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Early View

Original article

Early use of nitazoxanide in mild Covid-19 disease: randomised, placebo-controlled trial

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Take home message

This was the first study to evaluate the effect of early nitazoxanide therapy in mild Covid-19. Nitazoxanide did not accelerate symptom resolution after 5 days of therapy but did reduce viral load significantly with no serious adverse events.

Abstract

Nitazoxanide is widely available and exerts broad-spectrum antiviral activity *in vitro*. However, there is no evidence of its impact on SARS-CoV-2 infection.

In a multicenter, randomized, double-blind, placebo-controlled trial, adult patients presenting up to 3 days after onset of Covid-19 symptoms (dry cough, fever, and/or fatigue) were enrolled. After confirmation of SARS-CoV2 infection by RT-PCR on a nasopharyngeal swab, patients were randomized 1:1 to receive either nitazoxanide (500 mg) or placebo, TID, for 5 days. The primary outcome was complete resolution of symptoms. Secondary outcomes were viral load, laboratory tests, serum biomarkers of inflammation, and hospitalization rate. Adverse events were also assessed.

From June 8 to August 20, 2020, 1,575 patients were screened. Of these, 392 (198 placebo, 194 nitazoxanide) were analyzed. Median time from symptom onset to first dose of study drug was 5 (4-5) days. At the 5-day study visit, symptom resolution did not differ between the nitazoxanide and placebo arms. Swabs collected were negative for SARS-CoV-2 in 29.9% of patients in the nitazoxanide arm *versus* 18.2% in the placebo arm (p=0.009). Viral load was also reduced after nitazoxanide compared to placebo (p=0.006). The percent viral load reduction from onset to end of therapy was higher with nitazoxanide (55%) than placebo (45%) (p=0.013). Other secondary outcomes were not significantly different. No serious adverse events were observed.

In patients with mild Covid-19, symptom resolution did not differ between nitazoxanide and placebo groups after 5 days of therapy. However, early nitazoxanide therapy was safe and reduced viral load significantly.

Keywords: Covid-19; nitazoxanide; SARS-CoV-2; therapy; randomized trials

Introduction

The majority of patients infected with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) present mild symptoms of coronavirus disease 2019 (Covid-19) and recover with supportive care; however, in certain hosts, even mild disease may progress to clinical deterioration or a protracted course [1]. To date, no therapeutic interventions have proven effective in mild Covid-19.

Drug repurposing has been recognized as an important tool in the search for effective Covid-19 therapies, as it reduces development costs and timelines [2]. Nitazoxanide, a clinically approved and commercially available antiparasitic drug, has been found to have broad-spectrum antiviral activity, including against coronaviruses, influenza viruses, and hepatitis B and C viruses [3]. Furthermore, it has been shown to inhibit SARS-CoV-2 replication at low micromolar concentrations in Vero CCL81 cells [4]. In addition, nitazoxanide is orally bioavailable and broadly well-tolerated, thus representing a promising alternative for the management of Covid-19 were it to prove effective *in vivo* [3]. Efficacy in early treatment—initiated soon after symptom onset to reduce viral load and prevent disease progression—would be particularly appealing from a public health standpoint. However, there is no evidence of its safety or efficacy as therapy for mild Covid-19 patients.

In this context, a multicenter, randomized, placebo-controlled trial was carried out to evaluate whether early nitazoxanide therapy would be effective in accelerating symptom resolution. Secondarily, viral load, markers of inflammation, hospitalization rate, and the safety of nitazoxanide as compared with placebo were also assessed.

Methods

Study design

A double-blind, placebo-controlled trial was conducted at 5 freestanding urgent care centers and 2 hospitals across Brazil (**supplementary table S1**). The trial was designed by the executive committee and approved by the Brazilian National Commission for Research Ethics (CAAE: 32258920.0.1001.5257) and individual ethics committees of the participating sites. The trial was funded by the Brazilian Ministry of Science, Technology, and Innovation via the National Council for Scientific and Technological Development (CNPq). Nitazoxanide was provided free of charge by Eurofarma, which had no further role in the design or conduct of the trial. The executive committee assures the accuracy of the data and fidelity of the trial to the protocol, which was registered in the Brazilian Registry of Clinical Trials (REBEC) number RBR-4nr86m and ClinicalTrials.gov number NCT04552483. The independent Data and Safety Monitoring Board (DSMB), composed of experts in clinical trials and infectious diseases, was convened after 25%, 50%, and 75% of the participants had completed 14 days of follow-up and had access to information on adverse events and efficacy outcomes at every quartile.

The trial was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization (ICH) Good Clinical Practice Guideline (E6R2). Online clinical monitoring and quality control were outsourced to a contract research organization (ATCGen, Campinas, Brazil). This report follows the Consolidated Standards of Reporting Trials (CONSORT) guideline [5]. The final protocol, amendments and changes to the trial protocol, and the final statistical analysis plan are detailed in the Supplemental Methods.

Patients

Consecutive adult patients (aged 18 years or older) who presented with clinical symptoms of Covid-19 (defined for the purposes of this trial as dry cough, fever, and/or fatigue) of no longer than 3 days' duration were enrolled. The exclusion criteria were: negative reverse-transcriptase quantitative real-time polymerase chain reaction (RT-PCR) test for SARS-CoV-2 on an nasopharyngeal swab specimen; inability to swallow; preexisting conditions precluding the safe conduct of study procedures, including severe renal, heart, respiratory, liver, or autoimmune diseases, cancer in the last 5 years, or known allergy or hypersensitivity to nitazoxanide; therapy with nitazoxanide in the 30 days before presentation; and clinical suspicion of bacterial pneumonia or tuberculosis.

Randomization and masking

Patients were randomly assigned (1:1) using a computer-generated random number list to receive either placebo or nitazoxanide (500 mg oral solution, 20 mg/mL [25 mL], three times daily for 5 days), dispensed by the pharmacy of each study site. Placebo and nitazoxanide were color-matched to ensure that assessors were unaware of group allocation at all time points.

Procedures

On day 0 (baseline), patients were assessed for eligibility. Informed consent was obtained from each patient or, if the patient was unable to provide consent, from a healthcare proxy. A nasopharyngeal swab was then collected for RT-PCR testing. In order to mitigate any bias, all RT-PCR analyses were processed centrally at CT-VACINAS, Federal University of Minas Gerais, Brazil; specimens were sent on the day of collection by commercial courier. Patients who tested positive for SARS-CoV-2 (result obtained 1-2 days after RT-PCR) (figure 1) were contacted by telephone and

asked to return to the health facility to which they had originally presented. Site investigators then performed a comprehensive physical examination and measured body temperature, heart and respiratory rates, blood pressure, and peripheral oxygen saturation. Ethnicity, current medications, and date of symptom onset were self-reported by patients. Blood was then drawn for measurement of complete blood count, C-reactive protein (CRP), and serum biomarkers of inflammation (interleukin [IL]-6, IL-8, IL-1β, tumor necrosis factor [TNF]-α, and interferon [IFN]-γ) **(See Supplemental Methods for details of RT-PCR testing and biomarker assessment)**.

All patients took home a symptom journal designed to gather information on daily symptoms, new symptoms, and the date of resolution of each symptom (**supplementary table S2**). Study data were entered directly into an electronic database by an assigned staff member at each study site and further validated by external trial monitoring staff at ATCGen.

One day after completion of therapy, patients returned to the study sites to return their symptom journals and provide a new nasopharyngeal sample for RT-PCR and blood samples for complete blood count, CRP, and serum biomarkers. Data validation was done exclusively by the designated study site manager, with assistance from ATCGen staff as needed.

Primary Outcome

The primary outcome was complete resolution of the three symptoms of interest (dry cough, fever, and/or fatigue) after 5 days of therapy.

Secondary Outcomes

Secondary outcomes were reduction in viral RNA load on nasopharyngeal swab specimens (from baseline until the day after completion of therapy); improvement in

laboratory parameters (including serum biomarkers of inflammation); and incidence of hospital admission after completion of therapy. Patients who did not return to the study sites either to receive the therapy or after the end of therapy were contacted by telephone to understand the reasons for nonadherence and were then excluded from per-protocol analysis (**Supplemental Methods**). Adverse events, regardless of grade, were monitored throughout the trial by review of the electronic medical record, physical examination, vital signs, and laboratory tests from enrollment through day 14. The Medical Dictionary for Regulatory Activities® (MedDRA, version 23.0) was used for classification.

Statistical Analyses

We estimated a sample size of 392 patients (196 per arm, allocation ratio 1:1) would provide 85% power to detect an 11% increase in symptom-free days after nitazoxanide initiation compared to placebo at a two-sided significance level of α=0.05. Descriptive statistics were used for demographic, laboratory, and clinical data. For qualitative variables, Fisher's exact test was performed. The Mann-Whitney *U* and Wilcoxon tests were used for between and within-groups comparisons, respectively. Statistical analyses were performed in the SPSS Version 27 environment (IBM Corp, Armonk, NY), and a two-tailed *p*-value < 0.05 was considered significant **(Supplemental Methods).**

Results

Between June 8 and August 20, 2020, 1,575 patients were assessed for eligibility at the study sites. Of these, 475 tested positive for SARS-CoV-2 infection by RT-PCR and underwent randomization. **Figure 2** summarizes the enrollment and follow-up of study participants. Reasons for exclusion before randomization included negative RT-PCR

collected at day 0 and/or absence of Covid-19 symptoms (n=1,062), refusal to participate (n=27), hospitalization before the first dose of therapy (n=5), and other reasons (n=6). After randomization (n=475), patients were excluded due to discontinued intervention (n=41), moderate adverse events (n=7), and hospitalization (n=10). Six patients in the nitazoxanide arm and one patient in the placebo arm discontinued therapy due to moderate diarrhea and vomiting within the first 2 days; both had already experienced improvement of Covid-19 symptoms. Ten patients (5 from each arm) were hospitalized due to clinical deterioration; none had completed the 5-day course of therapy. Two patients, both in the nitazoxanide arm, required intensive care unit admission **(supplementary table S3)**. During analysis, 12 patients were excluded from the nitazoxanide arm and 15 from the placebo arm due to protocol deviation, missing data on the primary outcome, or non-evaluability, resulting in a studied population of 392 patients (194 in the nitazoxanide arm and 198 in the placebo arm).

