# A review on possible mechanistic insights of Nitazoxanide for repurposing in COVID-19

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ABSTRACT

The global pandemic of Coronavirus Disease 2019 (COVID-19) has brought the world to a grinding halt. A major cause of concern is the respiratory distress associated mortality attributed to the cytokine storm. Despite myriad rapidly approved clinical trials with repurposed drugs, and time needed to develop a vaccine, accelerated search for repurposed therapeutics is still ongoing. In this review, we present Nitazoxanide a US-FDA approved anti-protozoal drug, as one such promising candidate. Nitazoxanide which is reported to exert broad-spectrum antiviral activity against various viral infections, revealed good *in vitro* activity against SARS-CoV-2 in cell culture assays, suggesting potential for repurposing in COVID-19. Furthermore, nitazoxanide displays the potential to boost host innate immune responses and thereby tackle the life-threatening cytokine storm. Possibilities of improving lung, as well as multiple organ damage and providing value addition to COVID-19 patients with comorbidities, are other important facets of the drug. The review juxtaposes the role of nitazoxanide in fighting COVID-19 pathogenesis at multiple levels highlighting the great promise the drug exhibits. The *in silico* data and *in vitro* efficacy in cell lines confirms the promise of nitazoxanide. Several approved clinical trials world over further substantiate leveraging nitazoxanide for COVID-19 therapy.

## 1. Introduction

The COVID-19 pandemic has created huge panic and alarm in mankind across the globe. The absence of an assured cure, increase in number of afflicted and the fear of fatality (over half a million deaths worldwide) has created a chaotic ambiance (Sahebnasagh et al., 2020; WHO, 2020). The concept of repurposing drugs has become the norm for COVID-19 therapy, and clinical trials with repurposed drugs are being approved across the globe at unheard of speeds to find an immediate solution (Guy et al., 2020; Lam et al., 2020). Direct-acting antivirals, antimalarials, antiparasitics, corticosteroids and biologics singly or in combination are the major candidates being evaluated. The proposed convalescent plasma therapy would find application only in severe emergencies, but is certainly not an alternative for drugs, while vaccines need time to develop (Jin et al., 2020; Lam et al., 2020; Li et al., 2020). The antiviral drugs remdesivir (GS 5734) and favipiravir (Avigan®) have received restricted emergency use approval against COVID-19 (Ethiraj, 2020), however search for promising therapeutics is still ongoing.

The singular cause for fear is the mortality rate of up to 5%, which is ascribed majorly to the cytokine storm, a hyperimmune host reaction to the virus, which leads to acute respiratory distress syndrome (ARDS) with other major organ failures (Cao, 2020; Khan et al., 2020). The cytokine storm attack, known to occur generally in the second week is so sudden and intense, that patients could unexpectedly turn from mild/-moderate to severe, needing oxygen support and hospitalisation. If not tackled immediately severe lung damage that follows, often results in death. The approach of combining antiviral efficacy with interventions to curb the cytokine storm, with a single drug or drug combinations appears to be rational, promising and encouraging (Jamilloux et al., 2020; Nile et al., 2020). In this review we focus on one such promising drug candidate Nitazoxanide (Alinia®) for repurposing in COVID-19.

### 1.1. Repurposing "Nitazoxanide"

Nitazoxanide, a small-molecule (nitrothiazolyl-salicylamide) anti-protozoal drug marketed as tablets (500 mg) and suspension (100 mg/5 ml) (Rossignol et al., 2006), is currently approved for treating diarrhoea caused by the protozoa *Cryptosporidium* or *Giardia* in immunocompetent adults and children (Anderson and Curran, 2007; Fox and Saravolatz,

2005; Halsey, 2009). It has demonstrated broad spectrum antiviral efficacy *in vitro*, against a range of viruses including the respiratory syncytial virus, parainfluenza virus, coronavirus (CoV), rotavirus, norovirus, hepatitis B virus (HBV), hepatitis C virus (HCV), dengue virus, yellow fever virus, Japanese encephalitis virus, and human immunodeficiency virus (HIV). Furthermore, clinical trials suggested the potential role of nitazoxanide in gastroenteritis, hepatitis and influenza (Rossignol, 2014). A significant finding is the ability of nitazoxanide to promote balance between pro-inflammatory and anti-inflammatory responses in humans, which could play a crucial role in COVID-19 by curbing the hyperinflammatory cytokine storm (Jasenosky et al., 2019; Martins-Filho et al., 2020; Rossignol, 2016; Shakya et al., 2018a). Repurposing nitazoxanide against COVID-19 has been suggested in many reports (Alonso and Farina, 2020; Arshad et al., 2020; Bobrowski et al., 2020; Chibber et al., 2020; Mahmoud et al., 2020; Martins-Filho et al., 2020; Padmanabhan, 2020; Padmanabhan and Padmanabhan, 2020; Pepperrell et al., 2020; Rajoli et al., 2020). The approval of a number of clinical trials with nitazoxanide further substantiates this hypothesis (https://clinicaltrials.gov/) (Nitazoxanide clinical trials, 2020). This review presents mechanistic insights and unravels the promise of nitazoxanide as a repurposed drug candidate for comprehensively addressing COVID-19 pathogenesis.

## 2. Nitazoxanide

### 2.1. Structural features

Nitazoxanide [2-acetyloxy-N-(5-nitro-2thiazolyl)benzamide] a prodrug, consists of a nitrothiazole moiety and salicylamide moiety. In the body nitazoxanide is rapidly deacetylated to tizoxanide [2-hydroxy-N-(5-nitro-2thiazolyl)benzamide] (TIZ), the active form (Hemphill et al., 2006; Rossignol, 2009). Multiple activities of nitazoxanide are attributed to the structural moieties (Fig. 1), for instance, acetylbenzamide or

salicylamide moiety acts as an analgesic, antioxidant, anti-infective (against bacteria, fungi, parasite and viruses), affects interleukin production, regulates immune responses, antipolymerase activity against hepatitis virus, blocks influenza viral entry and host cell membrane fusion (Krátký and Vinšová, 2011). The nitrothiazole moiety exhibits antiprotozoal, antiproliferative, antiinfective effect (Colín-Lozano et al., 2017), control oxidative stress (El-Kowrany et al., 2019) and disrupts pyruvate: ferredoxin oxidoreductase dependent redox reactions essential for anaerobic protozoa and bacteria for their energy metabolism and survival (Hemphill et al., 2006; Kumar et al., 2014; Rossignol, 2009). Thus it can be postulated that nitazoxanide can inhibit various stages of the virus multiplication cycle and also potentiate host innate immunity (Martins-Filho et al., 2020; Padmanabhan, 2020). Furthermore, the potential of nitazoxanide to enhance natural host antiviral mechanisms, suggests lower possibility of developing antiviral resistance in comparison with drugs directly acting only on viral proteins (Krátký and Vinšová, 2011; Tilmanis et al., 2020).

### 2.2. Mechanism of action against SARS-CoV-2 pathogenesis

We now explain the pathways of SARS-CoV-2 entry and pathogenesis, systematically juxtaposing at each stage the role of nitazoxanide elucidating thereby the ability of the drug to exhibit direct antiviral action and host beneficial anti-inflammatory response to curb the cytokine storm. Importantly nitazoxanide is also reported to be useful in patients with co-morbidities (Elaidy et al., 2018; Fan et al., 2019; Ghusson and Vasquez, 2018; Nitazoxanide in fibrosis, 2020).

#### 2.2.1. Entry and fusion

SARS-CoV-2 can enter the host cell using two pathways, the endosomal pathway or the fusion based cell surface non-endosomal pathway. As early availability of cell surface proteases is important to facilitate fusion-based entry (Kupferschmidt and Cohen, 2020; Tang et al., 2020),
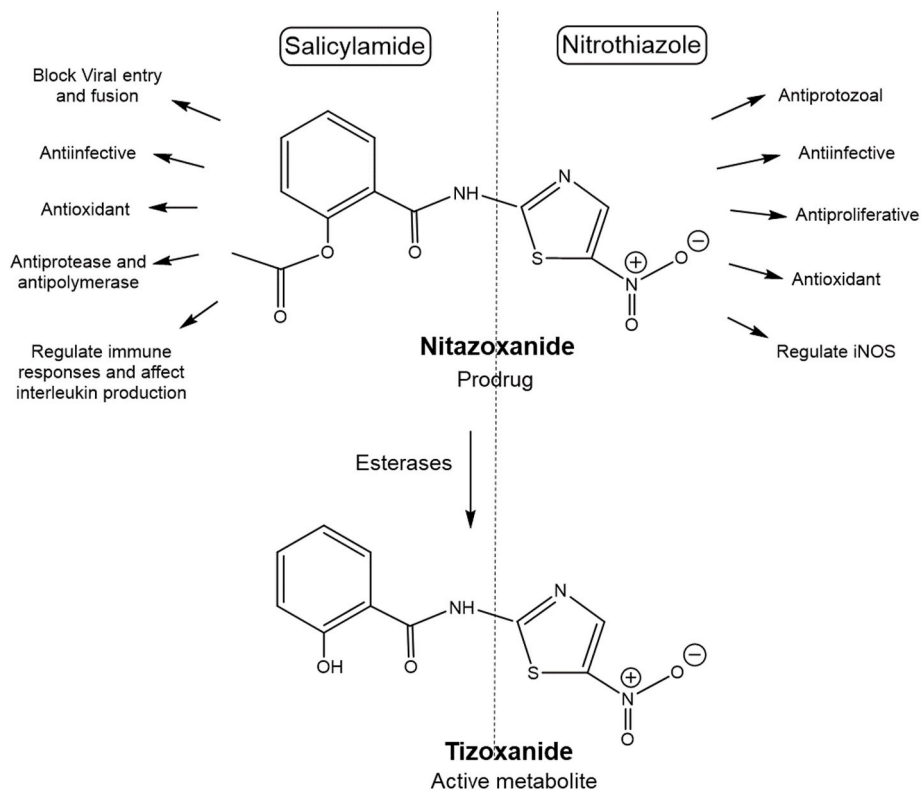


**Fig. 1. Nitazoxanide structural features.** Nitazoxanide a nitrothiazolyl benzamide is a prodrug metabolized to deacetylated active tizoxanide (TIZ). Both have the two major structural components a) salicylamide moiety and b) nitrothiazole moiety which are responsible for the multiple therapeutic actions. iNOS, instrinsic nitric oxide synthase.

the endosomal pathway appears to be more favoured.

