix analysis of receptor-ligand interaction positions, where all the unacceptable interaction parameters are rejected and finally remaining are ranked based on the different complementary score generated during geometric shape calculation. Among the scores, Atomic Contact Energy (ACE) value is most important one, as it relates atomic desolvation energy between one selected geometric shape and the ligand [16]. It indicates the possibilities of replacing a protein-atom/water contact, with a protein-atom/

protein-atom interaction. Initially atom-pairing frequencies of 18×18 possible positions are calculated using following statistical analysis, where predetermined effective contact energy between receptor atoms type *i* and ligand atom type *j* is calculated.

$$T[i,j] = -ln\frac{N_{i,j}/C_{i,j}}{(N_{i,0}/C_{i,0}) \times (N_{j,0}/C_{j,0})} \qquad (1)$$

where 0 represented the solvent, N represented the actual number of *i-j* ($N_{i,j}$) and *i*-0 ($N_{i,0}$) contact of known complex. Whereas, C represented the expected number of *i-j* ($C_{i,j}$) and *i*-0 ($C_{i,0}$) contact. Finally the ACE value is calculated using the given formula which is the summation of each atom pair of a particular position, within the threshold distance *d in Å between* two subunits as S_1 and S_2

$$E_{ACE} = \sum_{s \in S_1, t \in S_2, \|s-t\| \leq d} T[s,t] \qquad (2)$$

where $T[s,t]$ is the pre-calculated value of the atom pair *s* and *t* and $|s - t|$ is representing the Euclidean distance between *s* and *t*. The more favorable ACE value is the lower value which indicated the rigid complex formation between receptor and ligand. Here in this study nigellidine was docked with all the selected Angiotensin receptors and COVID 19 spike glycoproteins and analyzed using ACE value.

2.5. Molecular docking with Autodock

The molecular docking study between each Angiotensin receptors, spike glycoprotein with ligand nigellidine was again performed using Autodock software. Autodock is an automated docking tool for small molecule docking upon known receptors 3D structure and explores the ligands atomic affinity grids. Autodock have application in structure-based drug designing, lead optimization, X-ray crystallography or NMR structure analysis, virtual screening (HTS), combinational library design, protein-protein docking and chemical mechanism study. Autodock analyze the receptor-ligand docking from different angle through Binding energy, Ligand efficiency, Intermolecular energy, Desolvation energy, Electrostatic energy, Total energy, Torsion energy and Inhibition constant. The interactive energy calculation also helps to determine the affinity of a drug or inhibitor to competitive or non competitive inhibition also. Autodock follows the Monte Carlo Simulated Annealing (SA), Genetic Algorithm (GA) specifically Lamarckian Genetic Algorithm (LGA). Here LGA finds the best result for lowest energy calculation.

2.6. Docking result and docking position analysis

Each sets of molecular docking were analyzed using Pymol molecular visualization software [14]. The presentation of docking structures and interactive bonds were represented through technical and transparent surface analysis mainly. On the other hand the 2D interactions were analyzed through LIGPLOT software [17]. LIGPLOT basically reads the ligand binding sites of 3D structure in PDB format and then it unrolls the positions according to their rotatable bonds and becomes flattening then in 2D form.

2.7. Binding rigidity analysis of different docked molecules

To understand the binding between different docked molecules, each pairs were individually subjected to PRODIGY tool on HADDOCK server. Binding affinity analysis were analyzed between AT1-Nigellidine, AT2-Nigellidine, ACE1-Nigellidine, ACE1-Nigellidine, AT1-Angiotensin, AT2- Angiotensin, ACE1- Angiotensin, ACE1- Angiotensin.

3. Results

3.1. Nigellidine and nCoV binding

The nCoV is denoted as the spike glycoprotein of novel coronavirus or SARS CoV2. This protein is composed of homotrimeric unit. These units' together form a central channel which is more rigid and stabilized by inter and intra chain hydrogen bonding (Fig. 1). This central channel maintains the rigidity of the spike glycoprotein and directly attaches the protein to the viral envelope. On the other hand, central channel holds the most flexible part of the spike glycoprotein that is host receptor protein binding site. Spike glycoprotein initially binds to the Angiotensin Converting Enzyme 2 (ACE2) present at the host cell surface and then the viral entry gradually proceeds. This ACE2 attachments site remains folded until it reaches the receptor. Just before ACE2 attachment in nCoV spike protein's flexible part become unfolded and the attachment site become exposed (Fig. 1). This flexibility is maintained by a flexible hinge region as mentioned in Fig. 1. In our study nigellidine was found to interact with nCoV at the flexible hinge region with the highest ACE value of −34 0.50. ACE value indicated the affinity of drug to the specific receptor location. A nearby position was also observed with ACE value of −202.47 and a rigid bond (CB-O) formation was also observed with ALA 1020 of nCoV unit. Nigellidine binding to this site may hamper the proper exposure of host cell attachment side during ACE2 surface binding.

