Title: *N*-chlorotaurine, a novel inhaled virucidal antiseptic is highly active against respiratory viruses including SARS-CoV-2 (COVID-19)

Authors:

Michaela Lackner[1], Annika Rössler[2], André Volland[2], Marlena Stadtmüller[3], Brigitte Müllauer[2], Zoltan Banki[2], Johannes Ströhle[1], Angela Luttick[4], Jennifer Fenner[4], Heribert Stoiber[2], Dorothee von Laer[2], Thorsten Wolff[3],Carsten Schwarz[5], Markus Nagl[1]*

[1]Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria

[2]Institute of Virology, Medical University of Innsbruck, Innsbruck, Austria

[3]Unit 17-Influenza and Other Respiratory Viruses, Robert Koch-Institute, Berlin, Germany.

[4]360biolabs Pty Ltd, Melbourne, Australia

[5]Christiane Herzog Zentrum Berlin/Charité-Universitätsmedizin Berlin, Berlin, Germany

Co-senior-authors DvL, TW, CS, MN

*Corresponding author:

Markus Nagl, Assoc.Prof., MD

Institute of Hygiene and Medical Microbiology

Medical University of Innsbruck

Schöpfstr. 41

A-6020 Innsbruck / Austria

Tel. +43-(0)512-9003-70708

Fax +43-(0)512-9003-73700

E-mail: m.nagl@i-med.ac.at

ORCID: 0000-0002-1225-9349

Abstract

N-chlorotaurine (NCT) is a long-lived oxidant generated in activated cells of the innate immune system, namely neutrophilic and eosinophilic granulocytes and monocytes. NCT acts as an antiseptic agent that can be synthesized chemically and applied topically on different infected body sites. Even treatment of the lower respiratory tract via inhalation, which has been in development in the last years, was well tolerated in a recent phase I clinical trial. In this study, we demonstrate the activity of NCT against viruses causing acute respiratory tract infections, in fact severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenza viruses, and respiratory syncytial virus.

NCT revealed broad virucidal activity against all viruses tested. In the presence of organic proteinaceous material simulating body fluids, this activity was enhanced by transchlorination mechanisms so that significant inactivation of viruses could be achieved within 1 – 10 minutes.

Inhalation of 1.0% NCT as a prophylactic and therapeutic strategy against acute viral respiratory tract infections deserves comprehensive clinical investigation.

Introductory paragraph

Background

The coronavirus disease 2019 (COVID-19) pandemic is the major challenge of humanity presently. No highly effective treatment or a vaccine is available so far. Application of *N*-chlorotaurine (Cl-NH-CH_2-CH_2-SO_3Na, NCT), a safe, well tolerated, endogenous, mild antiseptic with anti-inflammatory properties may be a significant step forward to combat COVID-19 and other viral respiratory tract infections. NCT as an inhaled anti-infective has already demonstrated broad-spectrum microbicidal activity against bacteria, fungi, viruses and protozoa. Here, we aimed to establish the virucidal activity of NCT against three main viruses responsible for lower respiratory tract infections, namely severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenza A virus, and respiratory syncytial viruses (RSV).

The COVID-19 pandemic is caused by SARS-CoV-2. The pandemic is affecting individuals, populations, and health systems far beyond infection. The virus might persist globally and become a prolonged or permanent threat [1,2]. Up to date, there is no breakthrough regarding a highly sufficient and well tolerated prophylaxis, vaccine or therapy. The race for a cure is a global effort and different approaches have been developed and are currently studied [1,3]. Another major public concern is posed by influenza viruses, which annually cause 3 – 5 million cases of severe illness and about 290 000 to 650 000 of death worldwide [4]. Protection by the yearly influenza virus vaccine is unsatisfactory and resistance against existing antiviral drugs develops rapidly [5]. Therefore, new tools to combat influenza viruses are urgently needed.

One less known intervention is inhalation therapy with antiviral agents. An appeal for the inhaled route of administration has been published recently [6]. A first advantage is direct delivery of a high concentration of the medication to the lung, where the virus causes most of the severe problems [7]. Furthermore, topically applied therapies that are not systemically distributed avoid interactions with systemic medications, which are frequently necessary in

elderly or multimorbid patients who are particularly at risk for severe COVID-19 complications [8]. An ideal inhaled drug should have broad-spectrum antimicrobial activity to cover not only SARS-CoV-2, but also co-infections and superinfections with other respiratory viruses and microorganisms (bacteria and fungi) [9-11]. Antiviral drugs are often specific to distinct viruses, but identifying the virus causing an infection requires logistic and diagnostic efforts, which in the case of SARS-CoV-2 amounts to at best one to two days for a diagnosis[12]. Such an ideal inhaled broad-spectrum drug mentioned above could be applied instantly regardless of the pathogen causing the respiratory illness and would thus eliminate the need for time-consuming diagnostics. Another key requirement is anti-inflammatory activity of the compound to downregulate the 'cytokine storm', particularly for SARS-CoV-2, which causes hyper-inflammation in severe cases [13].

One molecule that fulfils the criteria of broad-spectrum antimicrobial (virucidal, bactericidal, fungicidal, protozoocidal) and anti-inflammatory activity [14,15], and good tolerability upon inhalation is *N*-chlorotaurine (Cl-NH-CH_2-CH_2-SO_3^-) [16]. It is known since the 1970's as a product of activated human granulocytes and monocytes and belongs to the long-lived oxidants and chloramines formed by the myeloperoxidase via hypochlorous acid to combat invading pathogens [17-19] (Fig. 1). Moreover, *N*-chlorotaurine is thought to be involved in the control of inflammation by downregulating of nuclear factor kappaB activation, chemokines and proinflammatory cytokines such as tumor necrosis factor alpha, some prostaglandins and interleukins like IL-6 [15,20,21]. The synthesis of the sodium salt of *N*-chlorotaurine (Cl-NH-CH_2-CH_2-SO_3Na, NCT) was successful in our laboratory [22], which enabled its development as an endogenous anti-infective and mild antiseptic in human medicine. As an active chlorine compound belonging to the class of chloramines, it has the typical broad-spectrum microbicidal activity without development of resistance against Gram-positive and Gram-negative bacteria including multi-resistant strains, yeasts and moulds, protozoa, and worm larvae (for review see [14,23,24]). Broad-spectrum activity was found against adenoviruses [25-27], herpes viruses 1 and 2 [26,27], human immunodeficiency virus [28], and it was shown *in vivo* against adeno and herpes viruses in epidemic keratoconjunctivitis up to a

phase II study as well as in herpes zoster in a case report, respectively [29-31]. Activity against coxsackievirus A24 and enterovirus 70 was found by the NCT-derivative *N,N*-dichloro-dimethyltaurine *in vitro* [32].

In the last years, inhalation of NCT has been investigated and developed in detail. Enhanced bactericidal and fungicidal activity has been found in the presence of lung epithelial cells [33]. Tolerability of repeatedly inhaled NCT has been confirmed in the normal lung and in a streptoccoccal inflammation model each in pigs, and in the normal lung of mice [34-36]. In humans, tolerability was confirmed in a placebo-controlled phase I clinical study [16]. Only minor and transient adverse effects were found, i.e. chlorine taste and occasional tickle in the throat [16]. NCT is not distributed systemically, which explains the absence of systemic adverse effects.

A safe, well tolerated, endogenous, inhaled substance with broad-spectrum activity against pathogens supported by anti-inflammatory properties may be a significant step forward for treatment of COVID-19 and other viral infections of the lower airways without the need of further diagnostics to discriminate between the infectious agents. In this regard, the aim of the present study was to establish and characterize the virucidal activity of NCT against three major viruses responsible for respiratory infections in humans, namely SARS-CoV-2, influenza viruses, and RSV *in vitro*.