### 2.2.1.1. Non-endosomal pathway:.

Fusion between virus and host cell membrane through the non-endosomal pathway results in the viral genetic material being released directly into the host cytosol. This route of entry involves cleavage of viral spike glycoproteins at (S1/S2) interface by host cell surface anchored proteases, transmembrane-protease-serine-2 (TMPRSS2) and furin, exposing fusion S2 peptide available for fusion with host cell membrane (Bestle et al., 2020; Jin et al., 2020; Omolo et al., 2020; Ou et al., 2020; Xiu et al., 2020). Such proteolytic cleavage is dependent on the thiol-disulfide exchange mechanism catalyzed by protein disulfide isomerase (PDI) (Diwaker et al., 2013; Hati and Bhattacharyya, 2020). PDI also regulates disulfide switch activity in the ectodomains of TMPRSS2 due to presence of cysteine-rich residues (Antalis et al., 2011; Curry and Rosewell, 2008; Rosewell et al., 2011; Turano et al., 2002). Nitazoxanide block this oxidoreductase mechanism (Fig. 2A) by interacting with cysteine residues of surface-bound PDI through S-nitrosylation (Bekendam et al., 2018; Flaumenhaft et al., 2015; Müller et al., 2008a; Piacentini et al., 2018). Further, inhibition of furin (Bonacci et al., 2011; Denault et al., 2002; McCarthy et al., 2008) by nitazoxanide can facilitate blocking of other protease signalling pathways, including Wnt/β-catenin (Miner et al., 2019b; Qu et al., 2018), MAPK/ERK (Shou et al., 2019), and PI3K/Akt/mTOR (Senkowski et al., 2015; Shou et al., 2020).

### 2.2.1.2. Endosomal pathway:.

The endosomal route of entry occurs through the angiotensin converting enzyme 2 (ACE2) receptor (Sharifkashani et al., 2020). The thiol-disulfide redox system governed by PDI assists disulfide bond (-S-S-) exchange between receptor binding domain (S-RBD of S1 fragment) of SARS-CoV-2 spike protein and ACE2 facilitating entry via the endosomal pathway. Reduction of disulfide bonds to thiol (–SH–SH-) impaired such binding (Hati and Bhattacharyya, 2020). Hence inhibition of the disulfide exchange can inhibit the interaction of S-protein RBD to ACE2, thereby hampering the receptor-mediated endocytosis route of SARS-CoV-2 entry (Fig. 2B) (Di Santo and Ehrisman, 2013; Diwaker et al., 2013; Müller et al., 2008a; Piacentini et al., 2018).

A newer entry pathway identified is the Cluster of differentiation 147 (CD147) receptor-mediated entry which proceeds through the activation of its catalytic domain by integrins and matrix metalloproteinases (MMPs), essential for S-protein binding (Gabison et al., 2005; Han et al., 2008; Liu and Zhu, 2020; Ulrich and Pillat, 2020). Nitazoxanide through its effect on PDI could inhibit activation of MMPs by blocking the cysteine switch mechanism (Bonacci et al., 2011; McCarthy et al., 2008; Van Wart and Birkedal-Hansen, 1990) to prevent such entry. Nitazoxanide can also inhibit other CD-147 mediated signalling pathways, like Wnt/β-catenin signalling (Miner et al., 2019a; Müller et al., 2008a; Qu et al., 2018), PI3K/Akt/mTOR signalling (Senkowski et al., 2015; Shou et al., 2020) and MAPK/ERK signalling (Shou et al., 2019).

### 2.2.2. Endosome vesicle formation and maturation

Receptor mediated entry follows the clathrin mediated endocytic pathway which typically results in early endosome formation and maturation to the late endosome before the contents are released in the cytosol. Such cytosolic release is a crucial step in furthering viral pathogenesis (Bayati et al., 2020; Glebov, 2020). Nitazoxanide can delay cytosolic release through its effect on multiple targets (Rossignol, 2014). Receptor mediated endocytosis dependent on oligomeric clathrin and regulated by two host cell adaptor protein complexes serine–threonine protein kinases, namely AP-2-associated protein kinase 1 (AAK1) and cyclin G-associated kinase (GAK) (Bekerman et al., 2017; Chibber et al., 2020; Stebbing et al., 2020). Nitazoxanide can inhibit Wnt/β-catenin signalling (El-Kersh et al., 2019; Qu et al., 2018; Shakya et al., 2018b), which in turn downregulates AAK1 thereby hampering clathrin mediated endocytosis (Agajanian et al., 2019). Another proposed mechanism is the ability of nitazoxanide to interact with cysteine residues through S-nitrosylation (Andrade and Reed, 2015; Bekendam et al., 2018; Goldman, 2010), which can hinder the irreversible inhibition of the kinases AAK1 and GAK by interaction with their cysteine residues Cys193 and Cys190 respectively (Sorrell et al., 2016). These two kinases along with phosphatidylinositol 3-phosphate5-kinase (PIKFYVE) initiates early endosome formation and causes synthesis of phosphatidylinositol-3,5-bisphosphate [PI(3,5)P2] (Phosphoinositides) (Kang et al., 2020; Ou et al., 2020), which activates two pore segment channel 2 (TPC2), a calcium channel expressed in lysosomal membranes (Filippini et al., 2020; Grimm and Tang, 2020). TPC2 maintains $Ca^{2+}$ homeostasis and the $Ca^{2+}$ release from this channel is controlled by nicotinic acid adenine dinucleotide phosphate (NAADP), phosphoinositide and mTOR (Jin et al., 2020; Petersen et al., 2020). The endolysosomal lumen serves as an important type of intracellular $Ca^{2+}$ store,
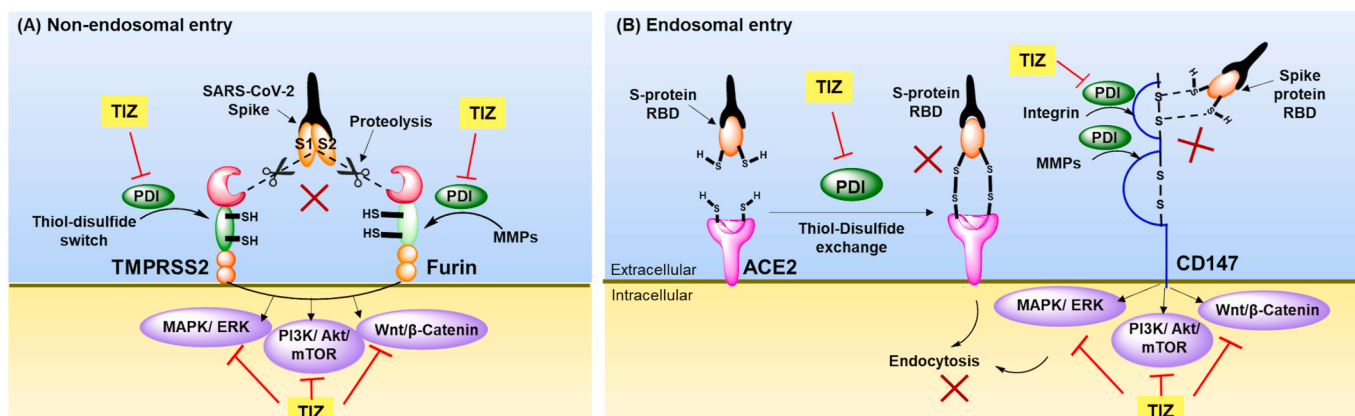


**Fig. 2. Schematic representation of possible role of tizoxanide (TIZ)-inhibition of SARS-CoV-2 fusion and entry.**
(A) Non-endosomal entry involves the proteolytic cleavage of SARS-CoV-2 spike proteins (S1/S2) by host cell proteases TMPRSS2 and furin. TIZ inhibits PDI and affects thiol-disulfide oxidoreductase switch in the ectodomains of TMPRSS2, and inhibition of PDI also inhibits MMPs activation required for furin action, (B) Endosomal entry involves the recognition and binding of RBD of SARS-CoV-2 spike proteins to the host cell receptors ACE2 and CD147 enabling receptor mediated endocytic entry. TIZ inhibits thiol-disulfide exchange mechanism essential for receptor interaction with RBD by inhibiting PDI. This PDI inhibition also hampers MMPs and intergrins activation required for CD-147 activity. TIZ also inhibits intracellular signalling MAPK/ERK, PI3K/Akt/mTOR and Wnt/β-catenin affecting SARS-CoV-2 fusion and entry. ACE2, Angiotensin converting enzyme 2; CD147, Cluster of differentiation 147; MAPK/ERK, Mitogen-activated protein kinase/extracellular signal-regulated kinase; MMPs, Matrix metalloproteinases; PDI, Protein disulfide isomerase; PI3K/Akt/mTOR, phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin; RBD, Receptor binding domain; TIZ, Tizoxanide; TMPRSS2, Transmembrane protease serine 2.