3.2. Nigellidine ACE2 binding

Angiotensin Converting Enzyme 2 are usually found as the outer surface protein of different cell types like lungs, arteries, heart, kidney and intestines. ACE2 acts on angiotensin II, an 8 amino acid long vasoconstrictor peptide, to convert it into angiotensin (1–7). These converted angiotensin function as vasodilator. Thus ACE2 controls high blood pressure. In present study ACE2 was analyzed for angiotensin II and nigellidine binding. As per the 3D structure of ACE2 it possesses an internal channel of angiotensin binding (Fig. 2). ACE2 functions as a carboxypeptidase and it cleaves a single residue from angiotensin I (AngI). That finally generates Ang1–9, and a single residue from angiotensin II (AngII) to generate Ang1–7 [18].

Angiotensin II is the main ligand of ACE2. As a result of which we also found the - 9.45 kcal/mol binding energy value of angiotensin II within the internal channel of ACE2. On the other hand the inhibition constant (Ki) value of angiotensin II to ACE2 was very high, i.e. 265.67 µmol at the same internal channel which indicated that no inhibition of ACE2 occurs during angiotensin processing as it is the substrate of it. Similar result was observed during the calculation on binding affinity and dissociation constant values. Here, Angiotensin showed higher binding affinity with ACE2 with a value of −11.6 ∆G (kcal mol-1) and it also showed some dissociation constant value of 6.20E-09. Whereas, molecular docking of ACE2 with nigellidine showed the lower binding energy of −7.54 and − 6.73 kcal/mol (S Table 1), lower binding efficiency of −5.6 ∆G (kcal mol-1) at the angiotensin binding site (S Table 3). But no dissociation constant value was observed for ACE2-nigellidine interaction. And the complete internal channel was found to block by the various posture of nigellidine (S Fig. 2). Even if the accumulation of nigellidine at the exit site of angiotensin was maximum. Nigellidine was also found to block the entry pocket of ACE2 with highest affinity and low binding energy value of −7.54 and −7.52 kcal/mol (Fig. 2). Though the binding energy of nigellidine (− 7.54 and −6.73) and the binding affinity (−5.6 ∆G (kcal mol-1)) were lower than angiotensin binding, it was found as a potent inhibitor of ACE2. The Ki value of nigellidine on ACE2 interaction was 2.99 and 11.64 µmol respectively at the Angiotensin binding site. Moreover the range of nigellidine Ki value (2.95–14.83) at different position of internal passage was also very low. It indicated that nigellidine can act as a potential inhibitor of ACE2. Nigellidine was also found to block the entry pocket
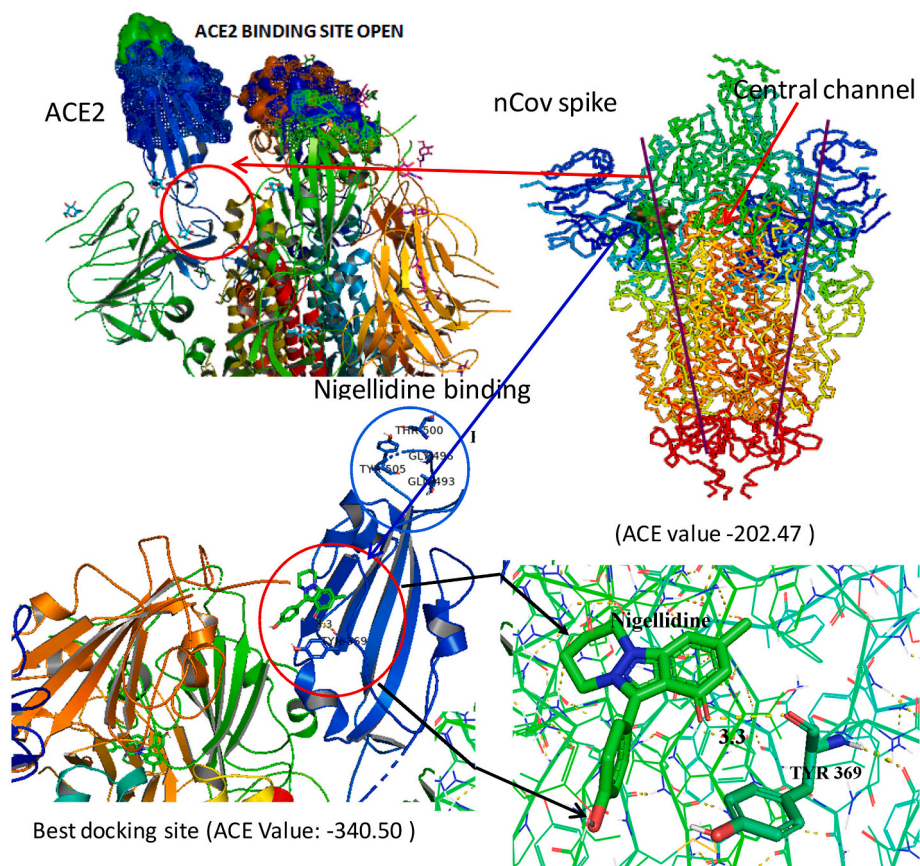
Fig. 1. Nigellidine and nCov binding was presented. The spike glycoprotein of COVID 19 has two parts; the central cannel and flexible attachment site. Attachment site are normally remain unexposed. During attachment with ACE2 receptor it becomes exposed. Nigellidine bound at the hinge region which controls the flexibility of spike glycoprotein attachment site.
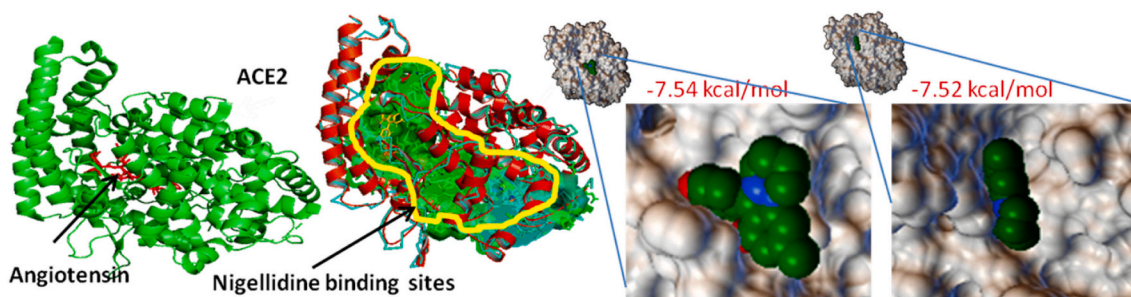