Results

NCT was incubated with SARS-CoV-2, influenza A virus or RSV, followed by assessment of virus inactivation using various readouts. NCT at a clinically relevant concentration of 0.1% - 1.0% demonstrated virucidal activity against SARS-CoV-2 (SARS-CoV-2 BavPat1, hCoV-19/Australia/VIC01/2020, clinical isolate 1.2 Innsbruck), influenza A virus, and RSV (RSV long strain). Longer NCT-exposure periods were required to inactivate SARS-CoV-2 than to inactivate influenza viruses or RSV. In the presence of organic matter, inactivation of viruses was even enhanced so that a significant reduction of plaque forming units and infected cells,

respectively, could be observed already after 5 min with SARS-CoV-2 by 1.0% NCT. Controls without NCT and specific inactivation controls showed full viral replication in all cases to warrant valid results. Detailed results are presented in the following paragraphs.

Virucidal activity of NCT against SARS-CoV-2

Inactivation of SARS-CoV-2 was assessed by incubating stock virus with NCT for indicated time periods at 37°C and then determining the remaining infectious particles using plaque assay or immunostaining as well as determining virus inactivation via RT-qPCR or TCID50. Exact incubation times of virus with NCT were ensured by adding met/his at the end of the incubation period, which inactivates NCT. All assays demonstrated a significant inactivation of SARS-CoV-2 with slight differences according to the individual test method and strain used. With plaque assay readout, a significant reduction in infectious particles was detected after 15 min of incubation, when incubating SARS-CoV-2 with NCT in a buffered aqueous solution (Fig. 2a). The mild oxidizing activity of the test antiseptic may explain why it took as long as 15 min to reduce infectious particles. In the presence of Vero cells (Fig. 2b) or particularly 5.0% peptone (Fig. 2a), however, a significant reduction of infectious virus particles occurred already after 5 min of incubation with 1.0% NCT. This remarkable enhancement of activity by organic load is typical for NCT and shown for viruses for the first time here and is explained most likely by transhalogenation (see discussion).

Virus inactivation assays with immunostaining readout showed a 50% reduction of infected cells after 1 min (not significant, $p = 0.085$), 20 – 80% reduction after 5 min ($p = 0.0102$), 81 – 91% after 7 min ($p < 0.01$), 81 – 97% after 10 min, 96 – 99% after 20 min, and > 99% after 30 min ($p < 0.0001$ for these values). A logarithmic scale with respective statistics is provided in Fig. 2c. The results found by RT-qPCR assay were similar with a highly significant reduction of genome copies (Fig. 2d). This was further confirmed by the TCID50 readout (Fig. 2e).

The antiviral activity was concentration-dependent. Inactivation controls demonstrated full inactivation of 1.0% NCT by 1.0% methionine / 1.0% histidine (met/his). This was valid for all tests and viruses in this study. Absence of cytotoxicity of inactivated NCT to the inoculated cell culture was proved by MTT testing with values of MTT reduction of 94.1 ± 8.5 (0.1% NCT plus 0.1% met/his) and 100.3 ± 5.6 (PBS control) ($p = 0.12$ by Student's unpaired t-test).

Virucidal activity of NCT against influenza viruses

Inactivation of influenza viruses was assessed like inactivation of SARS-CoV-2 by incubating stock virus with NCT for indicated time periods at 37°C and then determining the remaining infectious particles using plaque assay.

Virus inactivation as determined by plaque assay readout demonstrated an even faster inactivation of influenza viruses by NCT compared to SARS-CoV-2. All tested virus strains were inactivated rapidly with a 2 $\log_{10}$ reduction of the H3N2 virus within 5 min (Fig. 3a) and a 6 $\log_{10}$ reduction of H1N1 and H1N1pdm viruses within 1 min by 1.0% NCT (Fig. 3b and 3c). In general, H1N1 and H1N1pdm viruses were more susceptible than the H3N2 virus.

Addition of ammonium chloride (NH_4Cl) to NCT significantly enhanced its activity against influenza viruses (Fig. 3d). Ammonium chloride alone and the inactivation control with 0.1% NCT plus 0.1% ammonium chloride showed no antiviral effect at least up to 10 min incubation time.

Virucidal activity of NCT against RSV

Inactivation of RSV was assessed like inactivation of SARS-CoV-2 and influenza A viruses. As with SARS-CoV-2 and influenza A virus, inactivation of RSV as determined by plaque assay readout demonstrated a significant reduction of PFU/ml by NCT compared to mock treated controls. The incubation of RSV with 1.0% NCT resulted in a rapid drop of infectious virus titre with 4 $\log_{10}$ decrease within 5 minutes (Fig. 4). Almost no detectable amount of infectious RSV was measurable after 15 minutes. In the presence of 0.1% NCT, RSV titres

dropped in a time- and concentration-dependent manner reaching significant titre reduction after 15 minutes (Fig. 4).

Discussion

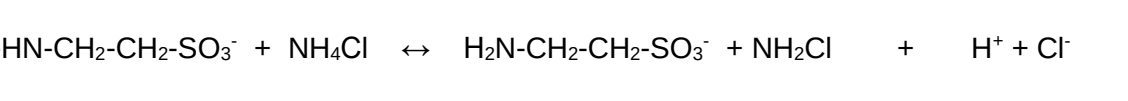
Safe, well tolerated, affordable, and effective medications are urgently needed against COVID-19 and would be beneficial for treatment of viral bronchopneumonia caused by other viruses such as influenza and RSV. As an endogenous mild long-lived oxidant [17], inhaled NCT has been demonstrated to be well tolerated and safe in animals (pigs and mice) and in a clinical phase I study in humans [16,34-36]. As an active chlorine compound belonging to the class of chloramines, it has broad-spectrum activity against pathogens without occurrence of resistance because of the oxidizing mechanism of activity with thio- and amino-groups as the main targets [14,22,23].

Actually, in the present study NCT had clear virucidal activity against three enveloped RNA viruses highly relevant for infections of the bronchopulmonary system. Depending on the NCT-concentration and test conditions, a rapid reduction of the number of infectious virus particles by several powers of ten within 1 - 10 minutes is achieved. Influenza A viruses of pre-pandemic and pandemic H1N1 subtype (H1N1 and H1N1pdm) were the most sensitive ones with reduction to the detection limit by 1.0% NCT within 1 min, followed by RSV, influenza (H3N2), and SARS-CoV-2. These differences can be explained by individual dynamics of oxidation and chlorination of proteins of the viral surface, and of penetration of NCT and attack on the viral nucleocapsid proteins. All these target sites have been shown with the NCT analogue *N,N*-dichloro-2,2-dimethyltaurine in adenovirus type 5 [37]. Thereby, chlorination of the surface proteins is the first step [38], which can be assumed to impact their function and therefore the attachment of viruses to body cells. Oxidation and chlorination of virulence factors of different pathogens by NCT and analogue chloramines with the consequence of their inactivation has been also shown for shigatoxin of *Escherichia coli* [39], several toxins of *Staphylococcus aureus* [40], aspartyl proteinases of *Candida spp*. and gliotoxin of *Aspergillus fumigatus* [41,42]. This indicates that inactivation of key proteins of all

kinds of pathogens is a central principle of the antimicrobial action of NCT and may underline such a function in innate immunity besides its anti-inflammatory one [15,20,23].

Accordingly, NCT has not only virucidal activity against enveloped viruses (herpes virus type 1 and 2 [26,27], human immunodeficiency virus 1 [28], and the viruses of the present study), but also non-enveloped ones. From the latter, a panel of adenoviruses has been tested mainly due to their importance in epidemic keratoconjunctivitis [25,27,30]. Similar to other active halogen compounds and other antiseptics such as tensidic compounds [43], adenoviruses are slightly less sensitive to NCT than the enveloped viruses [27]. Nevertheless, efficacy of NCT *in vivo* against adenoviruses in epidemic keratoconjunctivitis has been proven in the New Zealand White rabbit ocular model and in a phase II study in humans [30,31]. Application of NCT had a curative effect in a patient suffering from therapy-refractory herpes zoster infection in the upper thoracic area [29].

It must be taken into account that organic substances are omnipresent *in vivo* (in all human body fluids and tissues), and therefore we performed a part of the inactivation assays in the presence of organic matter as well. The results of Fig. 2a clearly show an enhancement of the virucidal activity of NCT in the presence of 5.0% peptone, which in the first view appears surprising since active chlorine compounds underlie a decrease of their oxidation capacity by chlorine-reducing substances of such organic load [23,44,45]. With NCT as a low-reactive chloramine compound, however, transchlorination as one of the reaction mechanisms becomes important [14,23]. Thereby, amongst others, monochloramine (NH_2Cl) is formed in equilibrium from NCT and ammonium chloride [14,17].