containing high $Ca^{2+}$ concentration of ~600 μM (Alharbi and Parrington, 2019). In the presence of high endosomal $Ca^{2+}$, the fusion proteins of coronavirus induces better membrane ordering, essential for fusion (Brailoiu and Brailoiu, 2016; Grimm and Tang, 2020). Nitazoxanide causes depletion of such ATP sensitive intracellular $Ca^{2+}$ stores, thus delaying endosome maturation (Ashiru et al., 2014). Additionally, TPC2 is also regulated by $Mg^{2+}$, the mitogen activated protein kinases (MAPKs), c-jun N-terminal kinase (JNK) and P38 (Alharbi and Parrington, 2019; Jha et al., 2014), which are inhibited by nitazoxanide (Senkowski et al., 2015; Shou et al., 2019; Tchouaffi-Nana et al., 2010), disturbing endosome maturation. The PIKFYVE and TPC2 functions are directly linked to Wnt/β-catenin (Miner et al., 2019a; Qu et al., 2018) and PI3K/Akt/mTOR signalling (Senkowski et al., 2015; Shou et al., 2020) which are via Vacuolar-type $H^{+}$ ATPase (V-ATPase) activates Cathepsin L, which promotes viral-host cell membrane fusion, causing viral genome release into the host cytosol (Collins and Forgac, 2020; Kupferschmidt and Cohen, 2020; Liu et al., 2020; Madadlou, 2020; Yang and Shen, 2020). Nitazoxanide can interact with cysteine residues (Andrade and Reed, 2015; Aqeel et al., 2015; Goldman, 2010) of Cathepsin L, preventing its activation, which leads to inhibition of viral and host cell membrane fusion and consequently the viral genome release (Madadlou, 2020; Nga et al., 2014; Otto and Schirmeister, 1997; Verma et al., 2016). Furthermore, TIZ which possesses weak acidic pKa~5.8 and lipophilic logP~3.15 (Jurgeit et al., 2012; Miner et al., 2019a) can also induce endosome neutralization analogous to niclosamide (Homolak and Kodvanj, 2020; Jurgeit et al., 2012; Xu et al., 2020).

### 2.2.3. Translation and proteolysis

Once in the cytosol, the viral genome undergoes translation of open reading frames ORF1a/b through host ribosomes into the large replicase polyprotein 1a (pp1a) and pp1ab, while other ORFs encode structural proteins. These polypeptides (PP1a/PP1ab) are proteolytically cleaved by 3-chymotrypsin like protease (3CLpro or Mpro) and papain-like protease (PLpro) to form several nonstructural proteins (nsp1-16) (Astuti, 2020; Omolo et al., 2020). The PLpro (nsp3) causes proteolytic processing of nsp1, nsp2, and nsp3 (Klemm et al., 2020), while 3CLpro (nsp5) processes nsp12, nsp13 and other subunits of the replicase–transcriptase complex (RTC) (Fong, 2020). Both proteases are key targets for virus multiplication and governing the host directed immune cell response; therefore, they are attractive targets for the antiviral interventions (Shereen et al., 2020). 3CLpro and PLpro are cysteine rich proteases, involved in peptide bond hydrolysis (Gil et al., 2020; Pillaiyar et al., 2016). The schematic representation of binding and interaction of the active metabolite TIZ with these proteases is depicted in Fig. 3.

**Inhibition of 3CLpro:** The active site of 3CLpro of SARS-CoV-2 contains Cys145 and His41 residues forming a catalytic dyad (He et al., 2020; Tahir ul Qamar et al., 2020), where cysteine thiol (-SH) functions as a common nucleophile (electron donating group) responsible for proteolytic cleavage (Yang et al., 2003). Structure activity relationship studies of pyrazolones and pyrimidines revealed that the presence of aromatic nitro ($-NO_2$) enhances cysteine protease inhibition activity, where the oxygen of the $NO_2$ forms a H-bond with side chains of Gly143 and Cys145, while the phenyl ring forms hydrophobic interactions (Pillaiyar et al., 2016). It is therefore proposed that the $NO_2$ group or the carbonyl carbon of amide bond of TIZ can interact with the catalytic cysteine nucleophile, while phenyl ring could exhibit hydrophobic interactions with the 3CLpro active site (Fig. 3A) (Piacentini et al., 2018). Recent molecular screening experiments based on linear free energy relationships reported activity of nitazoxanide against 3CLpro with a predicted $IC_{50}$ value of 4.36 μM which was comparable with known 3CLpro inhibitors ledipasvir, saquinavir and superior to lopinavir and ritonavir (Fong, 2020).

**Inhibition of PLpro:** The PLpro proteolytic site possess a catalytic triad of Cys112–His273–Asp287 (Báez-Santos et al., 2015; Klemm et al., 2020) where Cys112 acts as thiol nucleophile forming a H-bond with His273 which is paired with Asp287 causing deprotonation of Cys112 (Foster et al., 2003; Stoermer, 2020). Interaction of electrophilic $NO_2$ group of nitazoxanide with nucleophilic Cys112 residue at active site through S-nitrosylation could reversibly inhibit PLpro function (Foster et al., 2003; Hess et al., 2005; Xian et al., 2000). The SARS-CoV-2 PLpro has the thumb domain or a blocking loop, containing a critical tyrosine residue Tyr268 (Báez-Santos et al., 2015; Kandeel et al., 2020; Klemm



**(A) 3CLpro inhibition**
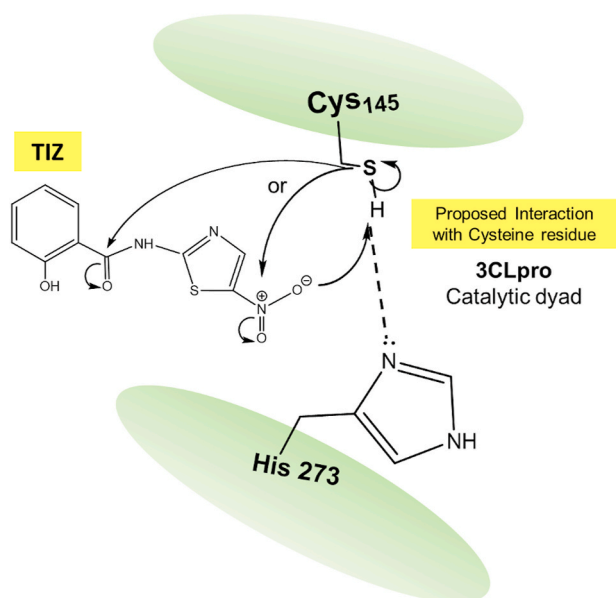
**(B) PLpro inhibition**
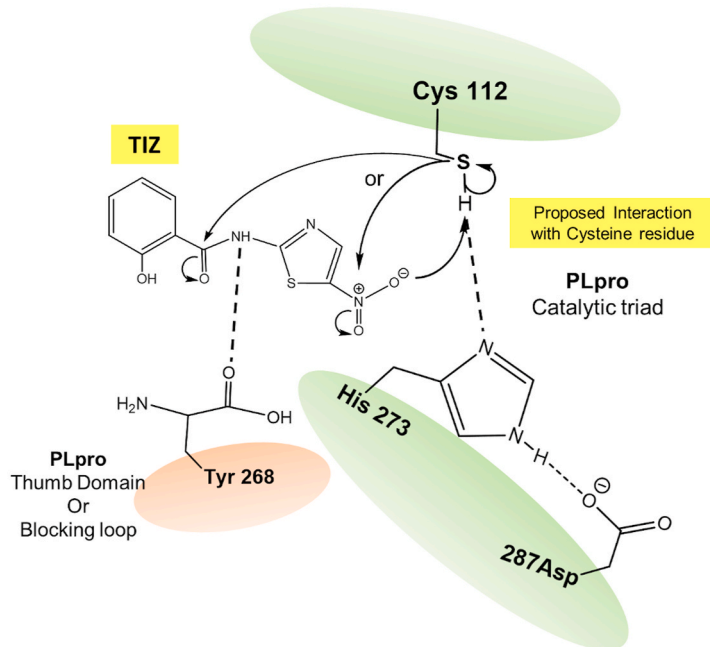
Fig. 3. **Schematic representation of nitazoxanide antiprotease action against 3CLpro and PLpro of SARS-CoV-2.** Tizoxanide (TIZ) (active metabolite of nitazoxanide) interacts with cysteine residue and thus inhibits the enzyme activity, (A) 3CLpro inhibition and (B) PLpro inhibition. Nucleophilic cysteine thiol interacts with electrophilic moieties of tizoxanide (TIZ). 3CLpro, 3-chymotrypsin like protease; PLpro, Papain like protease.

et al., 2020) which forms H-bond with the amide nitrogen of PLpro inhibitors, anchoring the inhibitor in the binding site (Báez-Santos et al., 2015; Xian et al., 2000; Yu et al., 2020). Nitazoxanide exhibits potential as a PLpro inhibitor as the amide nitrogen can form H-bond with Tyr268 (Fig. 3B) (Piacentini et al., 2018). Antiprotease activity of nitazoxanide against SARS-CoV-2 is further substantiated by recent *in silico* molecular modelling study which reported a binding energy score of $-110$ kJ/mol indicating strong binding and furthermore nitazoxanide shared $>70\%$ binding similarity with remdesivir (Abdul Kadhim et al., 2020).