Fig. 2. Nigellidine and ACE2 binding was presented. Angiotensine usually bound to ACE2 at an internal channel. Nigellidine was also found to block that internal channel. Nigellidine was also found to block the entry pocket of Angiotensine within ACE2.

of ACE2 with highest affinity and low binding energy value of −7.54 and −7.53 kcal/mol (Fig. 2; supplementary file, S-table1). This binding was compared with some known/reported binder epigallocatechin-3-gallate (EGCG) [19]; −4.53 kcal/mol with ki 479.87 μmol and Theaflavin gallate [TFDG]; −2.85 kcal/mol with ki value 8.2 μmol. Best ten binding values of nigellidine, EGCG and TFDG to ACE2 are presented in supplementary file, S-table2. Similar results were also found for ACE1, AT1 and AT2. Where binding affinity were lower than the Angiotensin binding (S table 3) but no dissociation constant was for nigellidine interaction.

Ligand efficiency represents the binding energy of per atom of ligand to its receptor [20]. Here all the interactions of ACE1, ACE2, AT1 and AT2 with nigellidine showed ligand efficiency at negative range like −0.27, (−0.3, −0.25), (−0.27, −0.24) and (−0.31, −0.34) respectively

(S Table 1). In relation to that intermolecular energy, representing the lower barriers between two docked structures [21], also supported the rigid binding with negative value of −6.56, (−7.11, −6.05), (−5.85, −5.72) and (−7.33, −8.14) respectively. The cumulative energy calculation of van der Waals interaction, Hydrogen bond and disolvation energy (vdw hb desolv energy) represented the negative value of −6.5, (−7.07, −6.01), (−5.83, −5.6) and (−7.24, −8.07) respectively. Electrostatic energy represents the potential energy of a system placed within the time-invariant electric field [22] where the positive value indicated the repulsion and negative value indicated the surface attraction between two molecules [23]. Which were also found negative for nigellidine interaction with ACE1 (−0.05), ACE2 (−0.04, −0.04), AT1 (−0.02, −0.13) and AT2 (−0.08, −0.07). Calculated RMS also showed the value less than 1, i.e. 0, (0, 1.1), (0,0) and (0, 0) for all

position which means the prediction was accurate with very less or nun error value [24]. From all aspects, nigellidine binding to ACE1, ACE2, AT1 and AT2 were non-erroneous and facilitate from all significant aspects.

3.3. Nigellidine ACE1 binding

Angiotensin converting Enzyme 1 or ACE1 has been classified into zinc metalloprotase, usually acts on Angiotensin I. It comprises of two catalytic domains (-N and -C). These two domains found side by side in two structural units. This enzyme converts Angiotensin I to active Angiotensin II, a vasoconstrictor and thus regulates the blood pressure. Just like ACE2, each unit consist of an internal channel. Molecular docking with nigellidine revealed that the internal channel could be blocked by nigellidine as well whereas, the outer surface of the single unit ACE1 could also be blocked by nigellidine with ACE value of −196.15 and binding energy of −5.48 (Fig. 3). The active site of ACE 1 comprises of different amino acids, i.e.T358, A332, E362, H365, H361, Y501, E389, H491, H331, K489, Y498 and Q259 with which interaction of D7-S8 were observed (Fig. 3). On the other hand two postures of nigellidine blocked this active site of ACE1 with binding energy of −5.25 and − 5.13 kcal/mol and ACE value of −59.66 and − 5.25 kcal/mol respectively. At this position nigellidine was bound with similar amino acids like ALA332, HIS331, TYR501, TYR498, HIS361, THR358 and GLU362 etc. This is all about the nigellidine binding with ACE1 monomeric binding unit. On the other hand, the affinity of nigellidine to ACE1 dimeric unit was much higher than monomeric unit (Supplementary file; S-fig. 1). We have observed the highest ACE value of nigellidine-ACE1 monomeric unit was −196.15. But nigellidine interaction with ACE1-dimer showed much lower ACE value of −224.79 and the interaction was found at the dimer attachment site mainly. During this attachment the binding energy of best autodock position was −6.95 and at the active site was −5.86 & -5.35 kcal/mol which was also more stable than monomeric unit (S-table1).