Monochloramine is more lipophilic than NCT and penetrates microorganisms more easily, which leads to enhanced inactivation by the reaction just mentioned [17,46]. The stronger activity of NCT in the presence of fluids containing proteinaceous material is a general

244 principle observed in different compositions, such as artificial sputum medium, different body
245 fluids, peptone, and plasma for bacteria and fungi (for review see [14,24,47]). In the present
246 study, it has been confirmed for viruses for the first time, too. The discrepancy between the
247 incubation time of 15 min (Fig. 2a) and of 10 min or less (Fig. 2b-e) needed for a significant
248 viral reduction in buffer solution in different tests may be explained by the presence of 1.0%
249 FCS in the tests depicted in Fig. 2c-e and organic matter in the presence of Vero cells in Fig.
250 2b. In agreement with these results, enhancement of the bactericidal and fungicidal activity of
251 NCT in the presence of different lung epithelial cells was observed recently [33].

252 Enhancement of antimicrobial and antiviral activity by organic material is of practical
253 relevance for topical treatment of infections with NCT, for instance bronchopulmonary ones.
254 The concentration of active chlorine after the end of an inhalation of 1.0% NCT decreases to
255 traces within 1 min and vanishes completely after further 10 min [16]. Inhalation for 10 min is
256 feasible and well tolerated, and within this time an impact on SARS-CoV-2 and on other
257 viruses can be expected *in vivo*, too, but remains to be evaluated in respective clinical
258 studies.

259 Also of practical relevance is the fact that NCT has broad-spectrum activity against
260 viruses, including important representatives relevant for bronchopulmonary infections
261 (SARS-CoV-2, influenza viruses, RSV). Topical treatment of all these virus infections by
262 inhaled NCT without the necessity of a diagnosis of the specific virus at hand is conceivable
263 and should urgently be investigated in clinical studies. Notably, the activity of NCT against
264 bacteria and fungi, including multi-resistant ones, may prevent super- and secondary
265 infections, which are a considerable problem in COVID-19 patients as well [9-11]. In addition,
266 the anti-inflammatory activity of NCT might have the potential to influence the aggressive
267 inflammatory response by downregulating the ‘cytokine storm’ and prevent airway damage in
268 severe ill patients with SARS-CoV-2 infection.

269 Further advantages of NCT would be high safety and high tolerability by human tissue [14],
270 absence of systemic absorption, of systemic adverse effects [16], of systemic interaction with

other medications, and of resistance development because of the oxidizing and chlorinating mechanism of action [14,23].

Conclusions

NCT demonstrated rapid activity against SARS-CoV-2, influenza A viruses, and RSV at a therapeutic concentration of 1.0% that can be safely inhaled. The activity is enhanced by an organic environment, which is omnipresent in human body fluids and tissues *in vivo*. Clinical efficacy of NCT in viral bronchopulmonary infections should be investigated in respective clinical studies.

Methods

Reagents

N-chlorotaurine sodium salt (NCT, molecular weight 181.57 g/l, lot 2020-03-17) was prepared in pharmaceutical quality as established at our Department and frozen at minus 20°C for storage [22]. For testing, it was freshly dissolved in phosphate-buffered saline (PBS) at pH 7.1 (7.0 – 7.2) to desired stock concentrations between 1.0% (55.08 mM) and 10%. As inactivation solution for NCT, a mixture of 1.0% methionine and 1.0% histidine (met/his, L-methionine and L-histidine, both from Carl Roth GmbH, Karlsruhe, Germany) in distilled water was used [48]. For tests in peptone, peptone enzymatic digest from Casein was applied (Fluka no. 82303, Sigma-Aldrich GmbH, Buchs, Switzerland). RPMI-1640 medium and fetal calf serum (FCS) were from Sigma-Aldrich GmbH, too.

Viruses, virus cell culture and preparation of viral suspensions

SARS-CoV-2

Robert Koch-Institute, Berlin (RKI)

SARS-CoV-2 BavPat1 strain was obtained from Christian Drosten's laboratory at the Institute of Virology at Charité Universitätsmedizin Berlin. Vero E6 cells were maintained in DMEM (supplemented with 10% FCS, 2 mM L-glutamine, non-essential amino acids, 1mM sodium pyruvate, 100 mg/ml streptomycin and 100 units/ml penicillin). For virus stock preparation, Vero E6 monolayer cultures grown in 75 cm^2 cell culture flasks were infected with a multiplicity of infection (MOI) of 0.01 in PBS (supplemented with 0.3% BA) for 2 days at 37°C and 5.0% CO_2. The supernatant was harvested and stored at minus 80°C until use.

Biolabs, Melbourne (Biolabs)

COVID-19 strain used was SARS-CoV-2 hCoV-19/Australia/VIC01/2020 (Melbourne's Peter Doherty Institute for Infection and Immunity, Melbourne, Australia). Parent stock of the virus was passaged twice in Vero cells. A working stock was generated at 360biolabs by two further passages in Vero cells in virus growth media, which comprised Minimal Essential Medium without L-glutamine supplemented with 1.0% (w/v) L-glutamine 1.0 µg/ml of TPCK-Trypsin, 0.2% BSA, 1 x Pen/Strep, and 1.0% Insulin Transferrin Selenium (ITS), then a further 2 passages in Vero E6 cells in growth media. This growth media comprised MEM supplemented with 1.0% (w/v) L-glutamine, 4.0 µg/ml of TPCK-Trypsin and 2.0% (v/v) heat inactivated FBS.

African Green Monkey Kidney (Vero E6) cells (ATCC-CRL1586) were sub-cultured to generate cell bank stocks in cell growth medium, which comprised Minimal Essential Medium without L-glutamine supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum and 1.0% (w/v) L-glutamine. Cell stocks were frozen at minus 80°C overnight and then transferred to liquid nitrogen. Vero E6 cells were passaged for a maximum of 13 passages, after which a new working cell bank stock was retrieved from liquid nitrogen for further use. Vero E6 cells were seeded into 96-well plates at 2 x 10^4 cells / well in 100 µl E6 seeding

media (Minimal Essential Medium supplemented with 1.0% (w/v) L-glutamine, 2.0% FBS). Plates were incubated overnight at 37°C, 5.0% CO_2.

Institute of Virology, Innsbruck

SARS-CoV-2 Isolate 1.2 was a clinical isolate from a patients' respiratory swab sample in Innsbruck, Austria. Virus stocks were produced on Vero/TMPRSS2 cells, kindly provided by Dr. Markus Hoffmann and Prof. Stefan Pöhlmann, Leibniz Institute for Primate Research, Göttingen, Germany [49]. Cells were cultured in DMEM plus 10% FCS and Pen/Strep. For Virus stock production, 80% confluent Vero/TMPRSS2 cells were infected with a MOI of 0.01 in DMEM plus 2.0% FCS. The supernatant was harvested 60 h post infection. Virus aliquots were stored at minus 80°C.

Influenza

Robert Koch-Institute, Berlin (RKI)

Influenza A/Panama/2007/1999 (H3N2) virus was grown in the allantois cavity of 11 day old embryonated chicken eggs for 2 days. Virus was harvested, clarified by centrifugation (300xg, 10 min) and stored at minus 80°C until use. Madin-Darby-Canine-Kidney (MDCK) II cells (ATCC) were maintained in MEM (supplemented with 10% FCS, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 units/ml penicillin) at 37°C and 5.0% CO2.

Institute of Hygiene and Medical Microbiology, Innsbruck

Influenza A/Singapore/Hongkong/2339/2000 (H1N1) was kindly provided by H. Katinger, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna, Austria. Influenza A/Swine Origin Virus (S-OIV)/California/2009 (H1N1pdm) was a clinical isolate from Innsbruck, Austria.