### 2.2.4. Viral genome synthesis

Synthesis of new genomic viral RNA relies on the multi-subunit replication transcription complex (RTC) which contains multiple enzymes viz. viral RNA-dependent RNA polymerase (RdRp) (nsp12), helicase (nsp13), exonucleases (nsp14) and endonuclease (nsp15) (Omolo et al., 2020; Ulferts et al., 2010). Such new viral genome synthesis occurs in the protective environment of double membrane vesicles (DMVs) or autophagosomes (Blanchard and Roingeard, 2015; Cottam et al., 2011; Omolo et al., 2020; Wong and Sanyal, 2020). Nitazoxanide not only exhibits the potential to inhibit all these major enzymes, but can also induce autophagy to disrupt protective environment of autophagosomes (Lam et al., 2012; Senkowski et al., 2015; Shou et al., 2020). Among the enzymes RdRp plays major role in replication of genomic viral RNA, and RdRp of SARS-CoV-2 shares a 96% sequence identity with SARS-CoV (Aftab et al., 2020; Gao et al., 2020). Recent molecular docking studies identified nitazoxanide amongst 10 most effective antipolymerase compounds with a docking score $-9.76$ indicating good binding affinity. Although the score was lower when compared with **remdesivir** ($-14.06$), galidesivir ($-12.53$), ribavirin ($-11.88$) and sofosbuvir ($-11.13$) it was comparable to nafamostat ($-9.76$), interferon ($-9.62$) and **favipiravir** ($-9.36$) (Aftab et al., 2020) proposing great promise. Helicase (nsp13) promotes the separation of double stranded RNA to single stranded RNA mediated through nucleotide triphosphate (NTP) hydrolysis-dependent manner (NTPase) (Shu et al., 2020). Helicase activity can be inhibited by hampering ATP binding or NTPase hydrolysis action, inhibiting nucleic acids interaction and helicase translocation, etc (Briguglio et al., 2011; Habtemariam et al., 2020). Reports suggests that, nitazoxanide inhibits oxidative phosphorylation leading to decreased ATP levels and causes viral protein misfolding, thereby inhibiting helicase ATP dependent action (Hemphill et al., 2006; Piacentini et al., 2018). The nsp14, exoribonuclease (ExoN) is a proofreading 3′–5′ exoribonuclease which help overcome errors caused in RNA replication, and helps modulate dsRNA levels and innate immune sensing (da Silva et al., 2020). ExoN activity depends on divalent metal ions $Mg^{2+}$ and low concentration of $Zn^{2+}$, while $Ca^{2+}$ inactivates ExoN (Chen et al., 2007; Minskaia et al., 2006). Loss of ExoN proofreading has been found to enhance the antiviral effect of remdesivir (Agostini et al., 2018; Shannon et al., 2020). As nitazoxanide affects $Mg^{2+}$ (Tchouaffi-Nana et al., 2010) and enhances intracellular $Ca^{2+}$ release (Ashiru et al., 2014), its action on ExoN is proposed. A recent molecular docking study revealed the partial binding affinity of nitazoxanide to NendoU with glide score of $-5.5$, which is comparable with known antiviral drug remdesivir score $-5.6$ (Joshi and Poduri, 2020) proposing action on the endoribonuclease (nsp15). Nsp15 encodes a specific protein called NendoU, which acts by cleaving single and double-stranded RNA catalyzed by $Mn^{2+}$ dependent manner (Senanayake, 2020), and helps the virus evade the host innate immune reaction (Kim et al., 2020). However, mutations in the nsps are an important cause of drug resistance, a major therapeutic challenge. Notably, nitazoxanide which is reportedly found to be less prone to such resistance, could thus serve as a safe, and effective antiviral intervention (Tilmanis et al., 2020). *In vitro* drug screening assay against coronavirus identified that nitazoxanide can inhibit viral N- protein expression, enhanced green fluorescent protein (EGFP) reporter gene expression and viral production (Cao et al., 2015). Artificial intelligence study exhibited better interaction score of nitazoxanide with the coatomer protein

complex 2 subunit (COPB2) gene, involved in replication of SARS-CoV-2, confirming potential of nitazoxanide for repurposing against COVID-19 (Avchaciov et al., 2020).

Protective environment of DMVs or autophagosomes concealing viral RNA is formed by the attachment of RTC with membrane derived from ER (Blanchard and Roingeard, 2015; Knoops et al., 2008), enabling it to escape the dsRNA-triggered host antiviral response (Astuti, 2020; Kumar et al., 2020; Margaritopoulos et al., 2017). SARS-CoV-2 escapes autophagy by inhibiting the activation of AMP-protein activated kinase (AMPK), and by promoting the mammalian activation target of rapamycin complex 1 (mTORC1) (Shojaei et al., 2020; Wong and Sanyal, 2020). Nitazoxanide promotes autophagy leading to degradation of DMVs contents by activating AMPK and inhibiting mTORC1, thereby adversely affecting virus genome synthesis (Fig. 4) (Cottam et al., 2011; Senkowski et al., 2015; Shou et al, 2019, 2020). The ability of nitazoxanide also increases intracellular $Ca^{2+}$ levels, induces ER stress and thereby induces protective unfolded protein response (UPR) activation, leading to autophagy (Ashiru et al., 2014; Di Santo and Ehrisman, 2013, 2014).

Out of many kinases, Beclin-1 acts as a master regulator of autophagy, which promotes the phagophore sequestration and enables the fusion of autophagosomes with lysosomes (Gassen et al., 2019; Lundin et al., 2014). While the conversion of Light chain LC3-I to LC3-II induces autophagosome maturation, the cargo receptor p62/sequestome1 (p62/SQSTM1) induces autophagic flux (Rubinsztein et al., 2012; Vakifahmetoglu-Norberg et al., 2015). TIZ enhanced Beclin1 expression, promoted the transformation of LC3-I to LC3-II, and the concentration-time based degradation of p62/SQSTM1 in RAW264.7, Vero, 293T, and HepG2 cells, inducing autophagosome maturation (Lam et al., 2012; Shou et al., 2020). Nitazoxanide therefore exhibits the ability to inhibit various targets of viral genome synthesis.

### 2.2.5. Packaging and virion release

The newly synthesized mRNAs are rapidly translated to the ER for producing and processing structurally associated proteins and budded in association with golgi bodies in the form of compartments called ER-golgi compartment. This compartment then encloses viral genome and promotes virion assembly. Finally, virions assembly are enclosed within smooth vesicles causing exocytosis of virion particles ready to infect neighbouring cells and other tissues (Fung and Liu, 2014; Zumla et al., 2016). $Ca^{2+}$ induces major post-translational changes in the recently synthesized viral proteins within ER lumen (Stutzmann and Mattson, 2011). Nitazoxanide induces reduction of ER $Ca^{2+}$ affecting post translational-modifications in proteins, and thereby hampering their release from ER (Ashiru et al., 2014; Perelygina et al., 2017; Rossignol et al., 2009).

### 2.2.6. Immunopathogenesis

COVID-19 causes a complicated clash between host antiviral innate immunity and continuous upsurge of inflammatory markers, the latter being responsible for a fatal condition called "Cytokine storm" (Coperchini et al., 2020; Samudrala et al., 2020). High systemic levels of interleukins, interferons, interferon inducible protein, tumour necrosis factor, growth factors, colony-stimulating factors, macrophage inflammatory protein, monocyte chemoattractant protein, chemokines are a hallmark of the SARS-CoV-2 cytokine storm (Costela-Ruiz et al., 2020; Henderson et al., 2020; Pearce et al., 2020; Samudrala et al., 2020). Nitazoxanide causes activation of innate immune responses and induction of host-directed antiviral mechanisms, thereby exhibiting great promise against the SARS-CoV-2 cytokine storm (Fig. 5) (Mahmoud et al., 2020; Padmanabhan, 2020; Padmanabhan and Padmanabhan, 2020).