Baseline characteristics

Patients' characteristics at baseline are given in **table 1**. Demographics and clinical characteristics were balanced in both groups. Age ranged from 18 to 77 years. Just over half (53%) were women and 69% were white. Regarding timing of therapy initiation, 8% of patients were enrolled on day 1, 25% on day 2, and 67% on day 3 after symptom onset. The median (IQR) time between symptom onset and first dose of nitazoxanide or placebo was 5 (4-5) days. Fever, dry cough, and fatigue were the most frequent clinical features present at enrollment. At baseline, the overall median (IQR) viral load in the nasopharyngeal swab at baseline did not differ significantly between the two arms Additional characteristics of the study population at baseline are given in **supplementary table S4**.

Primary Outcome

Complete resolution of symptoms (dry cough, fever, and fatigue) did not differ between the nitazoxanide and placebo arms after 5 days of therapy **(figure 3, supplementary figure S2)**.

Secondary Outcome

After 5 days of therapy, viral load was lower in the nitazoxanide arm compared to placebo (p=0.006) **(table 3)**. Moreover, the percentage of reduction in viral load from day 0 (baseline) **(table 1)** to day 5 **(table 2)** of therapy was significantly higher in the nitazoxanide arm (55%) than in the placebo arm (45%) (p=0.013). At the end-of-therapy visit, 29.9% of patients in the nitazoxanide arm *versus* 18.2% in the placebo arm were negative for SARS-CoV-2 on RT-PCR (p=0.009) **(table 3)**. Vital signs **(supplementary table S3)**, total leukocyte, neutrophil, lymphocyte, and platelet counts, CRP levels, and serum biomarkers of inflammation **(table 3)** did not differ between baseline and day 5 of therapy in the nitazoxanide arm, nor between the nitazoxanide and placebo arms. No patients that completed 5-day course of nitazoxanide were hospitalized (**supplementary table S3**).

No deaths or life-threatening adverse events were reported in either arm. Mild and moderate adverse events were experienced by patients in both arms (nitazoxanide, 30.9%; placebo, 30.4%) during the 5-day course of therapy **(table 4)**. The most common adverse events (grades 1 and 2) were diarrhea, headache, and nausea, with no significant differences between groups. Four patients reported severe adverse events (headache alone or with diarrhea); however, they were more frequent in the placebo arm. Discolored urine is a known side effect of nitazoxanide therapy, which might raise concerns about inadvertent unblinding. However, although this event was indeed more frequent in the treatment arm, it was also reported by patients receiving placebo (5.6% versus 1.5%, respectively) **(table 3).**

Discussion

In this multicenter, double-blind, randomized, placebo-controlled trial of patients with mild Covid-19, we found that symptom resolution (dry cough, fever, and fatigue) did not differ between the nitazoxanide and placebo after 5 days of therapy. Nitazoxanide was safe, significantly reduced viral load, and increased the proportion of patients testing negative for SARS-CoV-2 after 5 days of therapy compared to placebo. Nitazoxanide did not prevent hospitalization or effect any change in complete blood count, CRP levels, or serum biomarkers of inflammation. Moreover, nitazoxanide is inexpensive, widely available, and, to date, no other recommended therapies have been demonstrated to provide any benefit in this population.

There is an urgent need for evidence-based pharmacotherapeutics for Covid-19. In the challenging context of a pandemic, drug repurposing can reduce the time-consuming drug development process and allow rapid deployment of effective therapies to the population [2]. Before implementation of this trial, the NIH Clinical Collection (NCC) library, a collection of 727 FDA-approved drugs or drug-like compounds with a history of use in human clinical trials, was screened for potential *in vitro* antiviral activity against SARS-CoV-2 **(supplementary figure S1)**. Nitazoxanide and tizoxanide, its active metabolite, significantly reduced viral load in VeroE6, human embryonic kidney (HEK 293T), and lung epithelial (Calu-3) cells infected with SARS-CoV-2, without inducing loss of cell viability. The adverse effect profile of nitazoxanide is well-known, since it has been commercially available and used in the clinic since 1996; indeed, a commercial formulation was used in this trial. A dosage regimen of 500 mg every 12 hours is approved and commonly prescribed for treatment of intestinal parasitosis [5], with few reported adverse events. In our study, the same dose was administered, but every 8 hours, based on *in vitro* pharmacological studies by our group and published

data on plasma concentration [6-8], in order to maximize potential inhibition of SARS-CoV2 *in vivo* [7]. We assumed that high ratios of maximum serum concentration at doses safe in humans to maximal *in vitro* effective antiviral concentration would translate into higher potential to achieve viral suppression at approved doses [10].

Nitazoxanide failed to meet the primary outcome in patients with mild Covid-19 when evaluated after 5 days of therapy. Consistent with the *in vitro* data, we observed significant reductions in viral load after a 5-day course of nitazoxanide in patients with mild Covid-19. This effect may have epidemiological impact, potentially decreasing community spread of SARS-CoV-2 [9, 10], morbidity [11], and mortality [12].

We enrolled patients with mild Covid-19 as soon as possible after symptom onset because antivirals seem to be more effective at this stage of infection [13]. The cutoff point for enrollment was 3 days after onset of first symptom; however, as therapy was begun only after confirmation of SARS-CoV-2 infection, the median timing of treatment initiation was 5 (4-5) days after symptom onset **(table 1)**. In previous trials of putative antiviral agents, median time to initiation of treatment was 13 days [14], 9 days [15], 8-9 days [16], and 4-5 days [17] after symptom onset. Initiating therapy even earlier might be more effective, since the peak SARS-CoV-2 viral load usually occurs before symptom onset [18] and systemic hyperinflammation rather than viral pathogenicity dominates later stages of Covid-19, at which point antiviral therapy could be ineffective [18, 19]. Additionally, analyses were done 5 days after therapy since a longer period of time would result in symptom resolution in most patients independent of treatment; in previous studies, the median to time from onset to resolution of symptoms has been reported as 8 (6.25-11.5) days [20].

Besides timing, two additional points which may have biased our findings in favor of the nitazoxanide arm were the severity of Covid-19 at presentation and the favorable

demographic profile of the sample. Only patients with mild Covid-19 were enrolled; most were young adults (aged 18-39), few had comorbidities (12-18%), and use of concomitant medications was infrequent (less than 20% of the sample). Further studies are needed to evaluate whether nitazoxanide may play a role in more advanced Covid-19. In this line, Hung *et al.* recently reported the importance of combining antiviral therapies in patients with severe Covid-19, aiming to reduce viral load and mitigate symptoms [17].

The increase in dosage frequency of nitazoxanide did not result in any significant change in the adverse event rate. Neither frequency nor severity of adverse events differed significantly between the arms, except for urine discoloration (a known and harmless effect), suggesting nitazoxanide is safe for patients with mild Covid-19.

This study has several limitations. Only three symptoms (dry cough, fever, and fatigue) were considered for analysis of primary outcome, even though Covid-19 is now known to have protean manifestations and patients reported other symptoms. Patients were followed during the 5-day course of therapy; however, a long-term analysis of the effects of therapy should be performed. All patients were instructed to take their study medication 3 times daily as prescribed, return to the study site if symptoms worsened or adverse effects developed, and complete their symptom journals with data on any adverse events, the intensity of each Covid-19 symptom, and when symptoms abated and resolved. However, we cannot ascertain the extent to which patients followed these instructions. Nevertheless, given the placebo-controlled design, nonadherence may have occurred in both groups. In the nitazoxanide group, around 3% stopped therapy due to gastrointestinal upset after Covid-19 symptoms had already improved. Finally, no chest computed tomography scans or plain radiographs were obtained in these patients, since our work was mostly performed at freestanding mobile urgent care centers.

In summary, in patients with mild Covid-19 enrolled within 3 days of symptom onset, nitazoxanide as compared with placebo was not an effective therapy in terms of accelerating symptom resolution after 5 days of therapy, and did not modify clinically relevant secondary outcomes. However, nitazoxanide was safe, significantly decreased viral load, and increased the proportion of patients who tested negative for SARS-CoV-2 after completion of therapy.

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Data sharing

Qualified clinical researchers can request access to de-identified participant data set (PP and ITT), informed consent forms, and related documents including the study protocol that underlie this article through submission of a proposal with a valuable research question to the corresponding author. A contract should be signed.

Contributors

PRMR, PLS, FFC were responsible for the design, analyzing, and writing of the manuscript. MACMJ, PGGMMT, MAM, LFGO, CCL, EZS, WFJ, EM, NFM, JMJG, MNC, ISS, NFP, PVMM were responsible for recruitment and clinical care of the patients. APSMF, KGF, RPR, AFC, PAA, JLM, ATC, DBBT, REM were responsible for laboratory analysis. RRL, JRLS, and PP were responsible for the statistical analysis. KGF, JRLS, and PP were also responsible for writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflict of interest

Dr. Rocco reports personal fees from SANOFI as a DSMB member. The other authors declare no competing interests.

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Table 1. Characteristics of the Participants Testing Positive for SARS-CoV-2 on RT-PCR at Baseline

	Nitazoxanide (n=194)	**Placebo (n=198)**	***p* value**
Age range, n (%)			0.891
18 to 39 years	115 (59)	113 (57)	
40 to 59 years	68 (35)	74 (37)	
60 to 77 years	11 (6)	11 (6)	
Sex, n (%)			0.054
Men	101 (52)	83 (42)	
Women	93 (48)	115 (58)	
Ethnicity, n (%)			0.644
White	131 (68)	138 (70)	
Asian	5 (3)	2 (1)	
Indigenous Brazilian	0 (0)	1 (0.5)	
Black	31 (16)	32 (16)	
Mixed	27 (14)	24 (12)	
BMI (kg/m^2), n (%)			1.000
<29.9	134 (69)	136 (69)	
≥ 30.0	60 (31)	62 (31)	

Comorbidities, n (%)			0.091
No	171 (88)	162 (82)	
Yes*	23 (12)	36 (18)	
Time from onset of symptoms to enrollment, n (%)			0.241
1 day	14 (7)	18 (9)	
2 days	42 (22)	55 (28)	
3 days	138 (71)	125 (63)	
Time from onset of symptoms to the first dose of nitazoxanide or placebo, median (IQR), days	5 (4-5)	5 (4-5)	0.124
Concomitant medications, n (%)			
None	154 (80)	158 (80)	1.000
Angiotensin-II receptor antagonists	6 (3)	12 (6)	0.227
Angiotensin-conversing enzyme inhibitors	1 (1)	1 (0.5)	0.833
Metformin	1 (0.5)	4 (2)	0.372
Statins	0 (0)	4 (2)	0.123
Symptoms at baseline, n (%)			