3.4. Sequential and structural comparison of ACE1 and ACE2

ACE1 acts on Angiotensin I and converts it into Angiotensin II. This is being converted to Angiotensin 1–17 by ACE2. And thus the blood pressure of the body is being regulated. Though becoming functionally different the sequence of two proteins shares common amino acid combination and positions at maximum sites (S-fig. 1). Some major amino acid changes were also observed within two sequences. Whereas, the sequential diversity imprint vary less in structural diversity (data not shown).

3.5. Nigellidine binding with AT1 and AT2

AT1 and AT2 both were classified as the Angiotensin receptor. Though they are different subtypes they shares very close relation in respect to protein sequences and tertiary structural conformation (S-fig. 1). Both were mainly composed of alpha helix. In present study, AT1 (PDB ID:6OS1) and AT2 (PDB ID:5XJM) were analyzed for nigellidine binding affinity.

In AT1, Angiotensin binds to site 1 according to PDB structure 6OS1 (Fig. 4a). But another location (site 2) of Angiotensin binding was also observed during molecular docking study of AT1 and Angiotensin. At site1, amino acids of angiotensin TYR 4, LYS 5, HIS6, ARG2 and ALA8 formed bonds with PHE182, LYS199, TYR87, ASP1281, ASP1281 and LYS199 amino acids of AT1. During nigellidine docking, remarkable atomic contact energy value was observed at those two sites of Angiotensin binding (Fig. 4b). Highest ACE value of −227.09 was observed at site 2. According to autodock analysis also the site 2 was found with highest affinity and binding energy value of −7.12. Whereas, most of the nigellidine interactions were observed at site one. At site 1 maximum ACE value of −129.61 was found to block ASP1281, with which HIS6 molecule of Angiotensin usually binds (Fig. 4b). Other stable bond was formed with ARG167 and nearby amino acids were CYS180, TRP84, ALA21, ASP16, VAL179, TYR92 and TYR87. Among them LYS5 of Angiotensin usually interact with TYR87. According to autodock, out of
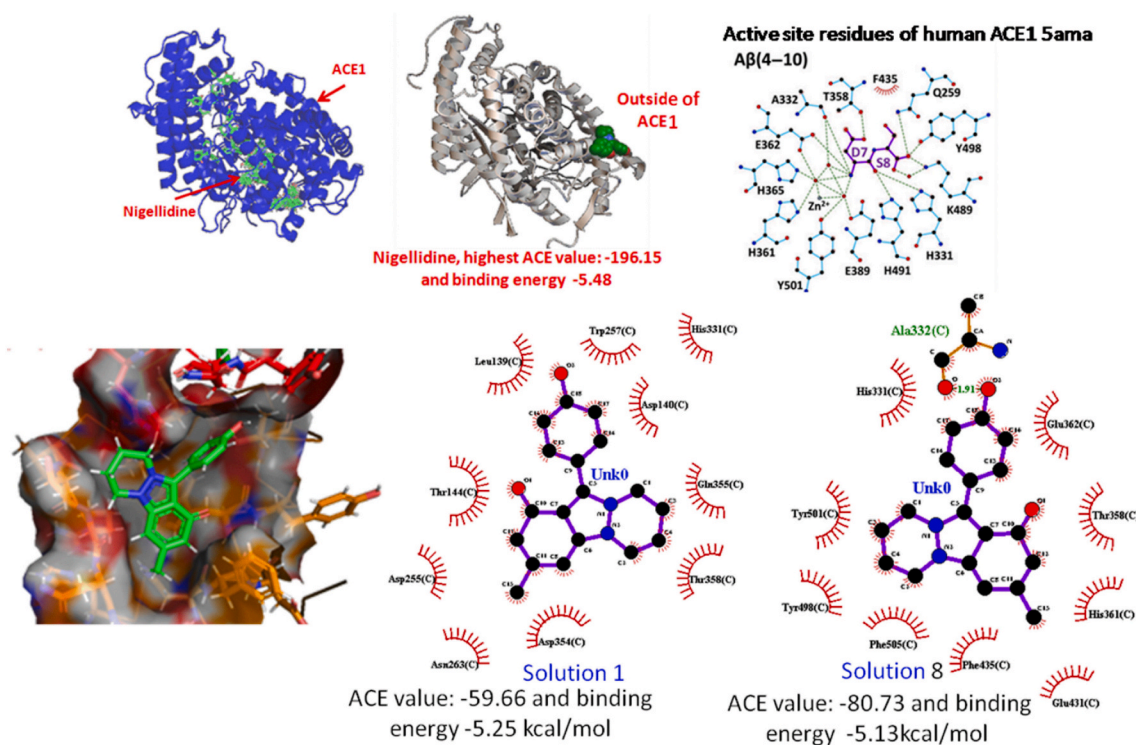