The viral RNA expresses pathogen-associated molecular patterns (PAMPs) sensed by pattern recognition receptors (PRRs) of host cells, including Toll-like receptors (TLR-3, -7, -8, -9), Retinoic acid Inducible Gene-I-(RIG-I) like receptors (RLRs), and the nucleotide-binding
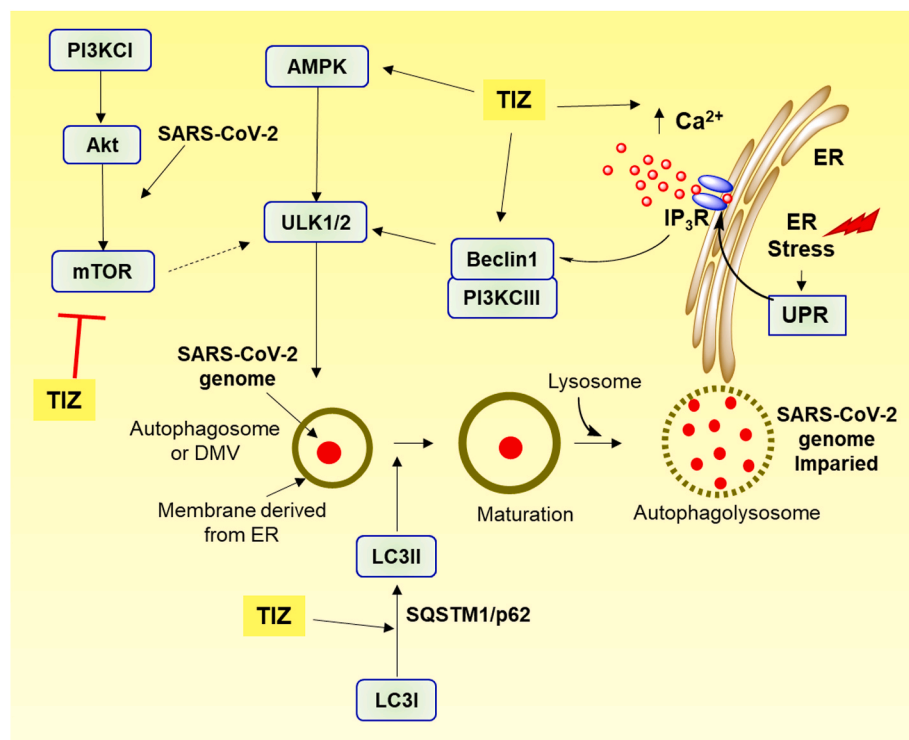
**Fig. 4. Schematic representation of tizoxanide induced autophagy hindering SARS-CoV-2 genome synthesis.** Tizoxanide (TIZ) activates Beclin1, PI3KCIII and AMPK which leads to activation of ULK1 inducing autophagosome formation. TIZ also increases cytosolic $Ca^{2+}$ concentration through $IP_3R$ by depleting ER $Ca^{2+}$ store inducing ER stress and activating protective UPR signalling. TIZ causes conversion of LC3I to LC3II, degradation of p62/SQSTM1 and inhibits PI3KCI/Akt/mTOR signalling, all together promoting autophagy. AMPK, AMP-protein activated kinase; ER, Endoplasmic reticulum; $IP_3R$, Inositol trisphosphate receptor; LC3, Light chain 3; p62/SQSTM1, cargo receptor p62/sequestome1; PI3KCI, Phosphatidylinositol-3-kinase class I; PI3K-CIII, Phosphatidylinositol-3-kinase class III; PI3KCI/Akt/mTOR, phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin; ULK1/2, Unc-51-like kinase-1/2; UPR, Unfolded protein response.

oligomerization domain-(NOD) like receptors (NLRs) (Fung and Liu, 2019; Lim et al., 2016). The TLR-7/8 senses single-stranded viral RNA in the endosomes, while the cytoplasmic viral RNA is noticed by TLR-3, RIG-I, melanoma differentiation-associated gene 5 (MDA5) and cyclic GMP-AMP synthase (cGAS) (Dixit and Kagan, 2013; Vanpouille-Box et al., 2019). Upon viral RNA recognitions these transducer molecules trigger adaptive proteins such as TIR-domain-containing adaptor protein including Interferon-β (TRIF), mitochondrial antiviral-signalling protein (MAVS) and stimulator of interferon genes protein (STING) to activate downstream signalling effectors, i.e. MyD88, the transcription factor nuclear factor-κB (NF-κB) and interferon regulatory factor 3/7 (IRF3/7) (Frieman and Baric, 2008; Guo et al., 2020; Lei and Hilgenfeld, 2017). SARS-CoV-2 prevents IRF3/7 signalling, suppresses Type I Interferon (IFN–I) release and inhibits JAK/STAT signalling (Gordon et al., 2020; Jamilloux et al., 2020; Zumla et al., 2016). While NF-κB activation leads to the secretion of pro-inflammatory markers (TNF-α, IL-1β, IL-6, IFN-γ, IL-17), IRF3/7 activation induces secretion of IFN-I, which suppresses viral replication and spread, helps cell recovery and boosts adaptive immunity (Costela-Ruiz et al., 2020). The IFN secretion triggers the JAK1/TYK2-STAT1/2 pathway, inducing production of numerous IFN stimulated genes (ISGs) in the nucleus (Jamilloux et al., 2020). Nitazoxanide treatment thus results in broad amplification of the host immune responses, including an increase in RLR activation, enhanced MAVS and IRF3 activities, and induction of the antiviral phosphatase GADD34 (Jasenosky et al., 2019). Also Nitazoxanide mediates phosphorylation of protein kinase RNA (PKR) and eukaryotic initiation factor 2α (eIF2α) leading to the enhanced IFN-I secretion (Ashiru et al., 2014; Haas et al., 2020). Moreover, the viral genome proteins ORF3a, ORF8b and E activate NLR signalling and JNK signalling, which then triggers caspase-1, NF-kB signalling, stimulate pro-inflammatory cytokines and induces cell apoptosis (Freeman and Swartz, 2020; Shah, 2020). Nitazoxanide inhibited the production of pro-inflammatory cytokines TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 in peripheral blood mononuclear cells (Rossignol, 2016), exhibited downregulation of IL-6/JAK2/STAT3 pathway and significantly modulated the p53/caspases-dependent signalling pathways affecting apoptosis (Tantawy et al., 2020). TIZ treatment reduced inflammation in

macrophage cell lines by suppressing NF-κB, MAPK and PI3K/Akt/m-TOR pathway (Shou et al., 2019), whereas in case of LPS induced inflammatory microglia, TIZ treatment decreased the release of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, chemokines like CCL-2 and CCL-3, intrinsic nitric oxide synthase (iNOS) and cyclo-oxygenase2 (COX2) expression (Li et al., 2020; Trabattoni et al., 2016). Thus in the case of COVID-19 patients, nitazoxanide could serve as a potential candidate, curbing the inflammation and activating host innate immune responses crucial for addressing SARS-CoV-2 pathogenesis (Martins-Filho et al., 2020).

*2.2.7. Lung damage*

In COVID-19 patients, lung damage is attributed to shedding of SARS-CoV-2 entry receptor ACE2 in the extracellular milieu mediated by A disintegrin and metallopeptidase domain 17 (ADAM17) (Costela-Ruiz et al., 2020; Krossa et al., 2018; Palau et al., 2020; Vitiello and Ferrara, 2020). ADAM17 activation, impaired IFN-I response and persistent lymphopenia (lymphocyte count $< 1.5 \times 10^9$/L) increases accumulation of lymphocytes in the lungs and other lymphoid organs with circulating higher levels of inflammatory cytokines (Venet et al., 2009; Yuki et al., 2020). The resulting inflammation, vascular leakage and enormous lung epithelial and endothelial cells damage, causes the acute respiratory distress syndrome (ARDS) (Costela-Ruiz et al., 2020; Hoffmann et al., 2020; Jamilloux et al., 2020; Venet et al., 2009; Vitiello and Ferrara, 2020; Zhang et al., 2020). This is further aggravated by downregulation of innate immunity and T-cell exhaustion, which makes COVID-19 patients immunocompetent (Diao et al., 2020; Samudrala et al., 2020; Tay et al., 2020). In one clinical case report, nitazoxanide treatment provided better relief to immunocompetent host infected with lung cryptosporidiosis, who presented with fever, cough, breathlessness, followed by diarrhoea, and had lung consolidation conditions similar to COVID-19 (Kumar et al., 2016).