Inclusion criteria			
Dry cough	141 (73)	148 (75)	0.574
Fever	109 (56)	115 (58)	0.760
Fatigue	95 (49)	95 (48)	0.920
Secondary symptoms			
Sore throat	82 (42)	68 (34)	0.119
Myalgia	60 (31)	53 (27)	0.374
Headache	64 (33)	81 (41)	0.117
Anosmia	17 (9)	18 (9)	1.000
Ageusia	20 (10)	17 (9)	0.607
Diarrhea	14 (7)	5 (3)	0.035
Nasopharyngeal swab RT-PCR viral load (Log_{10} copies/mL), median (IQR)	7.06 (5.77-8.13)	7.49 (6.15-8.32)	0.065
SpO_2, mean (SD)	97.3 (1.4)	97.4 (1.3)	0.835
WBC ($x10^3$/mL), median (IQR)	5.4 (4.3-6.6)	5.3 (4.6-6.5)	0.904
Neutrophils ($x10^3$/mL), median (IQR)	2.8 (2.1-3.9)	2.9 (2.2-4.0)	0.984
Lymphocytes ($x10^3$/mL), median (IQR)	1.9 (1.5-2.2)	1.8 (1.4-2.4)	0.423

Platelets (x10^3/mL), median (IQR)	245 (205-245)	213 (177-257)	0.243
CRP (mg/L), median (IQR)	6.0 (2.0-15.0)	4.5 (2.0-12.2)	0.190
Cytokine concentration (pg/dL), median (IQR)			
IL-6	0 (0-4.10)	0 (0-8.34)	0.471
IL-8	2.79 (0-12.48)	5.51 (0.38-17.91)	0.090
IL-1β	0 (0-0)	0 (0-19.45)	0.088
TNF-α	0 (0-0)	0 (0-0)	0.179
IFN-γ	0 (0-16.24)	0 (0-10.24)	0.526

BMI: body mass index; CRP: C-reactive protein; IFN: interferon; IL: interleukin; IQR: interquartile range; SD: standard deviation; SpO_2: peripheral oxyhemoglobin saturation; TNF: tumor necrosis factor. Interferon WBC: white blood cells. *systemic arterial hypertension, diabetes mellitus, asthma. No significant differences were observed between the two groups.

Table 2. Secondary outcomes after 5 days of therapy

	Nitazoxanide (n=194)	Placebo (n=198)	*p* value
Nasopharyngeal swab RT-PCR viral load (Log_{10} copies/mL), median (IQR)	3.63 (0-5.03)	4.13 (2.88-5.31)	**0.006**
RT-PCR status, n (%)			**0.009**
Positive	136 (70.0)	162 (82.8)	
Negative	58 (29.9)	36 (18.2)	
WBC ($x10^3$/mL), median (IQR)	6.1 (5.1-7.3)	6.4 (5.3-7.8)	0.080
Neutrophils ($x10^3$/mL), median (IQR)	3.4 (2.6-4.4)	3.6 (2.7-4.7)	0.327
Lymphocytes ($x10^3$/mL), median (IQR)	2.1 (1.7-2.5)	2.2 (1.7-2.6)	0.078
Platelets ($x10^3$/mL), median (IQR)	240 (209-288)	239 (198-285)	0.275
CRP (mg/L), median (IQR)	5.0 (1.0-16.2)	4.5 (2.0-13.0)	0.445
Cytokine concentration (pg/dL), median (IQR)			

IL-6	0 (0-0.03)	0 (0-0.03)	0.992
IL-8	2.73 (0-12.24)	2.70 (0-11.42)	0.855
IL-1β	0 (0-0)	0 (0-0)	0.399
TNF-α	0 (0-0)	0 (0-0)	0.627
IFN-γ	0 (0-12.54)	0 (0-3.25)	0.286

Table 3. Adverse Events

	Nitazoxanide (n=194)	**Placebo (n=198)**	***p* value**
Number of participants with at least one adverse event	60 (30.9%)	60 (30.4%)	0.913
Number of participants with two adverse events	22 (11.3%)	18 (9.1%)	0.507
Number of participants with three or more adverse events	16 (8.2%)	12 (6.1%)	0.438
Number of participants with severe adverse event	1 (0.5%)	1 (0.5%)	0.999
Number of participants discontinuing treatment because of moderate adverse events	6 (3.1%)	1 (0.5%)	0.065
Detailed adverse events			
Diarrhea	57 (29.4%)	49 (24.7%)	0.309
Headache	34 (17.5%)	32 (16.1%)	0.787
Nausea	28 (14.4%)	29 (14.6%)	0.999
Abdominal pain	10 (5.2%)	5 (2.5%)	0.197
Abnormal color of urine	11 (5.6%)	3 (1.5%)	**0.031**

Vomiting	9 (4.6%)	3 (1.5%)	0.085
Pruritus	4 (2.1%)	1 (0.5%)	0.212
Urticaria	1 (0.5%)	3 (1.5%)	0.623

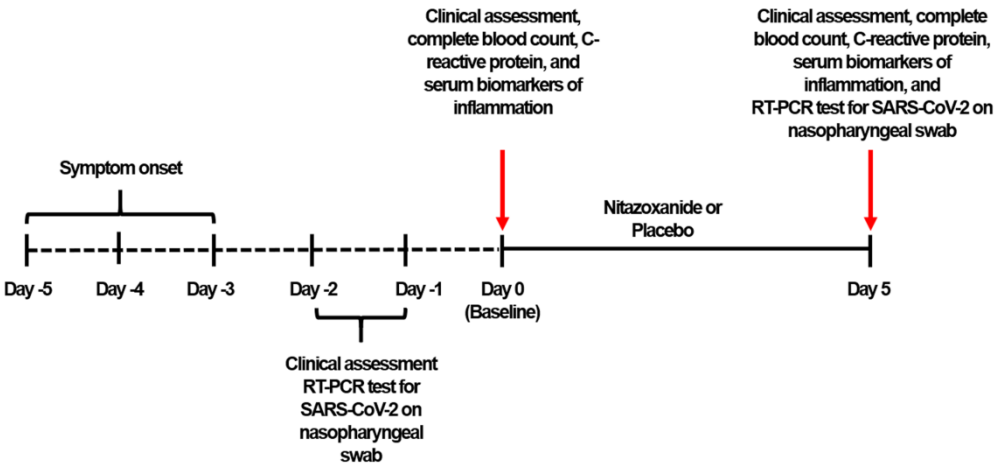
Data are n (%) and include all adverse events reported after nitazoxanide or placebo.

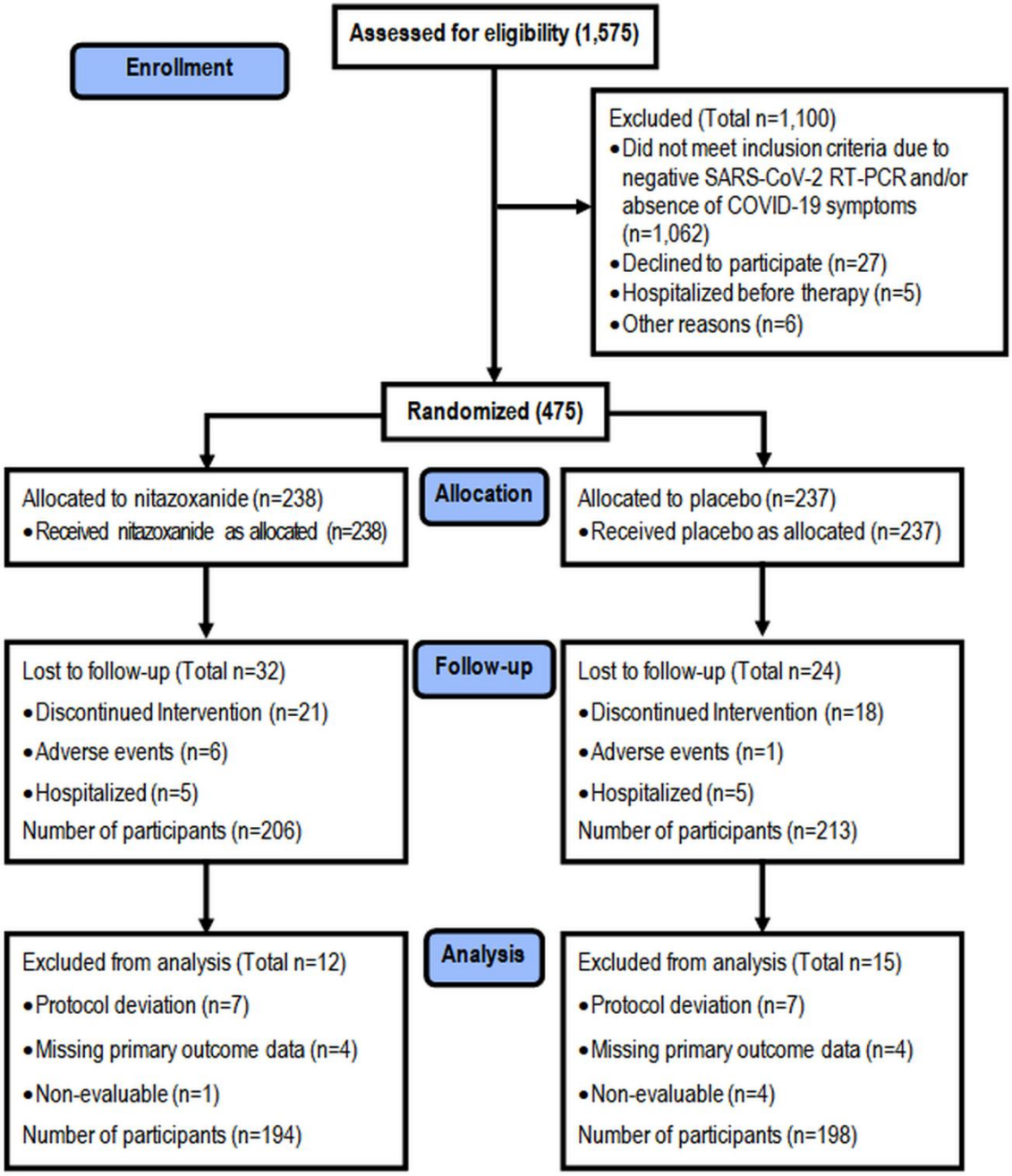
Figure Legends

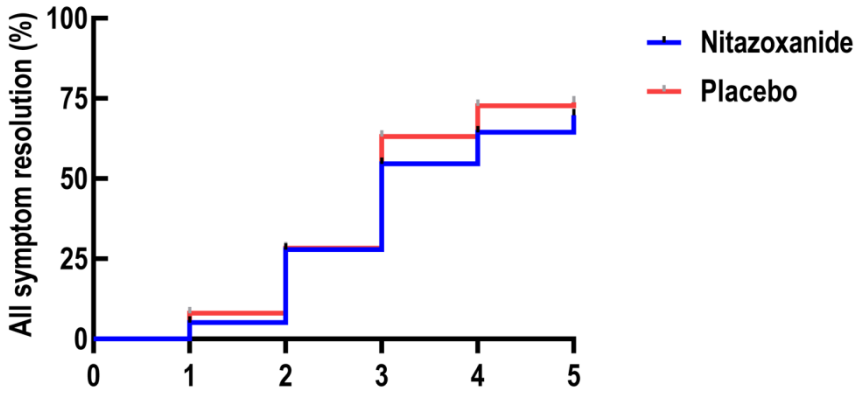
Figure 1. Timeline of study design. RT-PCR, real-time reverse transcription polymerase chain reaction.