Fig. 3. Nigellidine ACE mono-meric binding. Nigellidine was usually bound to ACE1 at its internal channel and outer surface of the ACE1 also. The active site location of ACE1 was represented which interacted with D7 and S8. The involved common amino acids were also blocked by nigellidine like ALA332.
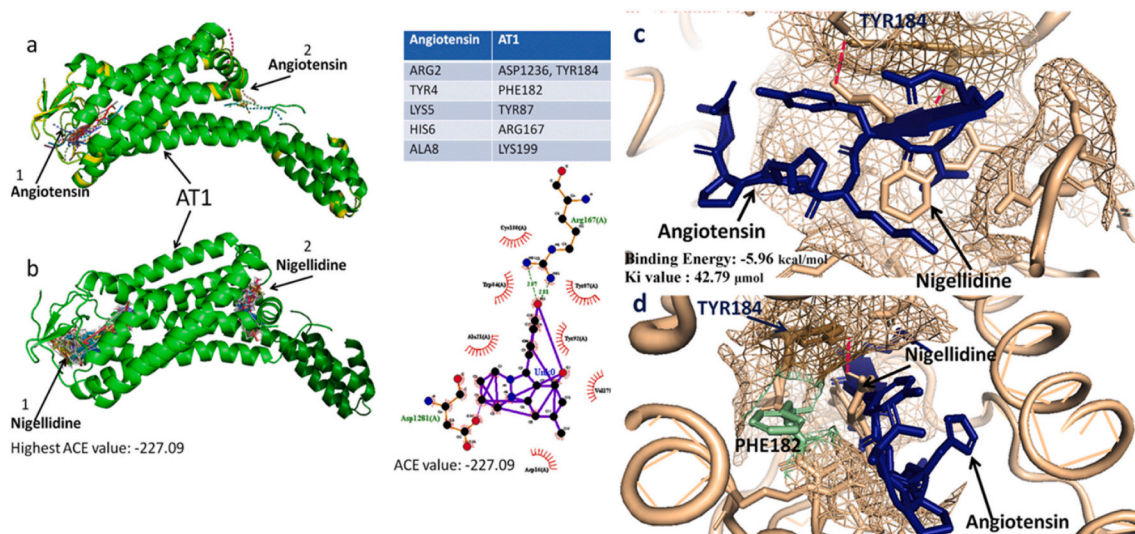
Fig. 4. Angiotensine interacted with AT1 at two different site 1 & 2. At site 1 specific amino acids involved in Angiotensine inding was reported (a). Same sites were occupied by nigellidine where amino acid ASP1281 was blocked by nigellidine at site 1 (b). According to autodock analysis, nigellidine bonded with TYR184 which may indirectly hamper the Angiotensin binding (c). Nigellidine also negatively influenced the PHE182- angiotensin binding (d).

10 best positions of nigellidine only one was found at site 1 with binding energy value −5.96 kcal/mol and Ki of 42.79 μmol. Where, nigellidine formed bond with TYR184 (Fig. 4c) which indirectly hampered the binding of Angiotensin TYR4 with PHE182 (Fig. 4d).

Likewise in AT2, site 1 and site 2 were observed with Angiotensin binding (Fig. 5a). According to PDB structure 5XJM, Angiotensin was found at site 1. Highest ACE value (−235.57) of nigellidine binding was at site 2. Maximum affinity of nigellidine binding was at site 1 (Fig. 5b). Nigellidine with ACE value −116.91 was found to interact with ARG182, the usual amino acid in Angiotensin-AT2 interaction. The other stable bond was found with MET128 and nearby amino acids were THR125, LEU124, ILE211, MET197, LYS215, PHE272 and TRP269. Among which LYS215 was another Angiotensin binding site. According to autodock, highest affinity of nigellidine was observed at site 1 with binding energy value of −6.61 kcal/mol (Fig. 5c). Other two postures of nigellidine with binding energy value of −6.51 and −6.46 kcal/mol were also found there (S-table1). Nigellidine was found to compete with Angiotensin upon binding with ARG182. On the other hand it also

hampered the Angiotensin - LYS215 binding. At this location, the Ki values were also lower (14.22, 16.79 & 18.36 μmol) than AT1-nigellidine binding (42.79 μmol). So, from the above analysis it could be concluded that AT2 is more prone to nigellidine then AT1.