In inflammatory lung conditions, there is downregulation of UPR and upregulation of PDI. In allergic airway disease model, ablation of PDI in lung epithelial cells, reduced apoptotic, inflammatory and fibrotic reactions (Chamberlain et al., 2019; Chamberlain and Anathy, 2020; Hoffman et al., 2016; Mohan et al., 2019; Stolf et al., 2011). However,
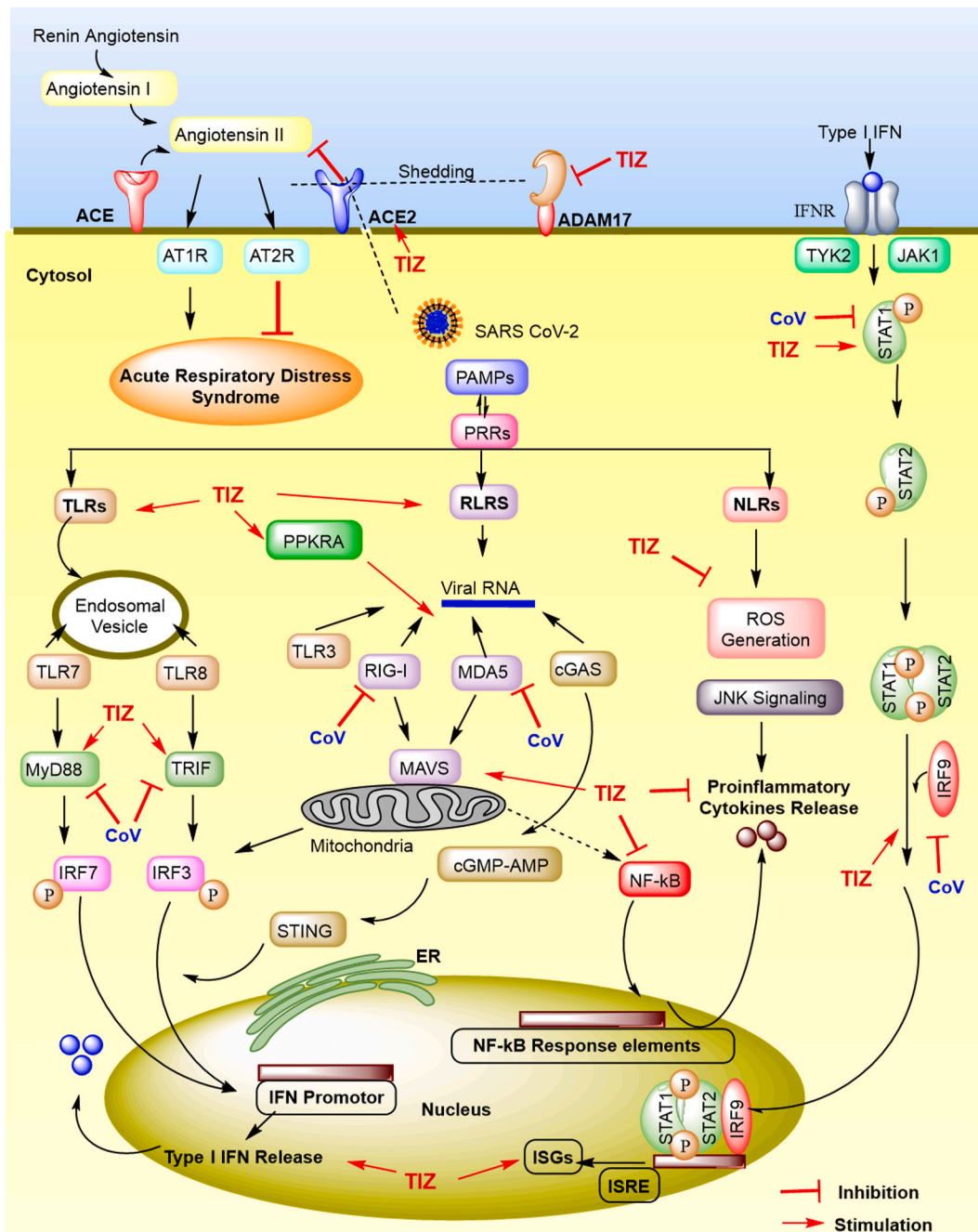
**Fig. 5. Evasion of host innate immune responses by SARS-CoV-2 and stimulation of host antiviral immune responses by tizoxanide (TIZ).** SARS-CoV-2 entry via ACE2 is hampered by TIZ which regulates renin-angiotensin system by blocking angiotensin II binding to its receptors (AT1R and AT2R), to provide protective responses. ADAM-17 mediated ACE2 shedding is prevented by TIZ due to PDI inhibition, thus curbing lung inflammation and consequently ARDS. Upon entry, SARS-CoV-2 pathogen-associated molecular patterns (PAMPs) evades recognition by host cells pattern-recognition-receptors (PRRs), like RLRs, TLRs and NLRs, which is opposed by TIZ. These PRRs cause downstream activation of NF-κB, IRF3/7 signalling, which leads to the secretion of proinflammatory cytokines and type I-IFN respectively. Secreted IFN binds to IFN receptor activating JAK/STAT signalling, which forms a complex with IRF9 and produces interferon stimulated genes (ISGs) promoted under IFN-stimulated response element (ISRE), boosting antiviral innate immune responses, enabling virus clearance. TIZ activates Type-I IFN secretion and curb proinflammatory responses. ACE2, Angiotensin converting enzyme 2; ADAM-17, A disintegrin and metallopeptidase domain 17; ARDS, Acute respiratory distress syndrome; AT1R, Angiotensin II receptor type 1; AT2R, Angiotensin II receptor type 2; cGAS, cyclic GMP-AMP synthase; IFN, Interferon; IFNR, IFNα/β receptor; IRF3/7, Interferon regulatory factor 3/7; JAK, Janus kinase; MAVS, Mitochondrial antiviral-signalling protein; MDA5, Melanoma differentiation-associated gene 5; NF-κB, Nuclear factor-κB; NLRs, Nucleotide-binding oligomerization domain-(NOD)-like receptors; PDI, Protein disulfide isomerase; PPKRA, Phopsphorylated protein kinase receptor; RLRs, Retinoic acid-inducible gene I protein (RIG-I) like receptors; ROS, Reactive oxygen species; STAT, Signal transducer and activator of transcription; STING, stimulator of interferon genes protein; TLRs, Toll-like receptors; TRIF, TIR-domain-containing adaptor protein including IFN-β; TYK2, Tyrosine kinase 2.

the prolonged UPR response, lead to cell death, secretion of proinflammatory cytokines and epithelial cell damage as observed in common lung diseases such as pulmonary fibrosis, asthma and chronic obstructive pulmonary disease (COPD) (Chamberlain and Anathy, 2020; Hoffman et al., 2016). Coronavirus can induce the UPR in cell culture models (Bechill et al., 2008; Siu et al., 2014). Nitazoxanide activates and or modulates UPR signalling and thus promotes antioxidant responses, curbs inflammatory cytokines, inhibits PDI and thereby prevents cell damage (Di Santo and Ehrisman, 2013, 2014). The UPR consists of three pathways regulating the ER stress, which decreases protein load by promoting folding or otherwise induces extended UPR to cause apoptosis. Each UPR pathway involves a particular signalling molecule, including inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6) (Ron and Walter, 2007; Sureda et al., 2020). The IRE1 causes mRNA splicing and activation of tumour necrosis factor associated factor 2 (TRAF2) triggering downstream inflammatory and apoptotic signalling (Rajapaksa et al., 2015). CoV induces prolonged IRE1 and activates TRAF2 (Fung et al., 2014; Fung and Liu, 2014; Lim et al., 2016). In contrast, nitazoxanide induces glutathione S-transferase P1 (GSTP1) which inhibits TRAF2 (Müller et al., 2008b). CoV induces ATF-6 to release ER chaperone proteins like PDI (Fung et al., 2014), whereas nitazoxanide being a PDI inhibitor, plays a crucial role in maintaining protective UPR response (Di Santo and Ehrisman, 2013, 2014). PERK which causes phosphorylation of the eIF2α and induces Activating Transcription Factor 4 (ATF4), protecting the cell against oxidative damage (Sureda et al., 2020), is inhibited by CoV, while nitazoxanide upregulates the ATF4 mechanism (Ashiru et al., 2014). Nitazoxanide mediated UPR signalling via PERK activation can exhibit antiprotease and antioxidant activity by stimulating nuclear factor erythroid 2-related factor 2 (Nrf2) through NADPH quinone oxidoreductase1, a critical response against respiratory viral infection, including coronaviruses (Antalis et al., 2011; Curry Jr and Rosewell, 2008; Rosewell et al., 2011; Victor et al., 2020). Furthermore, the calcium binding proteins, translationally controlled tumour protein (TCTP) and binding immunoglobulin protein (BiP) are activated within cytosol and ER respectively, during nitazoxanide therapy to balance $Ca^{2+}$ concentration, thus inhibiting apoptosis (Ashiru et al., 2014; Di Santo and Ehrisman, 2014). Another important aspect is the potent bronchodilation activity of nitazoxanide comparable to β-agonist isoproterenol, attributed to its role as antagonist to the $Ca^{2+}$-Activated-Cl$^-$ channel transmembrane member 16A (TMEM16A). This proposes a new mechanism of bronchodilation useful in treatment of severe lung inflammatory diseases, asthma and COPD. Nitazoxanide therefore exhibits great promise in alleviating lung damage (Miner et al., 2019a).

### 2.2.8. Nitazoxanide use in comorbidities

In COVID-19 patients, the elevated levels of C-reactive protein, erythrocyte sedimentation rate, serum amyloid A and ferritin, respiratory distress, vascular leakage and coagulation, neuronal damage result in fatalities often associated with multiple organ failures (Chibber et al., 2020; Kermali et al., 2020; Nile et al., 2020; Samudrala et al., 2020; Zeng et al., 2020). Interestingly, inhibition of PDI by nitazoxanide can play an important role in preventing multiple organ damage reported in COVID-19 patients. In case of cardiovascular diseases, PDI supports plaque formation and causes coagulation (Li et al., 2016), whereas in case of diabetes it hampers insulin release and prevents lowering of blood glucose (Mohan et al., 2019; Shergalis and Neamati, 2016). Similarly, in cancer patients, cell migration, invasion and metastasis is mediated through PDI activity (Lee, 2017; Xu et al., 2014). Moreover, in case of brain disorders, nitazoxanide inhibited neurodegeneration (Perri et al., 2016; Uehara et al., 2006; Verkhratsky and Petersen, 2002). Further, in the case of liver diseases activation of prolonged UPR signalling and PDI plays a crucial role in disease progression (Maiers and Malhi, 2019; Mohan et al., 2019). Renal fibrosis also exhibits high secretory level of PDI (ERP57) causing kidney damage (Dihazi et al.,

2013). Yet another mechanism by which nitazoxanide exhibits multiple organ protection is via phosphodiesterases (PDEs) inhibition (WO2018173069A1) (Deshpande et al., 2018). PDEs causes phosphodiesteric bond cleavage in cyclic nucleotides, cAMP and cGMP, leading towards fibrosis, memory loss, pulmonary hypertension, vasoconstriction, renal dysfunction and various other multiple organ damage (Mondaini, 2020; Santing et al., 2001; Sharma et al., 2013; Thomas et al., 2001). Thus inhibiting PDI, PDEs and simultaneously activating protective UPR signalling could be critical in effective therapy of patients with comorbidities like cardiovascular disease, cancer, diabetes, kidney, liver and brain disorders (Di Santo and Ehrisman, 2013; Müller et al., 2008a).