Figure 2. Enrollment, randomization, follow-up, and treatment. 1,575 patients were assessed for eligibility at the study sites. Of these, 475 tested positive for SARS-CoV-2 infection by RT-PCR and underwent randomization. Reasons for exclusion before randomization included negative RT-PCR collected at day 0 (baseline) and/or absence of Covid-19 symptoms (n=1,062), refusal to participate (n=27), hospitalization before the first dose of therapy (n=5), and other reasons (n=6). After randomization (n=475), patients were excluded due to discontinued intervention (n=39), moderate adverse events (n=7) (all gastrointestinal upset), and hospitalization (n=10) (5 patients from each group, none of whom completed therapy). During analysis, 12 patients were excluded from the nitazoxanide arm and 15 from the placebo arm due to protocol deviation, missing data on the primary outcome, or non-evaluability, resulting in a studied population of 392 patients (194 in the nitazoxanide arm and 198 in the placebo arm).

Figure 3. Kaplan–Meier curve of symptom resolution after 5 days of therapy.


Clinical assessment, complete blood count, C-reactive protein, and serum biomarkers of inflammation
Clinical assessment, complete blood count, C-reactive protein, serum biomarkers of inflammation, and RT-PCR test for SARS-CoV-2 on nasopharyngeal swab
Symptom onset
Nitazoxanide or Placebo
Day -5
Day -4
Day -3
Day -2
Day -1
Day 0 (Baseline)
Day 5
Clinical assessment RT-PCR test for SARS-CoV-2 on nasopharyngeal swab


Enrollment
Assessed for eligibility (1,575)
Excluded (Total n=1,100)
• Did not meet inclusion criteria due to negative SARS-CoV-2 RT-PCR and/or absence of COVID-19 symptoms (n=1,062)
• Declined to participate (n=27)
• Hospitalized before therapy (n=5)
• Other reasons (n=6)
Randomized (475)
Allocation
Allocated to nitazoxanide (n=238)
• Received nitazoxanide as allocated (n=238)
Allocated to placebo (n=237)
• Received placebo as allocated (n=237)
Follow-up
Lost to follow-up (Total n=32)
• Discontinued Intervention (n=21)
• Adverse events (n=6)
• Hospitalized (n=5)
Number of participants (n=206)
Lost to follow-up (Total n=24)
• Discontinued Intervention (n=18)
• Adverse events (n=1)
• Hospitalized (n=5)
Number of participants (n=213)
Analysis
Excluded from analysis (Total n=12)
• Protocol deviation (n=7)
• Missing primary outcome data (n=4)
• Non-evaluable (n=1)
Number of participants (n=194)
Excluded from analysis (Total n=15)
• Protocol deviation (n=7)
• Missing primary outcome data (n=4)
• Non-evaluable (n=4)
Number of participants (n=198)



Patients free of symptoms						
Nitazoxanide	0	10	54	106	125	135
Placebo	0	16	56	125	144	146

Log-rank test p= 0.277

Supplementary Appendix to the Manuscript

Early use of nitazoxanide in mild Covid-19 disease: randomized, placebo-controlled trial

Table of Contents:

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Information Technology Team: Antonio Jamisson Gonçalves Melo, Rodrigo Ramos Guimarães, José da Silva Junior.

Supplemental *In Vitro* Data to Support Clinical Trial Design

Supplemental *In Vitro* Methods

Cell lines

Vero CCL81 cells were acquired from the Banco de Células do Rio de Janeiro (BCRJ) repository. HEK293T cells were kindly supplied by Marcio C. Bajgelman (Center for Energy and Materials Research, Campinas, Brazil), and Calu-3 cells were provided by Patricia R.M. Rocco (Federal University of Rio de Janeiro, Brazil). Vero and HEK293T cell lines were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin and maintained at 37 ℃ in a 5% CO_2 atmosphere. Calu-3 cells were cultured in 1:1 DMEM F12 supplemented with 20% FBS, 1% L-glutamine and 1% penicillin/streptomycin, and maintained as described above.

Virus and preparation of viral stock

The SARS-CoV-2 strain HIAE-02 SARS-CoV-2/SP02/human/2020/BRA (GenBank accession number MT126808.1), isolated from a patient diagnosed with Covid-19 in Brazil, was kindly provided by Edison Luiz Durigon (University of São Paulo, Brazil). SARS-CoV-2 stocks were produced by passage in Vero CCL81 cells at 100% confluence using an approximate multiplicity of infection (MOI) of 0.01. Culture supernatant was harvested 36-40 h post-inoculation or on observation of 50% cytopathic effect (CPE), clarified by centrifugation, and stored at −80 ℃. Viral stock titer and identity were assessed by viral plaque assay and RT-qPCR.

Compounds

For the *in vitro* assays, the NIH Clinical Collection was obtained from the U.S. National Institutes of Health on a collaborative basis. Stock solutions were maintained at −20 ℃, (10mM concentration) in DMSO. Hit quality control was performed by UPLC-MS/MS. For high-throughput screening (HTS) and secondary assays, nitazoxanide was obtained from Sigma-

Aldrich. Tizoxanide (>90% purity by NMR) was obtained by hydrolysis of nitazoxanide using lithium hydroxide (1M) at room temperature, followed by neutralization with hydrochloric acid (1M). The hydrolysis product was filtered, washed, and dried under reduced pressure. Purified compound quality control was performed by melting point measurement, nuclear magnetic resonance (^{1}H and ^{13}C), and hyphenated ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS). Compound purity was assessed by NMR using TopSpin 3.6.2 software with trimethylsilyl propionate (TMSP-d_4, D_2O) as an internal reference, and checked against UPLC-PDA-MS data using the Bruker Data Analysis software.

SARS-CoV-2 cytopathic effect and cell viability assays

Vero CCL81 cells were dispensed in 384-well microplates in suspensions of 1700 cells per well in 45 µL of complete DMEM. Cells were incubated overnight at 37 °C/5% CO_2 for adhesion. NIH Clinical Collection stock solutions were used to prepare intermediary plates in complete DMEM at a concentration of 0.05 mM and 2% DMSO before transfer to assay plates. For SARS-CoV-2-induced cytopathic effect assay, 15 µL of compound solutions from intermediary plates were transferred to assay plates containing 45 µL of complete DMEM and cells and infected with SARS-CoV-2 in 15 µL of DMEM at a multiplicity of infection (MOI) of 0.1. For non-infected controls, the previous procedure was applied; however, the cells were mock-infected with 15 µL of DMEM only. Infected and non-infected culture plates were incubated for 60 h at 37 °C, 5% CO_2 before staining with Hoechst-33342 2µM and Mitotracker Deep Red 100nM for 45 minutes, following fixation with a 4% PFA solution in PBS.

Imaging and data processing

Plates were imaged with an Operetta automated microscope (Perkin-Elmer). Image segmentation and initial analysis were performed with the Columbus Image Data Storage and Analysis System

(Perkin-Elmer). For quantification of the SARS-CoV-2-induced cytopathic effect, one image per well was acquired using the 10× objective lens, and the number of Hoechst-33342 stained nuclei was used to determine the number of cells per well. Normalized inhibition of SARS-CoV-2-induced CPE was calculated by setting the mean values of cells in infected and non-infected control wells as 0 and 100%, respectively.

Antiviral activity assay

The antiviral activity of selected compounds was tested in 24-well plates containing either 2.5×10^5 Vero CCL81, 3.5×10^5 HEK293T cells, or 2.5×10^5 Calu-3 cells. One day (Vero or HEK) or 3 days (Calu-3) after plating, cells were infected with SARS-CoV-2 (MOI 0.01) for 1 h in 5% CO_2 at 37 °C. Virus inocula were removed and cell cultures were treated with compounds at 10 µM or 32 µM diluted in complete DMEM, or a serial dilution for concentration-response experiments. Samples were collected at 24 h post-infection (p.i.) in experiments using HEK cells and 48 h when using Vero or Calu-3 cells. Viral load was quantified by RT-qPCR and virus plaque-forming assays.

MTT assay

Cell viability experiments were identical to the antiviral activity assays, except for infection. Cell cultures were grown in 24-well plates, treated with compounds or vehicle, and incubated with the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3 hours in 5% CO_2 at 37 °C. The supernatant was removed, and tetrazolium crystals were solubilized in DMSO. Absorbance was measured in an EnSpire® Multimode Plate Reader at 490 nm. Cell culture viability data were normalized to vehicle-treated (DMSO) cell culture values and expressed as percentage relative to control.

Virus plaque-forming assay

Supernatant samples were assessed for the presence of infective SARS-CoV-2 viral particles using a plaque assay in Vero CCL81 cells. Confluent cell cultures in 24-well plates were incubated with a 10-fold serial dilution of the sample and plated at 37 °C, 5% CO_2, for 1 h. Samples were replaced with a semisolid overlay medium (1% w/v carboxymethylcellulose in DMEM supplemented with 5% FBS) for 3-4 days. The overlay medium was discarded, plates were fixed in 8% w/v paraformaldehyde, and stained with a 1% w/v methylene blue solution. Viral lysis plaques were counted, corrected by the sample dilution factors, and expressed as plaque-forming units (PFU) per mL of supernatant.