Influenza viruses were grown on MDCK cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald,

Germany). MDCK cells were grown in 25 cm^2 cell culture flasks (Sarstedt, Inc. Newton, NC, USA) in RPMI plus 10% FCS to a monolayer. The medium was replaced by 5 ml RPMI without FCS, and 10 µl of 1 mg/ml trypsin (final concentration 0.002 mg/ml) was added to activate neuraminidase. Viral suspension deep frozen at minus 80°C in RPMI (200 µl) was added. After 60 h of incubation at 37°C, a cytopathic effect was seen in all cells, and the supernatant was taken and centrifuged at 275 × g. The supernatant again was used as viral suspension for the tests.

RSV

RSV long strain, (kindly provided by T. Grunwald, Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany), was generated by infection of HEp2 cells at low MOI as described previously [50]. Virus titers were determined in plaque assays by infection of HEp2 with serial dilutions of the virus followed by immunocytochemical staining with polyclonal goat antibody against RSV (Gt X RSV, Merck) and HRP-conjugated rabbit polyclonal anti-goat IgG (Novusbio). 3-Amino-9-ethylcarbazole (AEC, Sigma) was used as a chromogen in immunohistochemistry to visualize the RSV infected cells.

Virus inactivation tests (quantitative killing assays) with NCT

General overview of the test method

The viral suspension was mixed with NCT (final concentration 0.1% to 1.0%) and incubated at 37°C for 1, 5, 7, 10, 15, 20, 30, 45, and 60 min. At the end of each incubation time, aliquots were removed and diluted in 1.0% met/his to inactivate NCT and to warrant exact incubation times. Virus inactivation was assessed by subsequent plaque assay, immunostaining or RT-qPCR as detailed below.

Controls were done in PBS or PBS with 5.0% peptone without NCT in parallel as well as inactivation controls. For the latter, 1.0% NCT was mixed with met/his before the addition of

the respective virus. The virus must survive in the inactivation solution to obtain reliable results, which was the case in all tests.

SARS-CoV-2

Virus inactivation assay with plaque assay readout (RKI Berlin)

Three µl of concentrated virus suspension (4.76×10^9 PFU/ml) were added to 400 µl of NCT (1.0% or 0.1% in PBS), NCT with peptone (1.0% NCT, 5.0% peptone in PBS) or PBS and incubated at 37°C. After 5, 15, 30 and 60 min, 100 µl were removed and added to 100 µl of met/his. As inactivation control, 100 µl of 1.0% NCT or 1.0% NCT with 5.0% peptone were added to 100 µl of met/his and thereafter 0.75 µl of virus suspension were added. Infectious virus particles in all suspensions were determined with plaque assay. Briefly, a serial tenfold dilution of the virus suspension in PBS with 0.3% bovine albumin was added to confluent Vero E6 cells in 12-well plates, which were washed with PBS immediately before. After incubation at 37°C for 1h, the inoculum was removed followed by a washing step with PBS. Avicel-overlay medium (double-strength DMEM supplemented with 10% FCS, 1.0% DEAE dextran, 5.0% sodium bicarbonate and 1.25% Avicel) was added and plates incubated at 37°C and 5.0% CO_2 for 3 days before staining with crystal violet for visualization of plaques. Counted plaques are expressed as plaque forming units per ml (PFU/ml).

Virus inactivation assay with plaque assay readout (GLP lab 360 biolabs, Melbourne)

Cell seeding media were removed from a pre-seeded plate (assay plate) and cell monolayers were washed with PBS twice. A volume of 50 µl of non-supplemented MEM was added to all wells except the isopropanol positive control wells. A volume of 50 µl of (4.0% or 0.4%) NCT was added to NCT-treated wells, 50 µl of non-supplemented MEM was added to virus only wells and 100 µl of isopropanol (≥ 99.5%, Sigma-Aldrich) added to positive control wells. A 100 µl volume of SARS-CoV-2 (B3) that had been pre-diluted 1:10 in non-supplemented MEM was added to all wells. Plates were incubated for 5, 10, 20, and 60 min at 37°C, 5.0%

411 CO_2. An additional 100 µl of virus growth media containing TPCK trypsin required for virus
412 growth (MEM supplemented with 1.0% (w/v) L-glutamine, 2.0% FBS and 8 µg/ml TPCK
413 trypsin) was added to plates pre-seeded with Vero E6 cells that samples were to be titrated
414 onto.

415 At each time point, 100 µl of either 1.0% or 0.1% NCT media was removed from the
416 assay plate and added to either 100 µl of met/his in distilled water to inactivate the NCT. The
417 positive control and virus only controls were also diluted 1:2 into distilled water. Each sample
418 was diluted a further 1:10 into virus growth media, MEM supplemented with 1.0% (w/v) L-
419 glutamine and 2.0% FBS containing 4.0 µg/ml of TPCK trypsin (i.e. 100 µl of inactivated
420 sample + 900 µl of virus growth media). The remaining virus after NCT inactivation was
421 quantified by addition of 100 µl volume of 1:10 diluted inactivated NCT to triplicate wells of
422 96-well plate pre-seeded with Vero E6 cells. Plates were incubated at 37°C, 5.0% CO_2 for 4
423 days. Virus-induced CPE was scored visually. The $TCID_{50}$ of the virus suspension was
424 determined using the method of Reed-Muench [51]. The virucidal effect was quantified as the
425 log_{10} reduction in virus titre compared to the SARS-CoV-2 control. Isopropanol (≥ 99.5%) was
426 used as the assay positive control.

427 For inactivation controls, 100 µl of NCT at 4.0% and 0.4% was added to 100 µl of
428 4.0% met/his or 0.4% met/his prior to addition of virus. A volume of 100 µl of this inactivation
429 mix was added to wash pre-seeded Vero E6 cells. To this, 100 µl of virus pre-diluted 1:10 in
430 non-supplemented MEM was added and incubated at 37°C, 5.0% CO_2 for 10 min or 60
431 minutes. Following the incubations, 100 µl was diluted into 900 µl of virus growth media
432 containing 4 µg/ml of TPCK trypsin (1:10). The remaining virus was quantified as outlined
433 above.

434

435 Absence of cytotoxicity of inactivated NCT to the inoculated cell culture.
436 As a cytotoxicity control, the 1.0% NCT and 0.1% NCT was set up the same and inactivated
437 by met/his as outlined above but instead of 100 µl of virus being added, 100 µl of non-
438 supplemented MEM was added. These samples were treated exactly the same as above

and titrated across pre-seeded cells to ascertain any cytotoxicity observed by the NCT. For cell viability staining, a 100 µl volume of a 3 mg/ml solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to cytotoxicity control plates and incubated for 2 h at 37°C in a 5.0% CO_2 incubator. Wells were aspirated to dryness using a multichannel manifold attached to a vacuum chamber and formazan crystals solubilised by the addition of 200 µl 100% 2-Propanol at room temperature for 30 minutes. Absorbance was measured at 540 – 650 nm on a plate reader. Absorbance values were averaged and reported as % reduction of MTT to formazan.

Virus inactivation assay with immunostaining and RT-qPCR (Virology Innsbruck).
Each 150 µl of NCT (2.0% in PBS) and of virus suspension (in DMEM plus 2.0% FCS plus 2 mM glutamine, plus Pen/Strep) were mixed and incubated at 37°C. After each incubation time, 50 µl were removed and transferred to an equal volume of met/his. Controls were done in PBS without NCT. As inactivation controls, 75 µl of 2.0% NCT in PBS were added to 150 µl of met/his, followed by addition of 75 µl virus suspension. After serial tenfold dilution of this suspension, 50 µl each were added to 90% confluent Vero/TMPRSS2 or Vero/TMPRSS2/ACE2 cells in 96-well plates, from which the medium was removed immediately before. After incubation of 1 h at 37°C, the supernatant was removed, and after a washing step with 100 µl of medium, 100 µl of fresh medium was added. After further 9 h incubation, cells were fixed for immunostaining or total RNA was extracted for RT-qPCR as described below.

Immunostaining (detection by antibodies and peroxidase-marked second antibody)
After fixation for 5 min with 96% EtOH, cells were blocked for 15 min with PBS containing 0.1% FCS. Subsequently, cells were stained using serum from a SARS-CoV-2 recovered patient and horse radish peroxidase (HRPO)-conjugated anti-human secondary antibody. The signal was developed using a 3-amino-9-ethylcarbazole (AEC) substrate. Infected cells were visible as red spots and the number of infected cells was counted using an

ImmunoSpot S6 Ultra-V reader and CTL analyser *BioSpot*® 5.0 software (CTL Europe GmbH, Bonn, Germany).