3.6. Active site analysis of different Angiotensin receptors

In the present study, different Angiotensin receptors were analyzed for nigellidine binding or inhibition criterion. Where nigellidine was found to block different amino acids at which Angiotensin usually binds. Here the Angiotensin binding amino acids at different receptors like AT1, AT2 and ACE2 were analyzed (Fig. 6). The angiotensin attached in the NMR structure of ACE2 was of 8 amino acids (DRVYIHPF). Whereas, Angiotensin attached with AT1 and AT2 were of 7 amino acids i.e. RVYKHPA and RVYIHPI respectively. The sequential differences between AT1 and AT2 were observed at the 4th (K/I) and 6th (A/I) position. For ACE2 position 1 (D) was extra amino acid and last one was F instead of A/I. Though AT1, AT2 showed structural similarity and ACE2
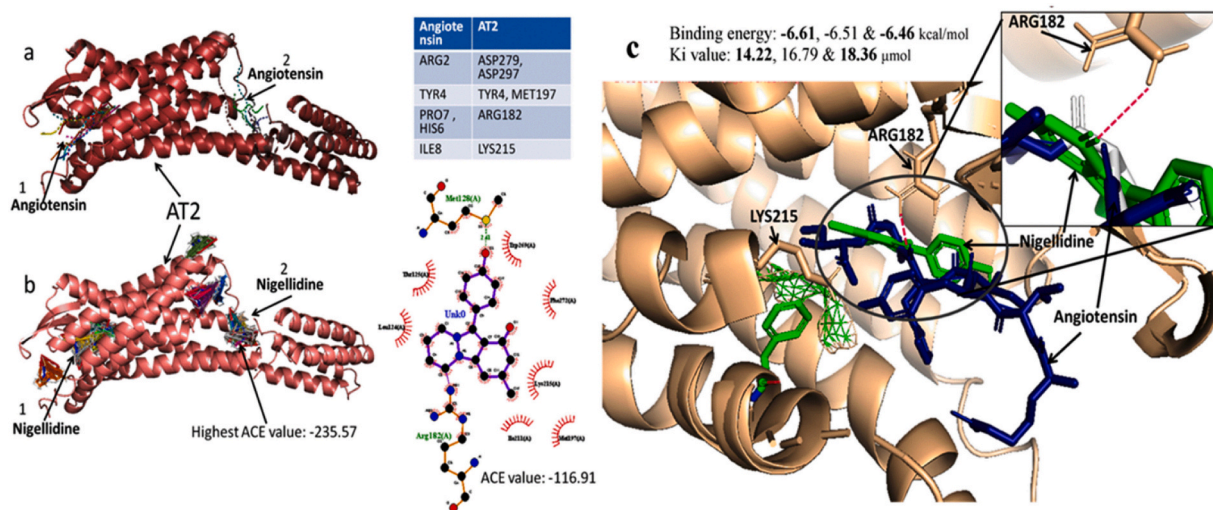