Viral RNA extraction and quantification by RT-qPCR

Cell supernatants were collected. Viral RNA extraction was performed using the PureLink RNA Mini Kit (Invitrogen), following manufacturer recommendations, and analyzed with a Nanodrop One spectrophotometer (Thermo Fisher Scientific) before use. SARS-CoV-2 RNA quantification was performed by One-step RT-qPCR according to the Charité protocol[7] using primers and probes for the E gene (forward: 5′-ACA GGT ACG TTA ATA GTT AAT AGC GT-3′, reverse: 5′-ATA TTG CAG CAG TAC GCA TAC GCA CAC A-3′, probe: 5′-6FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-QSY-3′). All reactions were assembled in a final volume of 12 µL with 3 µL of TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), 800nM and 400nM of primers and probe, respectively, and 6 µL of 100-fold diluted RNA in ultrapure water. The cycling algorithm used in this study was: 1 cycle at 50 °C for 10 minutes, 1 cycle at 95 °C for 2 minutes, followed by 45 cycles at 95 °C for 5 seconds, and 60 °C for 30 seconds in a QuantStudio3 System (Applied Biosystems). All applicable measures were taken to prevent cross-contamination of samples, and negative and positive control samples were included in all RT-qPCR plates.

For concentration-response curves, the data were normalized and fitted to the normalized log inhibitor vs. concentration-response curve [Y=100/(1+10^((LogIC50-X)*HillSlope)] in GraphPad Prism v8. Curves were plotted as mean ± SEM triplicate values of each concentration point, constituting one independent experiment. The EC_{50} and EC_{90} values (mean ± SEM) reported in this work were calculated from concentration-response curves from 5 independent experiments.

Statistical analysis

For HTS experiments, the Z factor was calculated as $Z = 1 - 3*(SD_{NI\text{-}controls} + SD_{Inf\text{-}controls})/|<NI_{controls}> - <Inf_{controls}>|$ and Spearman correlation applied for two HTS datasets in the Datawarrior software (openmolecules.org). Datasets from experiments involving viral quantification by RT-qPCR or plaque assays were analyzed using the non-parametric Kruskal-Wallis test coupled to Dunn's multiple comparison test, in which experimental groups were compared to the virus-infected, vehicle-treated control group. Data were expressed as mean + 95% confidence interval (CI). All tests were performed in GraphPad Prism v8.4.0 (GraphPad Software, La Jolla, CA, USA). Significance was established at $P < 0.05$.

Supplemental *In Vitro* Results

Nitazoxanide and tizoxanide are candidates for Covid-19 drug repurposing

We explored the repurposing potential of existing drugs for anti-SARS-CoV-2 activity and selected nitazoxanide, a broad-acting antiparasitic and antiviral compound [1, 2], as a candidate for clinical testing amongst more than 700 compounds (**Figure S1A-B**). Virology studies relevant to clinical translation were performed in preclinical infection systems using human embryonic kidney (HEK293T) (**Figure S1D-F**) and human pulmonary epithelial (Calu-3) cell

lines (**Figure S1G-I**). Both nitazoxanide and tizoxanide have specific antiviral activity against SARS-CoV-2 (**Figure S1C-I**) with an appropriate therapeutic index (**Figure S1J-L**). Notably, the concentrations of nitazoxanide and tizoxanide with *in vitro* anti-SARS-CoV-2 activity were within the concentrations attained with therapeutic dose ranges in healthy volunteers [3], implying that they are likely to achieve target concentrations to suppress SARS-CoV-2 under safe dosing conditions [4]. Beyond its well-documented preclinical antiviral activity, additional advantages of nitazoxanide as a repurposing candidate include its favorable oral bioavailability and tolerability in doses well in excess of the usual therapeutic dose range [3-5]. Nitazoxanide is available worldwide, inexpensive to produce and procure, and safe, with a vast body of clinical data accumulated in clinical trials and postmarketing experience, including over 75 million doses with no serious adverse effects reported [6].

Screening of the NIH Clinical Collection for anti-SARS-CoV-2 active compounds

To begin exploring the repurposing potential of existing drugs against SARS-CoV-2 infection, we developed a cell-based infection assay in Vero CCL81 cells scaled for image-based high-throughput screening in a 384-well microtiter plate format. The Vero CCL81 cell line derives from African green monkey kidney cells and has been commonly used as an *in vitro* model for viral infections. We used this assay to explore the potential *in vitro* antiviral activity of the NIH Clinical Collection (NCC) library, a collection of 727 FDA-approved drugs or drug-like compounds with a history of use in human clinical trials (**Figure S1A**). Each compound was screened at 10 mM for reduction of the SARS-CoV-2 cytopathic effect (CPE), which is a surrogate readout for viral infection and replication in Vero cells. The primary screen was repeated in two separate replicates (performed on separate days) for assay validation, resulting in a Z-factor > 0.6 and Spearman correlation between independent runs of 0.79 (**Figure S1B**).

Compounds were ranked on the basis of reduction of SARS-CoV-2-induced CPE (mean values from both HTS runs). Five compounds reduced CPE by more than 60% and were considered hit candidates, including nitazoxanide (which was selected for follow-up *in vitro* studies) and its active metabolite, tizoxanide.

Nitazoxanide and tizoxanide reduce SARS-CoV-2 replication *in vitro*

To examine if the anti-cytopathic effect of nitazoxanide reflected a reduction in SARS-CoV-2 replication, we developed a secondary assay in which Vero CCL81 cells were seeded in 24-well plates. Both infected and non-infected cells were treated with DMSO, nitazoxanide, or tizoxanide, added immediately after SARS-CoV-2 adsorption. SARS-CoV-2 replication was assessed by quantifying viral RNA levels (viral load) in cell-culture supernatants at 48 h post-infection using RT-qPCR. Six-point (0.1 to 32 mM) dose-response curves of nitazoxanide and tizoxanide confirmed the inhibition of SARS-CoV-2 replication in Vero CCL81 cells (**Figure S1C**). Curves were fitted to extract the EC_{50} and EC_{90} values. Nitazoxanide and tizoxanide presented comparable EC_{50} (6.1 ± 1.1 µM and 4.9 ± 3.3 µM, respectively) and EC_{90} (18.8 ± 4 and 19.0 ± 4, respectively) values against SARS-CoV-2.

Because our primary purpose was to find compounds that could proceed to clinical trials, nitazoxanide and tizoxanide were selected for further *in vitro* testing in Covid-19-relevant human cell lines. Therefore, we examined the ability of these compounds to inhibit SARS-CoV-2 replication in the human cell lines HEK-293T (embryonic and kidney-derived) and Calu-3 (epithelial and lung-derived). Cell cultures were seeded in 24-well plates, inoculated with SARS-CoV-2, and treated with nitazoxanide and tizoxanide at 10 and 32 mM, or with the vehicle DMSO. Data were obtained from RT-qPCR and virus plaque-forming assays performed with

supernatant samples, which provide information on the abundance of viral RNA and infectious viral titers, respectively. Our results indicated that nitazoxanide and tizoxanide reduced SARS-CoV-2 load in both human cell lines (**Figure S1D-I**).

Experiments performed with these compounds at 10 and 32 mM reduced viral RNA levels by at least 10-fold on average **(Figure S1D)**. The reduction in viral RNA levels in cell cultures treated with nitazoxanide or tizoxanide was accompanied by a more significant reduction in infectious SARS-CoV-2 in supernatant samples, in which nitazoxanide or tizoxanide treatment reduced viral titers by approximately 100-fold at 10 µM or to undetectable levels at 32 µM (**Figure S1E**). Representative SARS-CoV-2 plaque-forming assay images in **Figure S1F** illustrate the decrease in number of SARS-CoV-2 lysis plaques (white dots) in samples from experimental groups treated with nitazoxanide or tizoxanide in comparison to the DMSO (vehicle)-treated group. Briefly, virus lysis plaques are observed in vehicle-treated groups up to a 10^{-4} sample dilution factor, while nitazoxanide or tizoxanide treatment causes virus lysis plaques to disappear from samples diluted 10^{-2} and forward.

Following the results in the HEK293T cell line, treatment of infected Calu-3 cell cultures with nitazoxanide or tizoxanide at 10 µM reduced RNA levels by 3- to 5-fold, while treatment with 32 µM resulted in an at least 18-fold reduction (**Figure S1G**). The antiviral effect of nitazoxanide and tizoxanide was more pronounced on the infective SARS-CoV-2 load, in which treatment with either compound at 10 µM resulted in a 10- to 20-fold reduction, while treatment at 32 µM reduced viral load by approximately 1000-fold (**Figure S1H**). Representative images of the plaque-forming assays used to determine the infective viral load in experiments using Calu-3 cells illustrate the decrease in the number of viral lysis plaques in groups exposed to nitazoxanide or tizoxanide (**Figure S1I**). Samples from untreated SARS-CoV-2 infected groups

show virus lysis plaques up to the 10^{-4} dilution factor, which indicates high viral titers. Samples from groups receiving nitazoxanide or tizoxanide at 32µM show fewer lysis plaques at the lower dilution factor (10^{-1}), indicating a reduced viral titer.

Non-infected Vero CCL81, HEK293T, and Calu-3 cell culture plates were prepared in parallel to the antiviral assay plates to assess potential *in vitro* toxicity (**Figure S1J-L**). Cell cultures were incubated with nitazoxanide, tizoxanide, or DMSO for 24h or 48h (depending on the cell type) and an MTT assay was performed to assess cell culture viability. The results showed that nitazoxanide and tizoxanide caused no significant reduction in Vero CCL81 (**Figure S1J**), HEK293T (**Figure S1K**), or Calu-3 (**Figure S1L**) cell viability, even at the highest concentration tested (32 µM), in comparison to the non-treated cells. Thus, nitazoxanide and tizoxanide are not toxic to Vero CCL81, HEK293T, or Calu-3 cells at the tested concentrations.

Altogether, our results show that treatment with nitazoxanide or tizoxanide results in a significant decrease in viral RNA levels and infective viral load in cell culture, consistent with inhibition of viral replication, indicating that nitazoxanide and tizoxanide have antiviral activity against SARS-CoV-2.

Supplemental Clinical Methods

Inclusion criteria:

1. Patients with one or more of three selected symptoms of Covid-19 (fever and/or dry cough and/or fatigue) of 1 to 3 days' duration.