## 3. Nitazoxanide promising *in vitro* efficacy against SARS-CoV-2

Nitazoxanide has demonstrated superior efficacy against coronavirus replication in cell culture assays at low micromolar concentrations (Rossignol, 2014). Recent reports on comparative *in vitro* efficacy of nitazoxanide hampering SARS-CoV-2 in Vero E6 cells at 48 h post-infection revealed low 50% effective concentration ($EC_{50}$) 2.12 μM. The *in vitro* antiviral efficacy found promising and was comparable with antiviral drug remdesivir ($EC_{50}$ 0.77 μM), antimalarial chloroquine ($EC_{50}$ 1.13 μM) and superior to favipiravir ($EC_{50}$ 61.88 μM) (Wang et al., 2020).

## 4. Nitazoxanide dosing and safety

Nitazoxanide is a hydrophobic drug with low aqueous solubility (~7.5 μg/ml) and high permeability, which dictates its dose-dependent therapeutic efficacy. Nitazoxanide exhibits oral bioavailability of about 30%, which can rise up to 50% with food (Rossignol, 2014; Stachulski et al., 2018). Its low bioavailability and poorly discovered lung concentrations, are major challenges that need to be addressed (Anderson and Curran, 2007; Padmanabhan, 2020; Padmanabhan and Padmanabhan, 2020; Rajoli et al., 2020). In adults, nitazoxanide dose of 500 mg twice-daily achieved peak concentration of 10 μg/ml, at approximately 2–4 h for TIZ, while 1 g twice daily dose achieved the peak concentration of 24 μg/ml (Fox and Saravolatz, 2005; Jr, 2004). Moreover, nitazoxanide controlled-release tablets 300 mg twice-daily dose exhibited peak plasma concentrations of 4.6 μg/ml which is well above its *in vitro* antiviral concentration (Haffizulla et al., 2014; Rossignol, 2014). The estimated $IC_{50}$ and $IC_{90}$ of nitazoxanide against SARS-CoV-2 are 0.68 μg/ml and 1.84 μg/ml respectively, which can be readily achieved using frequent dosing (Padmanabhan and Padmanabhan, 2020). The physiologically-based pharmacokinetic (PBPK) modelling reported the optimal doses of nitazoxanide as 1200 mg four times, 1600 mg three times, 2900 mg twice daily in the fasted state and 700 mg four times, 900 mg three times and 1400 mg twice daily when given with food, to provide its plasma and lung concentrations well above its reported *in vitro* 90% effective concentration ($EC_{90}$) against SARS-CoV-2 (4.64 μM or 1.43 μg/ml) (Rajoli et al., 2020). Furthermore, one study utilized the ratio of $C_{max}/EC_{90}$ based on approved human dose of nitazoxanide, which exhibited the ratio value above 1 indicating plasma $C_{max}$ concentrations exceeded those necessary to inhibit 90% of SARS-CoV-2 replication (Arshad et al., 2020). Interestingly, nitazoxanide is well tolerated up to 4 g daily dose and its $LD_{50}$ is higher than 10,000 mg/kg, indicating high safety. Nitazoxanide high dose of 1 g two times daily administered in AIDS patients suffering from diarrhoea, devoid of any severe side effects (Fox and Saravolatz, 2005; Jr, 2004). Thus nitazoxanide is effective and safe therapeutic intervention and its estimated generic pricing is $1.41 for a 14-day COVID-19 treatment at 500 mg twice daily dose (Mahmoud et al., 2020; Martins-Filho et al., 2020; Pepperrell et al., 2020), which makes therapy inexpensive.

## 5. Nitazoxanide and COVID-19 severity

The clinical pathology of COVID-19 is characterized as mild, moderate, severe and critical (Siddiqi and Mehra, 2020). The life-threatening challenges emerge in severe and critical phases attributed to the cytokine storm (Chibber et al., 2020; Samudrala et al., 2020; Soy et al., 2020; Wang et al., 2020). While, favipiravir, remdesivir, umifenovir and hydroxychloroquine may act at early and mild to moderate phases, nitazoxanide holds promise to act even in the severe phase due to the potential to curb the cytokine storm (Krishna et al., 2020; Nyarko et al., 2020; Omolo et al., 2020; Sanders et al., 2020). Dexamethasone also found effective against cytokine storm (Horby et al., 2020; Robinson, 2020). Furthermore, the possibility of nitazoxanide in therapy of patients with comorbidities is certainly exciting. Fig. 6 depicts the various stages of COVID-19 and compares nitazoxanide with some of the promising emergency use approved drug candidates for COVID-19.

## 6. Clinical trials of nitazoxanide against COVID-19

Number of clinical trials registered using nitazoxanide as the only drug or in combination with other antivirals further substantiates the hypothesis on the high promise of nitazoxanide (Mahmoud et al., 2020; Martins-Filho et al., 2020). A total of 19 new clinical trials for COVID-19, have been registered in the USA, Egypt, Mexico, Argentina, Nigeria and Brazil in the past five months, between April to August 2020 (https://clinicaltrials.gov/) (Nitazoxanide clinical trials, 2020). Of these registered trials ten trials are nitazoxanide alone while the other nine are combinations of nitazoxanide with antivirals (Table 1). Notably, in the majority of registered clinical trials, the nitazoxanide dose is increased to 500 mg or 600 mg twice a day and more. Moreover, nitazoxanide has also been proposed in combination with other potential drugs such as hydroxychloroquine, ivermectin and ribavirin. These trials indeed emphasise the promise of the drug for COVID-19. A recent study identified 73 combinations of potential 32 drugs against SARS-CoV-2 using *in silico* modelling and further validated them through *in vitro* studies. The study reported high synergy of nitazoxanide with three antivirals remdesivir, amodiaquine and umifenovir. The study also reports that the combination of remdesivir and hydroxychloroquine demonstrated strong antagonism (Bobrowski et al., 2020). The combination use of nitazoxanide with macrolide antibiotic azithromycin (Kelleni, 2020)

and antimalarial drug hydroxychloroquine has been proposed (Padmanabhan, 2020).

## 7. Limitations and challenges

Although nitazoxanide demonstrated efficacy in clinical studies against Influenza, HBV, HCV, rotavirus, rhinovirus/enterovirus infections, it was never approved/marketed for these indications, due to poor significance over the placebo group. Although the clinical study against uncomplicated influenza demonstrated efficacy, the results were not convincing given only 1–2 log reduction in viral titer and minor difference in time for discharge between placebo (116.7 h) and test arms (nitazoxanide 300 mg twice daily 95.5 h and nitazoxanide 300 mg once daily 109.1 h) (Haffizulla et al., 2014). We contend that the dose was probably low. The differences would have probably been evident with further increase in dose to 500 mg twice a day or through the design of a bioenhanced formulation. The Mexico trial involving nitazoxanide against severe acute respiratory illness (SARI), found discouraging, with highlighted reasons for the same. It said, negative results might be associated with low plasma drug levels, which attributed to low dose and poor bioavailability. They also highlighted other limitations of their study, which included the heterogeneous patient population, a variety of factors affecting decisions related to the primary endpoint (hospital discharge), and limited sample size. Also, the maximum dose selected was 300 mg twice daily for five days (Gamiño-Arroyo et al., 2019).

The phase 3 global study data on uncomplicated influenza involved a higher dose of nitazoxanide 600 mg twice daily (1.2 gm/day) alone or in combination with oseltamivir 75 mg twice daily (NCT01610245). The results are however not available yet. This study substantiates our premise on the need for a higher dose. Nitazoxanide requires further translational development and de-risking studies to conclude its effective use in clinical situation to provide benefit to COVID-19 victims.