RT-qPCR

For RNA extraction, the supernatant was removed, and the cell monolayer was washed twice with PBS. The cells were lysed 5 min at room-temperature using 100 µl in-house direct lysis buffer (10 mM Tris-HCL pH 7.4, 25 nM NaCl, 0.5% IGEPAL, 10 Units RiboLock RNase Inhibitor in DEPC-treated water) [52]. Subsequently, 5 µl RNA was used in a one-step RT-qPCR assay using the iTaq™ RT-PCR (BIO-RAD) kit and previously published primers and probes specific for detection of the SARS-CoV-2 E Gene on a CFX96™ real-time system (BIO-RAD) [53].

Virus titration by $TCID_{50}$

Virus titrations were performed by tenfold serial dilution and end-point titration on 10^4 Vero/TMPRSS2/ACE2 cells per well in 96-well microtitre plates. Four days after inoculation, the CPE was analysed and the $TCID_{50}$ titre was calculated.

Influenza

Virus inactivation assay with plaque assay readout (RKI)

Eight µl of virus suspension (A/Panama/2007/1999 (H3N2), 1.3×10^8 PFU/ml) were added to 400 µl of NCT (1.0% or 0.1% in PBS) or PBS and incubated at 37°C. After 5, 15, 30 and 60 min, 100 µl were removed and added to 100 µl of met/his. As inactivation control, 100 µl of 1.0% NCT were added to 100 µl of met/his and thereafter 2 µl of virus suspension were added. Infectious virus particles in all suspensions were determined with plaque assay. Briefly, a serial tenfold dilution of the virus suspension in PBS with 0.3% bovine albumin was added to confluent MDCK II cells in 12-well plates, which were washed with PBS immediately before. After incubation at 37°C for 1 h, the inoculum was removed followed by

a washing step with PBS. Avicel-overlay medium (double-strength MEM supplemented with 0.2% BA, 1.0% DEAE dextran, 5.0% sodium bicarbonate, 1 mg/ml TPCK-trypsin and 1.25% Avicel) was added and plates incubated at 37°C and 5.0% CO_2 for 2 days before staining with crystal violet for visualization of plaques. Counted plaques are expressed as plaque forming units per ml (PFU/ml).

Virus inactivation assay with plaque assay readout (Hygiene and Medical Microbiology Innsbruck)

MDCK cells (2 x 10^4 / well) were grown in 96- well flat microtitre plates (Becton Dickinson Labware and Company, Franklin Lakes, NJ USA) for 24 h in RPMI plus 10% FCS. Subsequently, the medium was replaced by 100 µl of plain RPMI per well.

Each viral strain (H1N1 and H1N1pdm) was tested separately. Tenfold concentrated NCT (10.0%, 5.0%, and 1.0%, respectively) in distilled water (50 µl; water without NCT for controls) was added to 450 µl of virus suspension in RPMI (pH 7.2) to a final concentration of 1.0%, 0.5%, and 0.1%, respectively, and incubated for 1, 5, and 10 min at 22°C. A separate series of experiments was done with a final concentration of 0.1% NCT (5.5 mM) plus 0.1% ammonium chloride (18.7 mM) (Merck) and 1 min incubation time. At the end of the incubation time, aliquots of 100 µl were removed and mixed with 100 µl of met/his to inactivate NCT. Aliquots of 11 µl of this viral suspension in inactivated NCT were added to the MDCK cells in 96-well microtitre plates containing 100 µl RPMI per well. A series of tenfold dilutions in microtitre plates was performed. Inoculated plates were incubated at 37°C and 5.0% CO_2 and evaluated for plaques after 5 days. As inactivation controls, 100 µl each of 1.0% NCT and met/his were mixed. An aliquot of 50 µl was added to 450 µl of virus suspension.

RSV

RSV was incubated in the presence or absence of NCT at a final concentration of 0.1% and 1.0% for 5, 10, 15, 30 and 60 minutes at 37°C. Virus (24 µl in DMEM plus 1.0% FCS and 2

mM L-glutamine) was mixed with 24 µl of NCT in PBS. After indicated time-points, 48 µl of met/his was added to stop the reaction. As inactivation control, NCT was preincubated with equal amount of met/his for 10 minutes at RT prior to incubation with RSV. Infectious virus particles in all samples were titrated in plaque assay using HEp2 cells as described above. Aliquots of 25 µl were serially diluted in 100 µl of medium (DMEM plus 10% FCS and 2 mM L-glutamine) in microtitre plates, and 100 µl of Hep2 cells were added.

Statistics

Results are presented of mean values and standard deviation (SD) of generally at least three independent experiments each. Detection limits are indicated by dotted lines in the figures. Student's unpaired t-test, in cases of two groups, and one-way analysis of variance (ANOVA) and Dunnett's multiple-comparison test, in cases of more than two groups, were used to test for differences between the test and control groups. P values of < 0.05 were considered significant for all tests and indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Calculations were performed with GraphPad Prism 7.00 software (Graph- Pad, Inc., La Jolla, CA, USA).

References

1 Dhama, K. *et al.* Coronavirus Disease 2019-COVID-19. *Clinical microbiology reviews* **33**, doi:10.1128/cmr.00028-20 (2020).

2 Malik, Y. S. *et al.* Coronavirus Disease Pandemic (COVID-19): Challenges and a Global Perspective. *Pathogens (Basel, Switzerland)* **9**, doi:10.3390/pathogens9070519 (2020).

3 Fierabracci, A., Arena, A. & Rossi, P. COVID-19: A Review on Diagnosis, Treatment, and Prophylaxis. *International journal of molecular sciences* **21**, doi:10.3390/ijms21145145 (2020).

4 World Health Organization. *Influenza (Seasonal)*, <https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)> (2018).

5 Smith, D. J. *et al.* Mapping the antigenic and genetic evolution of influenza virus. *Science* **305**, 371-376, doi:10.1126/science.1097211 (2004).

6 Mitchell, J. P. *et al.* Urgent Appeal from International Society for Aerosols in Medicine (ISAM) During COVID-19: Clinical Decision Makers and Governmental Agencies Should Consider the Inhaled Route of Administration: A Statement from the ISAM Regulatory and Standardization Issues Networking Group. *Journal of aerosol medicine and pulmonary drug delivery* **33**, 235-238, doi:10.1089/jamp.2020.1622 (2020).

7 Arentz, M. *et al.* Characteristics and Outcomes of 21 Critically Ill Patients With COVID-19 in Washington State. *Jama*, doi:10.1001/jama.2020.4326 (2020).

8 Zhou, F. *et al.* Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*, doi:10.1016/s0140-6736(20)30566-3 (2020).

9 Arastehfar, A. *et al.* COVID-19 Associated Pulmonary Aspergillosis (CAPA)-From Immunology to Treatment. *Journal of fungi (Basel, Switzerland)* **6**, doi:10.3390/jof6020091 (2020).

10 Koehler, P. *et al.* COVID-19 associated pulmonary aspergillosis. *Mycoses* **63**, 528-534, doi:10.1111/myc.13096 (2020).

11 Zhu, X. *et al.* Co-infection with respiratory pathogens among COVID-2019 cases. *Virus Res* **285**, 198005, doi:10.1016/j.virusres.2020.198005 (2020).

12 Aufklärung, B. f. g. *Test auf eine Infektion mit dem Coronavirus SARS-CoV-2* <https://www.infektionsschutz.de/coronavirus/basisinformationen/test-auf-sars-cov-2.html#c13339> (2020).

13 Ye, Q., Wang, B. & Mao, J. Cytokine Storm in COVID-19 and Treatment. *The Journal of infection*, doi:10.1016/j.jinf.2020.03.037 (2020).

14 Gottardi, W. & Nagl, M. N-chlorotaurine, a natural antiseptic with outstanding tolerability. *J. Antimicrob. Chemother* **65**, 399-409 (2010).

15 Marcinkiewicz, J. & Kontny, E. Taurine and inflammatory diseases. *Amino Acids* **46**, 7-20, doi:10.1007/s00726-012-1361-4 (2014).