2. Age 18 years of age or older.

3. Willingness to take the study therapy.

4. Provision of written informed consent (by patient or a health care surrogate).

Exclusion criteria:

1. Negative result on RT-PCR for SARS-CoV-2 in a nasopharyngeal swab specimen collected at admission.
2. Inability to swallow.
3. History of severe liver disease.
4. Chronic kidney disease requiring renal replacement therapy.
5. Severe heart failure (NYHA class 3 and class 4).
6. Severe chronic obstructive pulmonary disease (COPD) (GOLD 3 and 4).
7. Any cancer in the last 5 years.
8. Any known autoimmune disease.
9. Known allergy to nitazoxanide.
10. Nitazoxanide treatment in the last 30 days.
11. Clinical suspicion of tuberculosis or bacterial pneumonia.

Additional Details on the Randomization Procedure

The trial statistician, not involved with patient enrollment or care, obtained a computer-generated randomization list (random.org). Participants were randomized (1:1 ratio) using this list to either the control arm (group B, placebo) or the intervention arm (group A, nitazoxanide). The study treatment (A or B) was revealed to the pharmacist only after patients were registered in the system, ensuring proper concealment of the allocation sequence. The designated pharmacist at each study site was the only person aware of group allocation throughout the trial.

Additional Details on Data Collection

A secure website was created by the information technology group (see Committees, Leadership, and Investigators) for data entry, validation, collection, and export. Site investigators, ACTGen monitors, and executive committee members were assigned a secure login and encrypted passwords (128-bit hash). The SARITA-2 system was built on ASP.NET MVC5 with an SQL Server database as the general system; ASP.NET MVC5 as the Web layer; DDD architecture with dependency injection and control inversion as the backend; jQuery with Bootstrap as the frontend; and SQL Server with Entity Framework (Migrations) for data entry and access. The system was hosted in Azure App Service, layer S1 (Microsoft Cloud).

Upon registration of a patient in the system, a unique trial identifier and barcode number (for laboratory tests) were generated and the patient was randomly allocated into group A or B, as mentioned above. Forms within the system were divided into sections that allowed registration of: 1 – demographic data (contact information, patient demographic data, general comments) and upload of informed consent forms; 2 – study day 1 (Baseline): symptoms, vital signs, swab collection data, and results; 3 – Patients who test positive for SARS-CoV-2 (result obtained 1-2 days after RT-PCR, returned to the health facility: clinical data, PCR results, blood test results; 4

– After 5 days of therapy: clinical information, PCR results, blood test results; and 5 – One week after completion of therapy: clinical information, when necessary.

Additional Details on Interventions

Timeline

At day 1 (baseline), a nasopharyngeal swab was collected from patients for molecular confirmation of SARS-CoV-2 infection by RT-PCR. Up to 48 hours later, RT-PCR results were displayed. If negative, patients would be excluded from the study. If positive, patients were invited to return to the study site for clinical and laboratory evaluation and initiation of treatment (nitazoxanide 500 mg or placebo, every 8 hours for 5 days, as per group allocation). Patients were given a thermometer and instructed to complete a self-administered questionnaire, which consisted of a list of symptoms and scales on which to record their intensity on each day of therapy (Table S2). Patients were also instructed to return to the study site if any adverse event occurred. One day before completion of therapy, patients received a phone call to remind them to come back to the study site on the next day for final evaluation.

Every included patient was followed according to the following data collection plan:

1. Clinical evaluation (fever, dry cough, fatigue): daily during the course of therapy (via self-administered questionnaire).

2. Nasopharyngeal swab collection for viral load assessment by RT-PCR: at baseline and 1 day after completion of the 5-day course of therapy.

3. Complete blood cell count and C-reactive protein (CRP): immediately before the first dose of study drug and 1 day after completion of the 5-day course of therapy.

4. Serum levels of selected proinflammatory mediators: immediately before the first dose of study drug and 1 day after completion of the 5-day course of therapy.

RNA extraction and real-time polymerase chain reaction (qPCR)

Nasopharyngeal swab samples obtained from each patient were collected in a single tube containing 2 mL of guanidine isothiocyanate transport solution, as previously described [7]. Extraction of the total RNA from collected specimens was performed using the QIAamp Viral RNA Mini Kit (Qiagen, USA), following manufacturer protocols. Quantitation of viral RNA was performed by reverse-transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) following the Berlin (Charité) protocol [8], using the Bio Gene Covid-19 PCR kit (Bioclin, Brazil) per manufacturer instructions. The RT-qPCR reaction was performed in a QuantStudio™ 3 or QuantStudio™ 5 Real-Time PCR System (Thermo Fisher, USA). Human RNase P mRNA was used as internal control and to correct the SARS-CoV-2 viral load in each sample by adjusting viral gene Ct values; to correct the Ct value of SARS-CoV-2 E-gene amplification of each sample, Ct values were normalized using the following equation: (sample SARS-CoV-2 E Ct value × sample RNaseP Ct value / plate mean RNaseP Ct value), as per Duchamp et al. [9] Standard curves were produced by using serial 10-fold dilutions of standard synthetic RNA transcripts of SARS-CoV-2 E gene, ranging from 2 to 2×10^5 copies/µL (Bioclin, Brazil). Absolute quantification of genomic viral load was performed by comparing sample Ct values to the standard curve. All samples were evaluated centrally at a single site (Centro de Tecnologia de Vacinas, Universidade Federal de Minas Gerais, Brazil).

Self-administered patient questionnaire

Throughout the 5-day course of therapy, patients were instructed to keep a symptom journal recording their body temperature and the presence and intensity (on a scale of 1 to 5) of dry cough, myalgia, sore throat, headache, dyspnea, diarrhea, and other symptoms if present.

Destination of blood for complete blood count and quantitation of CRP

Every study site collected blood from patients after the first positive RT-PCR result and at the end of the 5-day course of therapy. Complete blood count and CRP measurement were performed at the local laboratory of each site.

Destination of serum for quantification of proinflammatory mediators

Serum from patients (2 mL) was collected and stored in a -20 ℃ freezer at each study site. Cryotubes were labeled with the patient's unique trial identifier and the date of specimen collection. Samples were transported to the biorepository located at the Laboratory of Pulmonary Investigation, Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro. There, they were stored at -80 °C for molecular analysis of inflammatory mediators.

Molecular analysis

Serum from patients was evaluated for the following biomarkers: interleukin (IL)-6, IL-8, IL-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. All were measured with commercially available ELISA kits, following the manufacturer's recommendations (Peprotech Inc., Ribeirão Preto, São Paulo, Brazil).

Additional Details on Outcomes

This study evaluated efficacy and safety outcomes.

Primary outcomes:

1. Duration (in days) of fever and/or cough and/or fatigue in patients with confirmed Covid-19 treated with nitazoxanide or placebo.

Secondary outcomes:

1 - Evolution of viral load in nasopharyngeal swab specimens in patients with Covid-19 treated with nitazoxanide or placebo at baseline (i.e., at the time of enrollment) and 1 day after completion of the 5-day course of therapy.

2 - Hospitalization rate of patients with Covid-19 treated with nitazoxanide vs. those treated with placebo, over a 14-day period.

3 – Levels of inflammatory mediators (IL-6, IL-1β, IL-8, TNF-α, IFN-γ) in patients with Covid-19 treated with nitazoxanide vs. those treated with placebo, before the first dose of study drug and 1 day after completion of the 5-day course of therapy.

4 – Complete blood count of patients with Covid-19 treated with nitazoxanide vs. those treated with placebo, before the first dose of study drug and 1 day after completion of the 5-day course of therapy

5 - C-reactive protein (CRP) levels of patients with Covid-19 treated with nitazoxanide vs. those treated with placebo, before the first dose of study drug and 1 day after completion of the 5-day course of therapy

Safety outcomes:

1. Incidence of adverse events (AEs) throughout the study.

2. Rate of treatment discontinuation due to AEs.

All outcomes were assessed by blinded investigators. We conducted source data verification of the D8 assessment from study sites and laboratory forms for all patients at sites.

Additional Details on Changes in Protocol During Study

Initially, we planned on following patients until D8, regardless of final RT-PCR test result.

Additional Details on Sample Size Calculation

Calculation of the sample size was based on a previous study which demonstrated that 78% of Covid-19 patients in group 4 (Hospitalized without oxygen therapy), according to the WHO ordinal classification, experienced complete resolution of symptoms after receiving placebo [10]. In the present trial, patients were classified as group 2 (Symptomatic and independent), and a greater degree of recovery as measured by symptom-free days (80%) was expected even after placebo. Thus, assuming an 11% increase in symptom-free days in those patients who would receive nitazoxanide compared to placebo, we would need approximately 196 patients per experimental group, admitting a beta error of 15% and alpha error of 5%, for a total n of 392 patients.