## 8. Conclusion

Nitazoxanide an FDA approved antiprotozoal, safe, inexpensive and well-tolerated old drug, proposes great potential for repurposing against COVID-19. In this review we highlight proposed mechanism of nitazoxanide against numerous targets involved in SARS-CoV-2 pathogenesis affecting viral entry and multiplication. The potential of
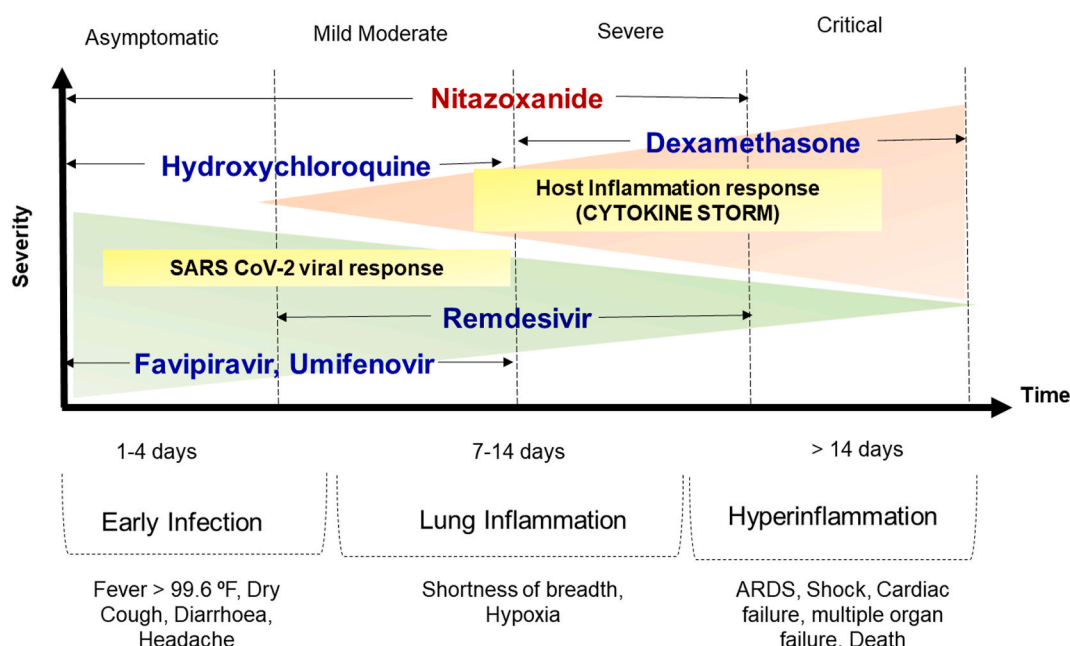


**Fig. 6. Different clinical phases of COVID-19 infection based on severity and recommended drug use.** ARDS, Acute respiratory distress syndrome.

**Table 1**

Clinical studies registered on Nitazoxanide/Nitazoxanide combinations against COVID-19 (Updated August 28, 2020).

| Sr. no. | Trial Number | Study Proposed | Drug Used & Dose | No of patients, Status & Date of Posting |
|---|---|---|---|---|
| **A** | **Nitazoxanide alone** | | | |
| 1 | NCT04486313 **Romark Laboratories L. C. USA** Phase III | Efficacy and safety in mild to moderate patients | Two nitazoxanide 300 mg tablets administered orally twice daily with food for 5 days with vitamin B complex versus Placebo and vitamin B complex | 800 Recruiting **July 24, 2020** |
| 2 | NCT04463264 **Austral University, Argentina** Phase II/III | Efficacy and safety in mild patients | Nitazoxanide orally 500 mg every 6 h for 14 days) orally with food versus Placebo | 135 Recruiting **July 9, 2020** |
| 3 | NCT04441398 **Azidus Brazil** Phase II/III | Efficacy and safety to treat mild ambulatory patients | Nitazoxanide 600 mg three times a day (total dose 1800 mg/day) for 7 days versus Placebo | 300 Not yet recruiting **June 22, 2020** |
| 4 | NCT04435314 **Azidus Brazil** Phase II | Efficacy and safety of post exposure prophylaxis | Nitazoxanide 600 mg three times a day (total dose 1800 mg/day) for 7 days versus Placebo | 200 Not yet recruiting **June 17, 2020** |
| 5 | NCT04423861 **Hospital Casa de Saúde - Vera Cruz - Campinas - SP – Brazil** Phase II | Efficacy in non-critical patients | Nitazoxanide 600 mg three times a day (total dose 1800 mg/day) for 7 days | 50 Not yet recruiting **June 9, 2020** |
| 6 | NCT04406246 **Materno-Perinatal Hospital of the State of Mexico, Mexico** Phase IV | Prophylactic treatment | Nitazoxanide 500 mg every 6 h for two days and then every 12 h for four days. | 150 Recruiting **May 28, 2020** |
| 7 | NCT04359680 **Romark Laboratories L. C. USA** Phase III | Efficacy and safety | Nitazoxanide 600 mg orally twice daily for six weeks versus Placebo | 800 Recruiting **April 24, 2020** |
| 8 | NCT04348409 **Hospital Vera Cruz Campinas, São Paulo, Brazil** Proof of Concept | Safety and efficacy in moderate condition | Nitazoxanide 600 mg twice daily for 6 weeks versus Placebo | 50 Recruiting **April 16, 2020** |
| 9 | NCT04343248 **Romark Laboratories L. C. USA** Phase III | Efficacy and safety | Nitazoxanide 600 mg twice daily for 6 weeks | 300 Recruiting **April 13, 2020** |
| 10 | NCT04343248 **Romark Laboratories L. C. USA** Phase III | Efficacy and safety | Nitazoxanide 600 mg orally twice daily for 6 weeks versus Placebo | 600 Not yet recruiting **April 13, 2020** |
| **B** | **Nitazoxanide combination** | | | |
| 11 | NCT04498936 **Assiut University** | Efficacy and safety | Nitazoxanide (500 mg, orally) four times per day for 14 days, plus | 240 Recruiting |

**Table 1** (*continued*)

| Sr. no. | Trial Number | Study Proposed | Drug Used & Dose | No of patients, Status & Date of Posting |
|---|---|---|---|---|
| | **Hospital, Assiut, Egypt** Phase IV | | the standard care treatment versus regimen combination of Sofosbuvir/ Ledipasvir (400 mg and 90 mg, orally) once daily for 14 days, plus the standard care treatment | **August 5, 2020** |
| 12 | NCT04459286 **Infectious Disease Hospital, Olodo Ibadan, Oyo State, Nigeria** Phase II | Efficacy and safety | Nitazoxanide 1000 mg tablets twice daily and 300/100 mg atazanavir/ritonavir tablets once daily with meal for 28 days versus Standard of care treatment | 98 Not yet recruiting **July 7, 2020** |
| 13 | NCT04392427 **Mansoura University, Mansoura, Egypt** Phase III | Combination efficacy | Nitazoxanide, ivermectin and ribavirin 200 mg or 400 mg for 7 days + zinc supplement versus Placebo | 100 Not yet recruiting **May 18, 2020** |
| 14 | NCT04382846 **Tanta University, Tanta, Egypt** Phase III | Efficacy novel regimens | **Nitazoxanide,** Ivermectin, Chloroquine, Azithromycin dose not mentioned | 80 Not yet recruiting **May 11, 2020** |
| 15 | NCT04360356 **Tanta University, Tanta, Egypt** Phase II/III | Combination therapy | Ivermectin 200 mcg/kg once orally on empty stomach plus **Nitazoxanide** 500 mg twice daily orally with meal for 6 days versus standard care oxygen via ventilators | 100 Not yet recruiting **April 24, 2020** |
| 16 | NCT04361318 **Tanta University, Tanta, Egypt** Phase II/III | Combination therapy | 200 mg of Hydroxychloroquine orally three times daily for 10 days plus 500 mg of **Nitazoxanide** orally twice daily for 6 days versus Standard care | 100 Not yet recruiting **April 24, 2020** |
| 17 | NCT04351347 **Tanta University, Tanta, Egypt** Phase II/III | Efficacy | Drug: Chloroquine Drug: Nitazoxanide + Chloroquine Drug: Ivermectin + Chloroquine | 60 Recruiting **April 17, 2020** |
| 18 | NCT04345419 **Tanta university hospital Tanta, Egypt** Phase II/III | Efficacy and safety | Chloroquine versus Favipiravir versus **Nitazoxanide** versus Ivermectin versus Niclosamide dose not mentioned | 100 Not yet recruiting **April 14, 2020** |
| 19 | NCT04341493 **Materno-Perinatal Hospital of the State of Mexico, Mexico** Phase IV | Safety and efficacy | Hydroxychloroquine 200 mg twice daily for 10 days versus Nitazoxanide 500 mg twice daily + Hydroxychloroquine 200 mg twice daily for 10 days | 86 Recruiting **April 10, 2020** |

Trials accessed from https://clinicaltrials.gov/.

nitazoxanide to inhibit the inflammatory cytokine storm while simultaneously stimulating innate immune responses presents great promise to prevent ARDS. The ability to protect the lung, avert associated multiple organ damage, and the beneficial effects in SARS-CoV-2 patients with comorbidities proposes nitazoxanide as a promising candidate for repurposing in COVID-19.

## 9. Future perspectives

Nitazoxanide presents exciting possibilities for repurposing in COVID-19. The drug is approved as an antiprotozoal and for localized action in the gastro-intestinal tract (GIT). Hence considering strategies to enhance bioavailability could be an important approach to enhance efficacy at possibly lower doses (Omolo et al., 2020). Furthermore, directing the drug in high concentration to the target site following oral administration could be achieved by lymphatic targeting of oral particulate carriers (Bachhav et al, 2017, 2018). With the myriad targets the drug exhibits potential to attack in the pathogenesis of COVID-19, let us hope it is the panacea the world seeks to overcome the fatality and the fear of COVID-19.

## Conflicts of interest/competing interests

The authors declare no competing financial interest.

## Ethical approval

Not required.

## CRediT authorship contribution statement

**Amit S. Lokhande:** Conceptualization, Data curation, Data analysis and/or interpretation, Writing- original draft, Visualization, Writing - review & editing. **Padma V. Devarajan:** Conceptualization, Supervision, Writing - review & editing. All authors have reviewed the manuscript and approved the submission.

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