16 Arnitz, R. *et al.* Tolerability of inhaled N-chlorotaurine in humans – a double-blind randomized phase I clinical study. *Ther. Adv. Resp. Dis.* **12**, 1-14, doi:10.1177/1753466618778955 (2018).

17 Grisham, M. B., Jefferson, M. M., Melton, D. F. & Thomas, E. L. Chlorination of endogenous amines by isolated neutrophils. *J. Biol. Chem.* **259**, 10404-10413 (1984).

18 Weiss, S. J., Klein, R., Slivka, A. & Wei, M. Chlorination of taurine by human neutrophils. *J. Clin. Investig* **70**, 598-607 (1982).

19 Zgliczynski, J. M., Stelmaszynska, T., Domanski, J. & Ostrowski, W. Chloramines as intermediates of oxidation reaction of amino acids by myeloperoxidase. *Biochim. Biophys. Acta* **235**, 419-424 (1971).

20 Kim, C. & Cha, Y. N. Taurine chloramine produced from taurine under inflammation provides anti-inflammatory and cytoprotective effects. *Amino. Acids* **46**, 89-100 (2014).

21 Park, E., Alberti, J., Quinn, M. R. & Schuller-Levis, G. Taurine chloramine inhibits the production of superoxide anion, IL-6 and IL-8 in activated human polymorphonuclear leukocytes. *Adv. Exp. Med. Biol* **442**, 177-182 (1998).

22 Gottardi, W. & Nagl, M. Chemical properties of N-chlorotaurine sodium, a key compound in the human defence system. *Arch. Pharm. Pharm. Med. Chem.* **335**, 411-421 (2002).

23 Gottardi, W., Debabov, D. & Nagl, M. N-chloramines: a promising class of well-tolerated topical antiinfectives. *Antimicrob. Agents Chemother.* **57**, 1107-1114 (2013).

24 Nagl, M., Arnitz, R. & Lackner, M. N-Chlorotaurine, a Promising Future Candidate for Topical Therapy of Fungal Infections. *Mycopathologia* **183**, 161-170, doi:10.1007/s11046-017-0175-z (2018).

25 Garcia, L. A. T., Boff, L., Barardi, C. R. M. & Nagl, M. Inactivation of adenovirus in water by natural and synthetic compounds *Food and Environmental Virology* **11**, 157-166, doi:10.1007/s12560-019-09370-8 (2019).

26 Huemer, H. P., Nagl, M. & Irschick, E. U. In vitro prevention of vaccinia and herpes virus infection spread in explanted human corneas by N-chlorotaurine. *Ophthalmic. Res.* **43**, 145-152 (2010).

27 Nagl, M., Larcher, C. & Gottardi, W. Activity of N-chlorotaurine against herpes simplex- and adenoviruses. *Antiviral Res* **38**, 25-30 (1998).

28 Dudani, A. K., Martyres, A. & Fliss, H. Short communication: rapid preparation of preventive and therapeutic whole-killed retroviral vaccines using the microbicide taurine chloramine. *AIDS Res. Hum. Retroviruses* **24**, 635-642 (2008).

29 Kyriakopoulos, A. M., Logotheti, S., Marcinkiewicz, J. & Nagl, M. N-chlorotaurine and N-bromotaurine combination regimen for the cure of valacyclovir-unresponsive herpes zoster comorbidity in a multiple sclerosis patient. *International Journal of Medical and Pharmaceutical Case Reports* **7**, 1-6, doi:10.9734/IJMPCR/2016/25476 (2016).

30 Romanowski, E. G. *et al.* N-chlorotaurine is an effective antiviral agent against adenovirus in vitro and in the Ad5/NZW rabbit ocular model. *Invest. Ophthalmol. Vis. Sci.* **47**, 2021-2026 (2006).

31 Teuchner, B. *et al.* Tolerability and efficacy of N-chlorotaurine in epidemic keratoconjunctivitis – a double-blind randomized phase 2 clinical trial. *J. Ocul. Pharmacol. Ther.* **21**, 157-165 (2005).

32 Jekle, A. *et al.* Broad-spectrum virucidal activity of (NVC-422) N,N-dichloro-2,2-dimethyltaurine against viral ocular pathogens in vitro. *Invest. Ophthalmol. Vis. Sci.* **54**, 1244-1251 (2013).

33 Leiter, H. *et al.* Microbicidal activity of N-chlorotaurine can be enhanced in the presence of lung epithelial cells *Journal of Cystic Fibrosis*, doi:10.1016/j.jcf.2020.03.005 (2020).

34 Geiger, R. *et al.* Tolerability of inhaled N-chlorotaurine in the pig model. *BMC Pulmon. Med.* **9**, 33 (2009).

35 Nagl, M. *et al.* Tolerability of inhaled N-chlorotaurine versus sodium chloride in the mouse. *J. Med. Res. Pract.* **2**, 163-170 (2013).

36 Schwienbacher, M. *et al.* Tolerability of inhaled N-chlorotaurine in an acute pig streptococcal lower airway inflammation model. *BMC Infect. Dis.* **11**, 231 (2011).

37 Yoon, J. *et al.* Virucidal mechanism of action of NVC-422, a novel antimicrobial drug for the treatment of adenoviral conjunctivitis. *Antiviral Res* **92**, 470-478 (2011).

38 Gottardi, W. & Nagl, M. Chlorine covers on living bacteria: the initial step in antimicrobial action of active chlorine compounds. *J. Antimicrob. Chemother* **55**, 475-482 (2005).

39 Eitzinger, C. *et al.* N-chlorotaurine, a long-lived oxidant produced by human leukocytes, inactivates Shiga toxin of enterohemorrhagic *Escherichia coli*. *PloS One* **7**, e47105 (2012).

40 Jekle, A. *et al.* NVC-422 inactivates *Staphylococcus aureus* toxins. *Antimicrob. Agents Chemother* **57**, 924-929 (2013).

41 Nagl, M. *et al.* Impact of N-chlorotaurine on viability and production of secreted aspartyl proteinases of *Candida* spp. *Antimicrob. Agents Chemother.* **46**, 1996-1999 (2002).

42 Reeves, E. P., Nagl, M., O'Keeffe, J., Kelly, J. & Kavanagh, K. Effect of N-chlorotaurine on *Aspergillus*, with particular reference to destruction of secreted gliotoxin. *J. Med. Microbiol* **55**, 913-918 (2006).

43 Sauerbrei, A. *et al.* Sensitivity of human adenoviruses to different groups of chemical biocides. *J Hosp. Infect* **57**, 59-66 (2004).

44 Kramer, A. *et al.* Consensus on Wound Antisepsis: Update 2018. *Skin pharmacology and physiology* **31**, 28-58, doi:10.1159/000481545 (2018).

45 McDonnell, G. & Russell, A. D. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev* **12**, 147-179 (1999).

46 Gottardi, W., Arnitz, R. & Nagl, M. N-chlorotaurine and ammonium chloride: an antiseptic preparation with strong bactericidal activity. *Int. J. Pharm.* **335**, 32-40 (2007).

47 Gruber, M., Moser, I., Nagl, M. & Lackner, M. Bactericidal and fungicidal activity of N-chlorotaurine is enhanced in cystic fibrosis sputum medium. *Antimicrob. Agents Chemother.* **61**, 1-10, doi:10.1128/AAC.02527-16 (2017).

48 Böttcher, B., Sarg, B., Lindner, H. H. & Nagl, M. Inactivation of microbicidal active halogen compounds by sodium thiosulphate and histidine/methionine for time-kill assays. *J. Microbiol. Methods* **141**, 42-47, doi:10.1016/j.mimet.2017.07.014 (2017).

49 Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e278, doi:10.1016/j.cell.2020.02.052 (2020).

50 Ternette, N., Tippler, B., Uberla, K. & Grunwald, T. Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. *Vaccine* **25**, 7271-7279, doi:10.1016/j.vaccine.2007.07.025 (2007).

51 Reed, L. J. & Muench, H. A simple method of estimating fifty percent endpoints. *American Journal of Epidemiology* **27**, 493-497, doi:10.1093/oxfordjournals.aje.a118408 (1938).