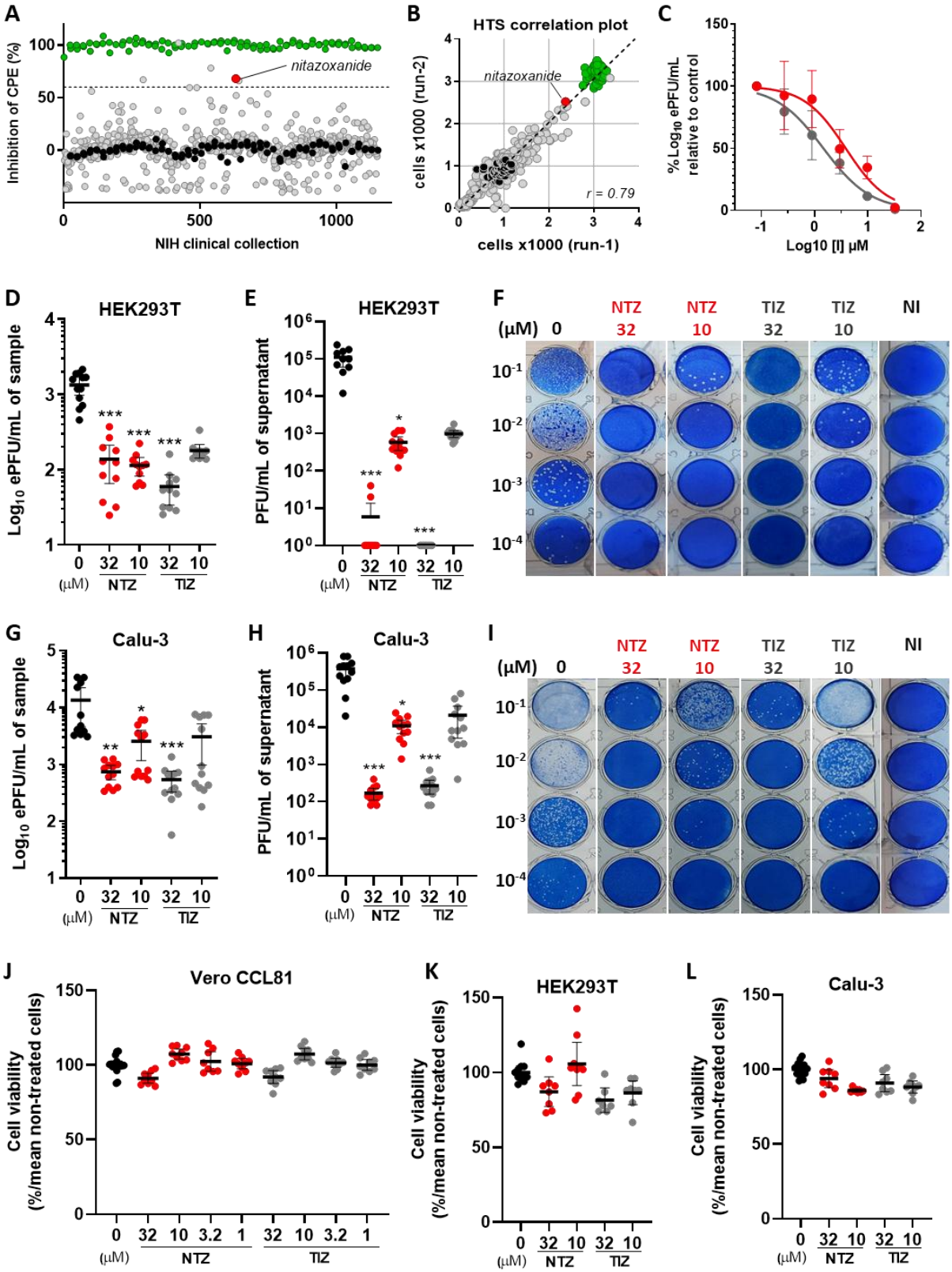
A
Inhibition of CPE (%)
nitazoxanide
NIH clinical collection
B
HTS correlation plot
nitazoxanide
r = 0.79
cells x1000 (run-2)
cells x1000 (run-1)
C
%Log10 ePFU/mL relative to control
Log10 [I] µM
D
HEK293T
Log10 ePFU/mL of sample
(µM) 0 32 10 32 10
NTZ TIZ
E
HEK293T
PFU/mL of supernatant
F
(µM) 0
NTZ 32
NTZ 10
TIZ 32
TIZ 10
NI
G
Calu-3
H
Calu-3
I
J
Vero CCL81
Cell viability (%/mean non-treated cells)
(µM) 0 32 10 3.2 1 32 10 3.2 1
NTZ TIZ
K
HEK293T
L
Calu-3

Figure S1: Nitazoxanide has antiviral activity against SARS-CoV-2 in cell culture. **(A)** Screening of the NIH Clinical Collection (NCC) for inhibitors of SARS-CoV-2-mediated cytopathic effect (CPE) in Vero CCL81 cells. The 727 NCC compounds (grey circles) were assayed and compared to non-infected (100%, green) and untreated (0%, black) controls to calculate the percentage of CPE inhibition. Compounds that inhibited CPE >60% were considered hit candidates (nitazoxanide shown in red). **(B)** Correlation plot between two independent HTS experiments. **(C)** Concentration-response curves of nitazoxanide and tizoxanide in SARS-CoV-2 infected Vero CCL81 cells (MOI 0.01). Viral load was assessed in the supernatant by RT-qPCR. **(D)** Viral load measured by RT-qPCR in supernatant samples from infected HEK293T cells treated or not with nitazoxanide or tizoxanide, at 24h post-infection (p.i.) **(E)** Infectious viral load assessed by plaque-forming assay in supernatant samples from infected HEK293T cell cultures. **(F)** Methylene blue-stained wells representative of the plaque-forming assay using HEK293T cell culture samples, which were serially diluted (10^{-1} to 10^{-4}) to visualize virus lysis plaques (white dots). **(G)** Viral load measured by RT-qPCR in supernatant samples from infected Calu-3 cells treated or not with nitazoxanide or tizoxanide, at 48 h p.i. **(H)** Infectious viral load was assessed by plaque-forming assay in supernatant samples from infected Calu-3 cell cultures. **(I)** Methylene blue-stained wells representative of viral plaque-forming assay in Calu-3 cell culture samples. which were serially diluted (10^{-1} to 10^{-4}) to visualize virus lysis plaques (white dots). Nitazoxanide and tizoxanide toxicity were assessed in **(J)** Vero CCL81, **(K)** HEK293T, or **(L)** Calu-3 cell cultures using the MTT assay. NI: non-infected; NTZ: nitazoxanide; TIZ: tizoxanide. *$p<0.05$, **$p<0.01$, ***$p<0.001$ relative to the virus-infected control group. Data in graphs are presented as mean + 95% CI. Data in **(C)** are presented as mean ± SEM and are representative of 5 independent experiments (n=9). Data in

(D, E, G, H) are representative of 2 independent experiments (n=12). Data in **(J-L)** are representative of at least 2 independent experiments (n=8-18).

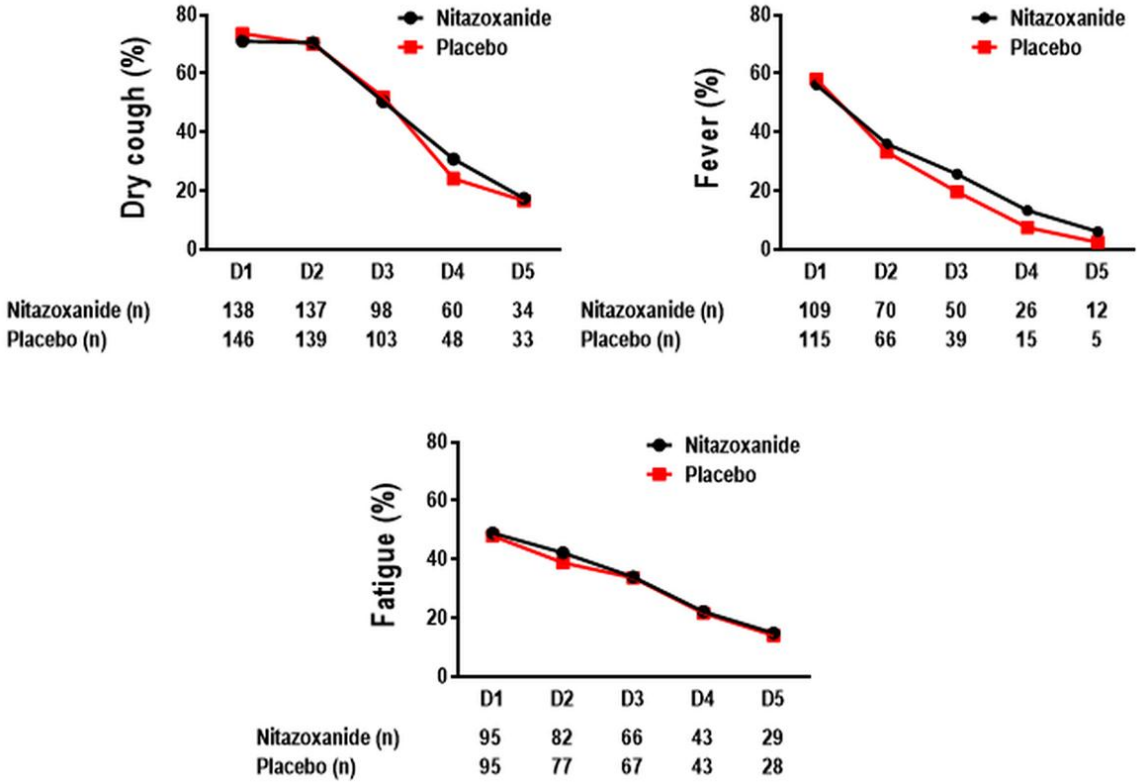

Figure S2. Time course of symptoms in patients who tested positive for SARS-CoV-2 treated with nitazoxanide (black line) and placebo (red line). Each symbol represents the percentage of patients with dry cough, fever, and fatigue during 5 days of therapy. n=absolute number of participants with each symptom at each day. Generalized estimating equation (GEE) was used to investigate the effect of time point and group on each variable. No significant differences were observed. Dry cough (p=0.879), fever (p=0.960) and fatigue (p=0.746).

Table S1. List of Sites and Number of Randomized Patients Per Site

	Site	Number of randomized patients
1	Hospital Municipal de Emergências Albert Sabin, São Caetano, São Paulo, Brazil	116
2	Hospital Municipal de Barueri Dr Francisco Moran, Barueri, São Paulo, Brazil	85
3	Hospital e Maternidade Therezinha de Jesus, Juiz de Fora, Minas Gerais, Brazil	71
4	Hospital Santa Casa de Misericórdia de Sorocaba, Sorocaba, São Paulo, Brazil	68
5	Secretaria de Estado de Saúde do Distrito Federal, Brasília, Distrito Federal, Brazil	33
6	Secretaria Municipal de Saúde de Bauru, Bauru, São Paulo, Brazil	10
7	Secretaria Municipal de Saúde de Guarulhos, Guarulhos, São Paulo, Brazil	9

Table S2 – Patient Self-Administered Clinical Questionnaire

DIÁRIO DO PACIENTE

#500VoluntariosJa #CombateCOVID19

Para que possamos estar perto de você durante o estudo clínico da NITAZOXANIDA no combate a COVID-19, iremos encaminhar, diariamente, 07 perguntas por SMS para acompanhar seus sintomas durante os próximos 05 dias.

Adicionalmente, pedimos que as mesmas informações que foram enviadas pelo SMS sejam anotadas (com um "x") aqui no seu Diário e que seja entregue no último dia na sua consulta final.