Fig. 5. Angiotensine interacted with AT2 at two different sites 1 & 2. At site 1 specific amino acids involved in Angiotensine inding was reported (a). Same sites were occupied by nigellidine where amino acid ARG182 was blocked by nigellidine at site 1 (b). According to autodock analysis, nigellidine bonded with ARG182 which compete with the Angiotensin binding directly(c). Nigellidine also negatively influenced the LYS215-angiotensin binding.
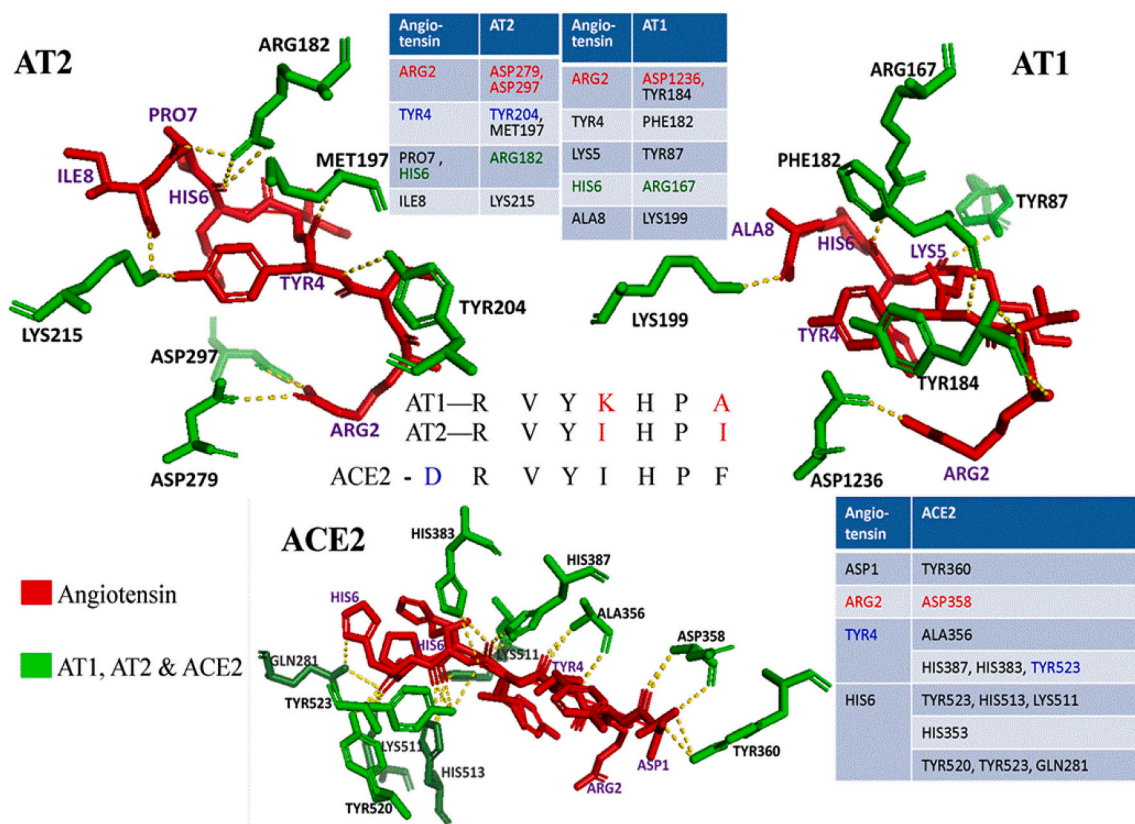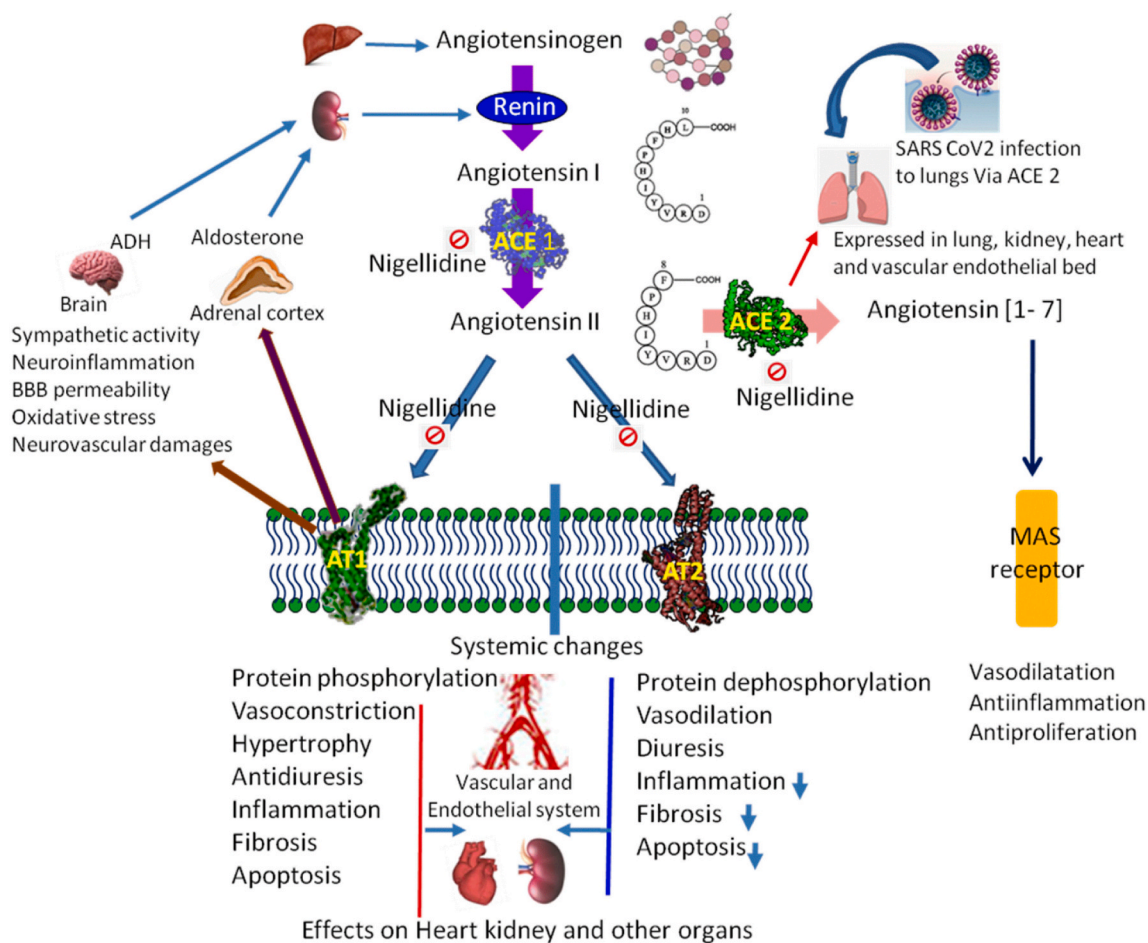
Fig. 7. Summarization of angiotensin regulations and its physiological actions. Influences on these regulations i.e. via SARS CoV2 infection may impair the ACE2 functions and its expressions. As a result, angiotensin function becomes severely altered that cleats vaso-turbulence and massive endothelial dysfunctions resulting in multiple organ failure. Further, this impairment is highly increased in co-morbid like hypertensive, cardiac, renal and diabetic patients.