52 Shatzkes, K., Teferedegne, B. & Murata, H. A simple, inexpensive method for preparing cell lysates suitable for downstream reverse transcription quantitative PCR. *Scientific reports* **4**, 4659, doi:10.1038/srep04659 (2014).

53 Corman, V. M. *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* **25**, doi:10.2807/1560-7917.Es.2020.25.3.2000045 (2020).

Acknowledgements

We are grateful to Andrea Windisch for excellent technical assistance in the production of NCT and to Thomas Grunwald and Leila Issmail, Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany, for provision of and technical support with the RSV. We thank Brian Monk (Otago University, Dunedin, New Zealand) and Ian Monk (Doherty Institute, Melbourne, Australia) for establishing the connection with 360biolabs, Melbourne, Australia.

Author Contributions

A.R., A.V., M.S., T.W., A.L., J.F. and H.S. performed the assays against SARS-CoV-2, M.S., T.W., J.S. and M.N. the assays against influenza viruses, B.M. and Z.B. the assays against RSV. M.L., M.N., C.S., D.v.L., planned the work, made the concept and guided the work. M.N. and M.L. wrote the manuscript under contribution of all other authors. All authors edited and approved the manuscript.

Funding

This study was supported by the German Federal Ministry of Education and Research (BMBF) grants ‘NUM Organo-Strat’ and ‘BUA Coronavirus Exploration project’.

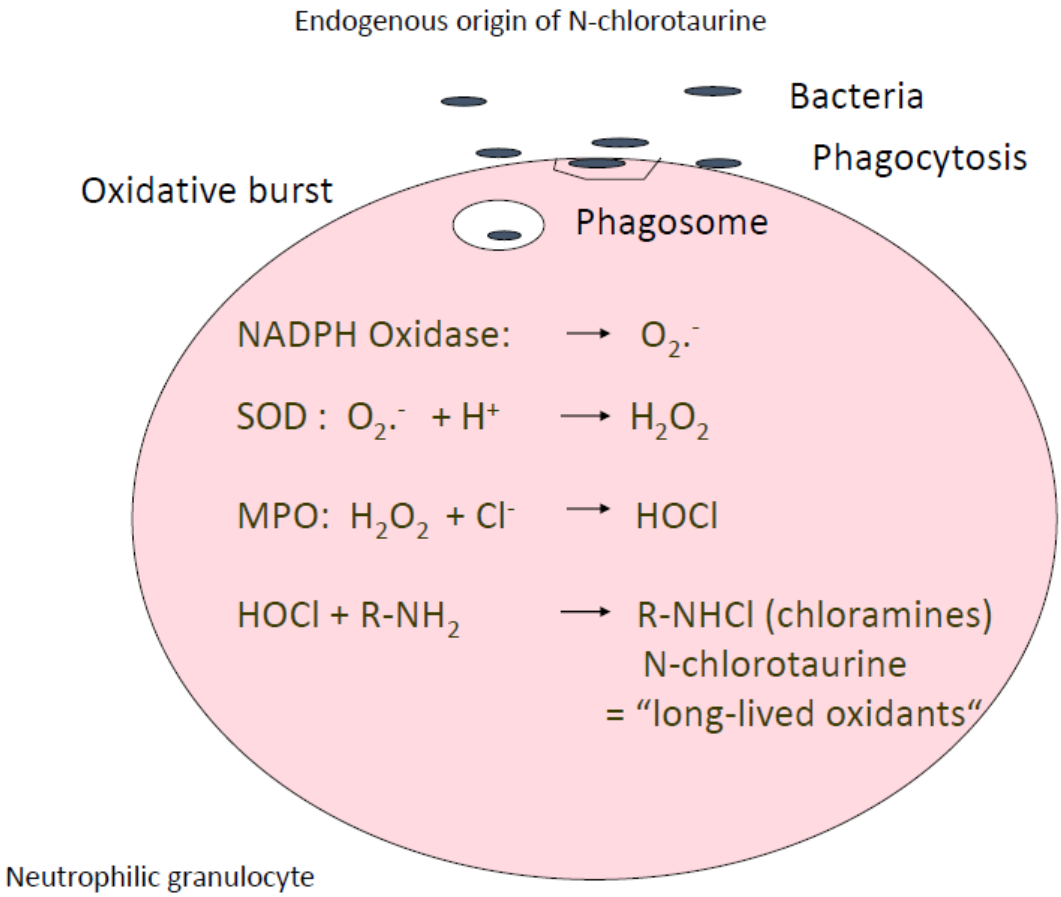
Competing Interests statement

M. Nagl is co-inventor of a patent on the application of NCT for inhalation (EP 2265267 B1). All other authors declare no conflict of interest.

718

719 **Figures**

720



721

722

723 Fig. 1

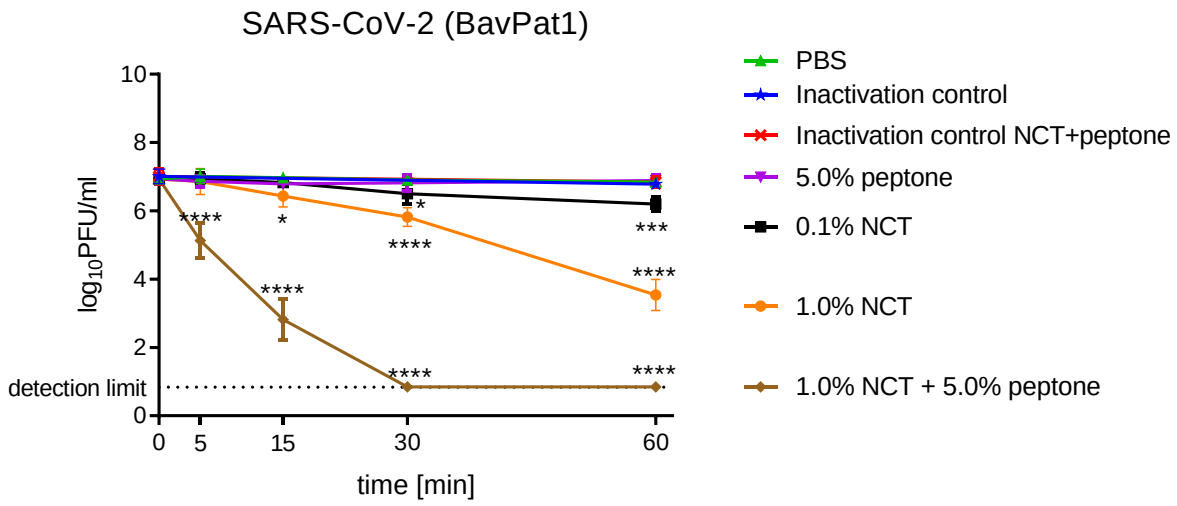
724

725 **Fig. 1: Endogenous origin of NCT.**

726 NCT is formed in activated human granulocytes and monocytes via an enzymatic cascade,
727 the oxidative burst. Subsequent to superoxide ($O_2.^-$) and hydrogen peroxide (H_2O_2), the
728 highly reactive hypochlorite (HOCl) is created by myeloperoxidase (MPO), which among
729 others reacts with amino compounds to form less reactive chloramines, also named long-
730 lived oxidants. NCT is the main representative of these chloramines. SOD superoxide
731 dismutase.

732

733



734

735 Fig. 2a

736

737 **Fig. 2: Inactivation of SARS-CoV-2 by NCT.**

738 **a**, Virus suspension (SARS-CoV-2 BavPat1) was incubated with 1.0% (55 mM) NCT, 0,1%

739 (5.5 mM) NCT, 1.0% NCT with 5.0% peptone or PBS or 5.0% peptone for 5 min, 15 min, 30

740 min, or 60 min at 37°C, after which samples were diluted 1:1 in met/his solution for

741 inactivation of NCT. Remaining infectious virus particles were determined using plaque

742 titration. To control for inactivation of NCT by met/his, virus was added after dilution of 1.0%

743 NCT with or without peptone in met/his. Mean values ± SD of three to eight independent

744 experiments in duplicates. The dotted line indicates the detection limit (0.84 $\log_{10}$). Data were

745 statistically analysed using a two-way ANOVA including a Dunnett's multiple comparison test

746 to PBS controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Of note, the

747 inactivation of the virus by NCT was markedly enhanced in the presence of peptone.