NOME: _____ CPF: _____

DATA DE CONFIRMAÇÃO POSITIVO PARA COVID-19: ____ / ____ / ____

USO DO MEDICAMENTO

500 mg de NITAZOXANIDA de 8 em 8 horas durante 5 dias consecutivos.
ATENÇÃO: Seguir instruções da embalagem do medicamento

DATA: ____ / ____ / ____ **D1**

1) Por favor, utilize o termômetro e informe a sua **temperatura** corporal?
( ) Menor que 37º ( ) 37º a 37,5º ( ) 37,6º a 38º ( ) acima de 38º

2) Qual a intensidade da sua **TOSSE SECA**?
Sendo (1) sem tosse seca e (5) tosse de intensidade insuportável.
(1) (2) (3) (4) (5)

3) Qual a intensidade da sua **DOR DE GARGANTA**?
Sendo (1) nenhuma dor e (5) dor de garganta de intensidade insuportável.
(1) (2) (3) (4) (5)

4) Qual a intensidade da sua **DOR DE CABEÇA**?
Sendo (1) nenhuma dor e (5) dor de cabeça de intensidade insuportável.
(1) (2) (3) (4) (5)

5) Qual a intensidade das suas **DORES MUSCULARES**?
Sendo (1) nenhuma dor e (5) dor de intensidade insuportável.
(1) (2) (3) (4) (5)

6) Qual a intensidade da seu **DESCONFORTO RESPIRATORIO**?
Sendo (1) nenhum desconforto e (5) desconforto de intensidade insuportável.
(1) (2) (3) (4) (5)

DATA: ____ / ____ / ____ **D2**

1) Por favor, utilize o termômetro e informe a sua **temperatura** corporal?
( ) Menor que 37º ( ) 37º a 37,5º ( ) 37,6º a 38º ( ) acima de 38º

2) Qual a intensidade da sua **TOSSE SECA**?
Sendo (1) sem tosse seca e (5) tosse de intensidade insuportável.
(1) (2) (3) (4) (5)

3) Qual a intensidade da sua **DOR DE GARGANTA**?
Sendo (1) nenhuma dor e (5) dor de garganta de intensidade insuportável.
(1) (2) (3) (4) (5)

4) Qual a intensidade da sua **DOR DE CABEÇA**?
Sendo (1) nenhuma dor e (5) dor de cabeça de intensidade insuportável.
(1) (2) (3) (4) (5)

5) Qual a intensidade das suas **DORES MUSCULARES**?
Sendo (1) nenhuma dor e (5) dor de intensidade insuportável.
(1) (2) (3) (4) (5)

6) Qual a intensidade da seu **DESCONFORTO RESPIRATORIO**?
Sendo (1) nenhum desconforto e (5) desconforto de intensidade insuportável.
(1) (2) (3) (4) (5)

7) Você apresenta **DIARREIA**?
Sendo (1) nenhum sintoma e (5) diarréia de alta intensidade.
(1) (2) (3) (4) (5)

8)
Sendo (1) nenhum sintoma e (5) diarréia de alta intensidade.
(1) (2) (3) (4) (5)

DATA: ____ / ____ / ____ **D3**

1) Por favor, utilize o termômetro e informe a sua **temperatura** corporal?
( ) Menor que 37º ( ) 37º a 37,5º ( ) 37,6º a 38º ( ) acima de 38º

2) Qual a intensidade da sua **TOSSE SECA**?
Sendo (1) sem tosse seca e (5) tosse de intensidade insuportável.
(1) (2) (3) (4) (5)

3) Qual a intensidade da sua **DOR DE GARGANTA**?
Sendo (1) nenhuma dor e (5) dor de garganta de intensidade insuportável.
(1) (2) (3) (4) (5)

4) Qual a intensidade da sua **DOR DE CABEÇA**?
Sendo (1) nenhuma dor e (5) dor de cabeça de intensidade insuportável.
(1) (2) (3) (4) (5)

5) Qual a intensidade das suas **DORES MUSCULARES**?
Sendo (1) nenhuma dor e (5) dor de intensidade insuportável.
(1) (2) (3) (4) (5)

6) Qual a intensidade da seu **DESCONFORTO RESPIRATORIO**?
Sendo (1) nenhum desconforto e (5) desconforto de intensidade insuportável.
(1) (2) (3) (4) (5)

7) Você apresenta **DIARREIA**?
Sendo (1) nenhum sintoma e (5) diarréia de alta intensidade.
(1) (2) (3) (4) (5)

DATA: ____ / ____ / ____ **D4**

1) Por favor, utilize o termômetro e informe a sua **temperatura** corporal?
( ) Menor que 37º ( ) 37º a 37,5º ( ) 37,6º a 38º ( ) acima de 38º

2) Qual a intensidade da sua **TOSSE SECA**?
Sendo (1) sem tosse seca e (5) tosse de intensidade insuportável.
(1) (2) (3) (4) (5)

3) Qual a intensidade da sua **DOR DE GARGANTA**?
Sendo (1) nenhuma dor e (5) dor de garganta de intensidade insuportável.
(1) (2) (3) (4) (5)

4) Qual a intensidade da sua **DOR DE CABEÇA**?
Sendo (1) nenhuma dor e (5) dor de cabeça de intensidade insuportável.
(1) (2) (3) (4) (5)

5) Qual a intensidade das suas **DORES MUSCULARES**?
Sendo (1) nenhuma dor e (5) dor de intensidade insuportável.
(1) (2) (3) (4) (5)

6) Qual a intensidade da seu **DESCONFORTO RESPIRATORIO**?
Sendo (1) nenhum desconforto e (5) desconforto de intensidade insuportável.
(1) (2) (3) (4) (5)

7) Você apresenta **DIARREIA**?
Sendo (1) nenhum sintoma e (5) diarréia de alta intensidade.
(1) (2) (3) (4) (5)

DATA: ____ / ____ / ____ **D5**

1) Por favor, utilize o termômetro e informe a sua **temperatura** corporal?

( ) Menor que 37º ( ) 37º a 37,5º ( ) 37,6º a 38º ( ) acima de 38º

2) Qual a intensidade da sua **TOSSE SECA**?
Sendo (1) sem tosse seca e (5) tosse de intensidade insuportável.
(1) (2) (3) (4) (5)

3) Qual a intensidade da sua **DOR DE GARGANTA**?
Sendo (1) nenhuma dor e (5) dor de garganta de intensidade insuportável.
(1) (2) (3) (4) (5)

4) Qual a intensidade da sua **DOR DE CABEÇA**?
Sendo (1) nenhuma dor e (5) dor de cabeça de intensidade insuportável.
(1) (2) (3) (4) (5)

5) Qual a intensidade das suas **DORES MUSCULARES**?
Sendo (1) nenhuma dor e (5) dor de intensidade insuportável.
(1) (2) (3) (4) (5)

6) Qual a intensidade da seu **DESCONFORTO RESPIRATORIO**?
Sendo (1) nenhum desconforto e (5) desconforto de intensidade insuportável.
(1) (2) (3) (4) (5)

7) Você apresenta **DIARREIA**?
Sendo (1) nenhum sintoma e (5) diarréia de alta intensidade.
(1) (2) (3) (4) (5)

Table S3. Detailed Profile of Hospitalized Patients

	Arm	Hospitalization setting	Day of treatment initiation	Days elapsed between treatment initiation and hospitalization
1	Nitazoxanide	General ward	07/16/2020	2 days
2	Nitazoxanide	ICU	07/23/2020	1 day
3	Nitazoxanide	General ward	07/05/2020	1 day
4	Nitazoxanide	General ward	07/11/2020	1 day
5	Nitazoxanide	ICU	07/24/2020	4 days
6	Placebo	General ward	07/11/2020	Never took study drug
7	Placebo	General ward	07/11/2020	1 day
8	Placebo	General ward	07/22/2020	Never took study drug
9	Placebo	General ward	08/06/2020	4 days
10	Placebo	General ward	08/02/2020	5 days

ICU, intensive care unit.

Table S4. Additional Characteristics of the Population at Baseline

	Overall (392)	Nitazoxanide (194)	Placebo (198)	*p* value
Age in years, median (IQR)	37 (29-45)	37 (28-45)	37 (29-45)	0.772
Previous use of medications, n (%)				
Steroids	23 (5.9%)	12 (6.2%)	11 (5.6%)	0.833
NSAIDs	8 (2.0%)	5 (2.6%)	3 (1.5%)	0.499
Azithromycin	39 (9.9%)	19 (9.8%)	20 (10.1%)	1.000
Ivermectin	8 (2.0%)	5 (2.6%)	3 (1.5%)	0.499
Vital signs, mean (SD)				
Temperature, °C	36.4 (0.5)	36.4 (0.6)	36.4 (0.5)	0.494
Systolic blood pressure, mmHg	127.6 (14.8)	126.5 (14.1)	128.8 (15.4)	0.159
Diastolic blood pressure, mmHg	81.7 (11.5)	81.9 (11.8)	81.5 (11.3)	0.591
Heart rate, bpm	85.1 (13.0)	85.9 (12.5)	84.3 (13.4)	0.181
Respiratory rate, bpm	18.4 (1.8)	18.5 (1.9)	18.3 (1.7)	0.509

IQR, interquartile range; NSAIDs, non-steroidal anti-inflammatory drugs; SD, standard deviation

Table S5. Adverse Events

Adverse event	Total (n=392)		Group			
			A = Nitazoxanide (n=194)		B = Placebo (n=198)	
	n	%	n	%	N	%
Headache						
None	326	83.2	160	82.5	166	83.8
Mild	50	12.8	25	12.9	25	12.6
Moderate	11	2.8	8	4.1	3	1.5
Severe	4	1.0	1	0.5	3	1.5
Diarrhea						
None	287	73.2	138	71.1	149	75.3
Mild	68	17.3	36	18.6	32	16.2
Moderate	35	8.9	19	9.8	16	8.1
Severe	2	0.5	1	0.5	1	0.5
Nausea						
None	335	85.5	166	85.6	169	85.4
Mild	41	10.5	19	9.8	22	11.1
Moderate	14	3.6	8	4.1	6	3.0
Severe	2	0.5	1	0.5	1	0.5
Vomiting						
None	381	97.2	186	95.9	195	98.5
Mild	9	2.3	6	3.1	3	1.5
Moderate	2	0.5	2	1.0	0	0
Severe	0	0	0	0	0	0
Anorexia						
None	388	99.0	191	98.5	197	99.5
Mild	3	0.8	2	1.0	1	0.5
Moderate	1	0.3	1	0.5	0	0
Severe	0	0	0	0	0	0
Pruritus						
None	387	98.7	190	97.9	197	99.5
Mild	3	0.8	3	1.5	0	0
Moderate	1	0.3	1	0.5	0	0
Severe	1	0.3	0	0	1	0.5
Urticaria						
None	388	99.0	193	99.5	195	98.5
Mild	4	1.0	1	0.5	3	1.5
Moderate	0	0	0	0	0	0
Severe	0	0	0	0	0	0

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