blood vessels which is noticed in the large number of Covid patients and also in the autopsy studies [37,38]. Studies have revealed that 71·4% of non-survivors of COVID-19 matched the grade of overt disseminated intravascular coagulation (≥5 points according to the International Society on Thrombosis and Haemostasis criteria) [39]. Taken together, the synergistic effects of all these events put enormous pressure and malfunctioning in the endothelial cells (Fig. 7). The peripheral vascular bed mostly comprises of huge amount of endothelial cells these break down in SARS CoV infections and results in the decisive failure to the vasculature-dependant organs (Fig. 7).

AT2 receptor acts like a double sided sword. In a very narrow window it antagonizes angiotensin-induced AT1 functions. Though AT1 induced vasoconstriction may be outperformed by the AT2 regulated vasodilatation but in several conditions over-activation of AT2 may results in severe hypotonic shock [40]. Moreover, AT2 bound with angiotensin induces smooth muscle cell apoptosis via GATA-6 activation and FasL-Fas involvement [41]. GATA activation by MEK/ERK1/2 may result in growth and differentiation [41] and the Fas signalling may activate Caspase 8 and result in extrinsic apoptosis [42]. All these could have been occurring in the current situation in patients of severe Covid condition. Our present study suggests that nigellidine has an affinity for binding to the AT2-angiotensin binding site and may terminate this series of adverse events. AT2 activation may also stimulate bradikynin and NO dependant cGMP signalling [43]. And phospholipase A 2 dependant arachidonic acid production and K+ release from the cells that result in hyper-polarization and decreased excitability [44].

Initially it was surprising to us that how a single molecule can bind

effectively in so many places. The interesting point what we noticed that Angiotensin binding site of all these proteins have a very common amino acid environment and the physical architecture of those binding locations are rationally analogous. And nigellidine is preferred just by that specific locations and amino acid environment. Nevertheless, Angiotensin regulations imparts direct or indirect influence on several physiological factors like aldosterone/salt balance, immunological; T cells, macrophage, dendritic cells functions and also has influence on inflammatory factors like Fas/FasL, CD40/ CD40L and TNF and TNFR signalling. And all these factors are the main determinant of the severity of Covid pathophysiology especially in the elderly/comorbid patients. So restoring the angiotensin regulations at its basal level could have been the best choice at this time point besides searching some other better drug target. In this respect our finding is very much important. Previous literature suggests that nigellidine and its derivative compounds are used in the control of arterial blood pressure, anticholinergic, antihistaminic, tracheal relaxation, control of asthma and in the treatment of other allergic diseases [45,46].

Nigellidine interact with spike protein near the hinge region and opposite of RBD of S1 domain with TYR369 with a binding energy value of −5.31, Ligand efficiency value of −0.24 and Ki value of 127.39 according to Autodock analysis (Fig. 1). Another location was also found where nigellidine formed rigid bond with ALA1020 with an ACE value of −202.47 and at the hinge region with an ACE value of −340.50 (Fig. 1, lower panel). Whereas, nigellidine bind with ACE2 with higher binding energy value of −7.54 and −6.73 with ligand efficiency of −0.34 and −0.31, Ki value of 2.95 and 11.64 respectively. As a result of which, it

could be concluded that, nigellidine can hamper the ACE2-spike RBD domain interaction indirectly. But, indirect yet crucial site-binding of Nigellidine should hamper spike to ACE2 binding (Fig. 1). But during high viral load or a higher rate of viral replication, over dose of nigellidine will control the sudden fluctuation of blood pressure and spike binding to ACE2. Nigellidine mainly act as the inhibitor of angiotensine conversion by ACE2 or inhibits the carboxypeptidase activity of ACE2 which ultimately controls the vasodilatation and may control blood pressure fluctuation.

In our current study, we were trying to screen a drug which has wide range of affinity towards the angiotensin binding proteins (enzymes and receptors). So that it can block the proteins and arrest the angiotensin II enzymatic and receptor signalling at the sub threshold levels (Fig. 7). In Covid patients, the angiotensin II induced vaso-dysfunction and impaired vascular events will be strictly restored. Not only that, Angiotensin II and its receptor AT1/2-mediated downstream inflammatory signalling will also be blocked. Here, for the first time we demonstrated that nigellidine an indazole alkaloid strongly bind to the ACE2 and then ACE1 enzymes. Moreover, it can block the AT1/2 receptors.

Data availability

All data are available upons request.

Authors' statement

The authors declare they read the article and agreed to published its current revised form in the Journal Vascular Pharmacology.

In silico Nigellidine (*N. sativa*) bind to viral spike/active-sites of ACE1/2, AT1/2 to prevent COVID-19 induced vaso-tumult/vascular-damage/comorbidity.

Conflict of interests

None.

Acknowledgements

Institutional members.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2021.106856.

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