748

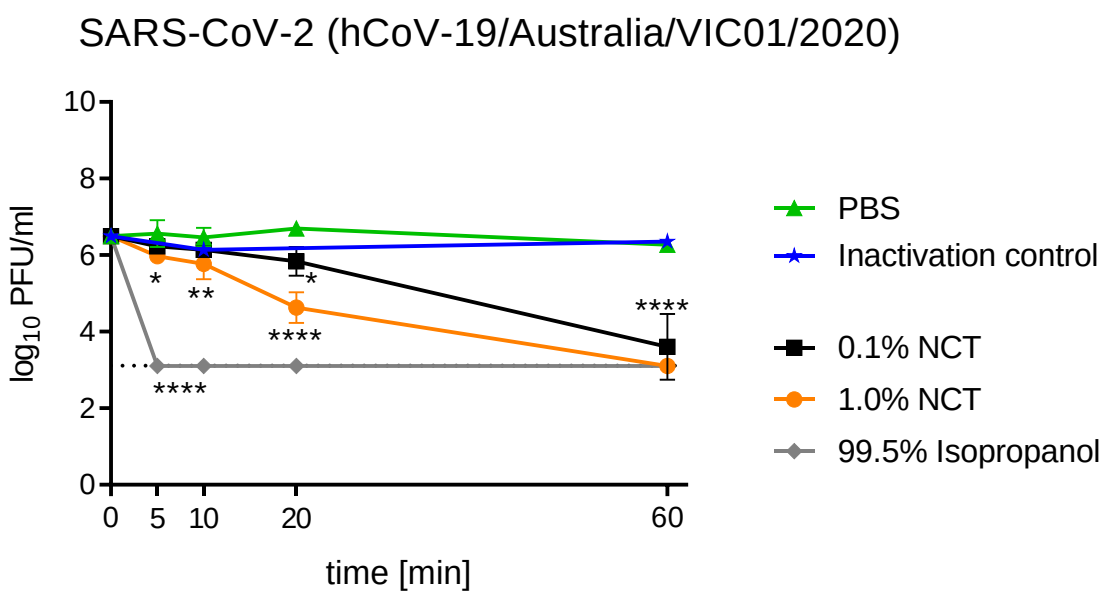
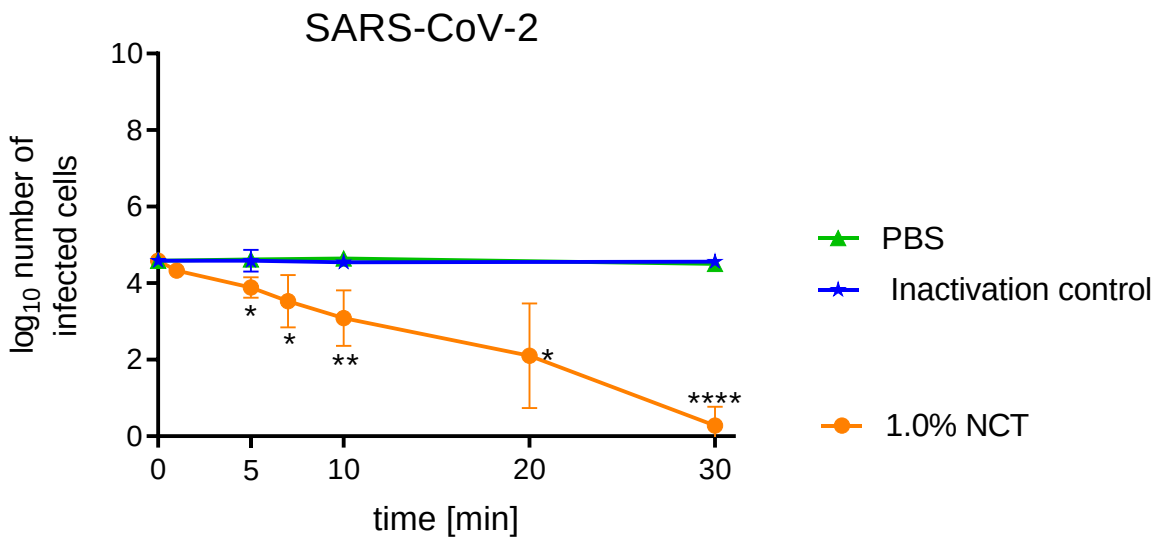


Fig. 2b

Fig. 2: Inactivation of SARS-CoV-2 by NCT.

b, Virus suspension (SARS-CoV-2 h CoV-19/Australia/VIC01/2020) was incubated with NCT or PBS or isopropanol (positive control) for 5 min, 10 min, 20 min, and 60 min at 37°C and then diluted 1:1 in met/his for inactivation of NCT, followed by plaque titration. Mean values ± SD of three independent experiments. Detection limit 3.11 $\log_{10}$ (dotted line).



762

763

764 Fig. 2c

765

766 **Fig. 2: Inactivation of SARS-CoV-2 by NCT.**

767 **c**, Virus suspension (SARS-CoV-2, clinical isolate) was incubated with 1% NCT or PBS for 1

768 min, 5 min, 7 min, 10 min, 20 min, and 30 min at 37°C. After inactivation of NCT and serial

769 dilution, aliquots were added to Vero/TMPRSS2/ACE2 cells for 1 h in 96-well plates. Cells

770 were washed, incubated for further 9 h, and fixed for Immunostaining (**c**) or RT-qPCR (**d**). In

771 immunostaining, infected cells were visible as red spots and counted using an ImmunoSpot

772 S6 Ultra-V reader and CTL analyser *BioSpot*® 5.0 software. Mean values ± SD of three

773 independent experiments.

774

775

776

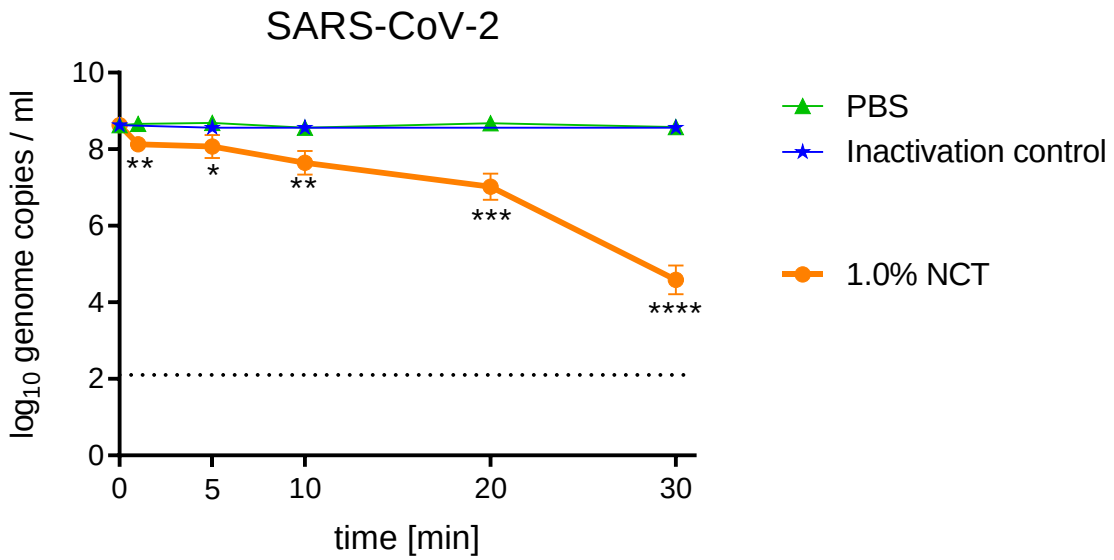


Fig. 2d

Fig. 2: Inactivation of SARS-CoV-2 by NCT.

d, After cell lysis and RNA extraction, one-step RT-qPCR assay was performed using the iTaq™ RT-PCR (BIO-RAD) kit and previously published primers and probes specific for detection of the SARS-CoV-2 E Gene on a CFX96™ real-time system (BIO-RAD). Mean values ± SD of genome copies of three independent experiments. Detection limit 2.10 $\log_{10}$ RNA copies/ml (dotted line).

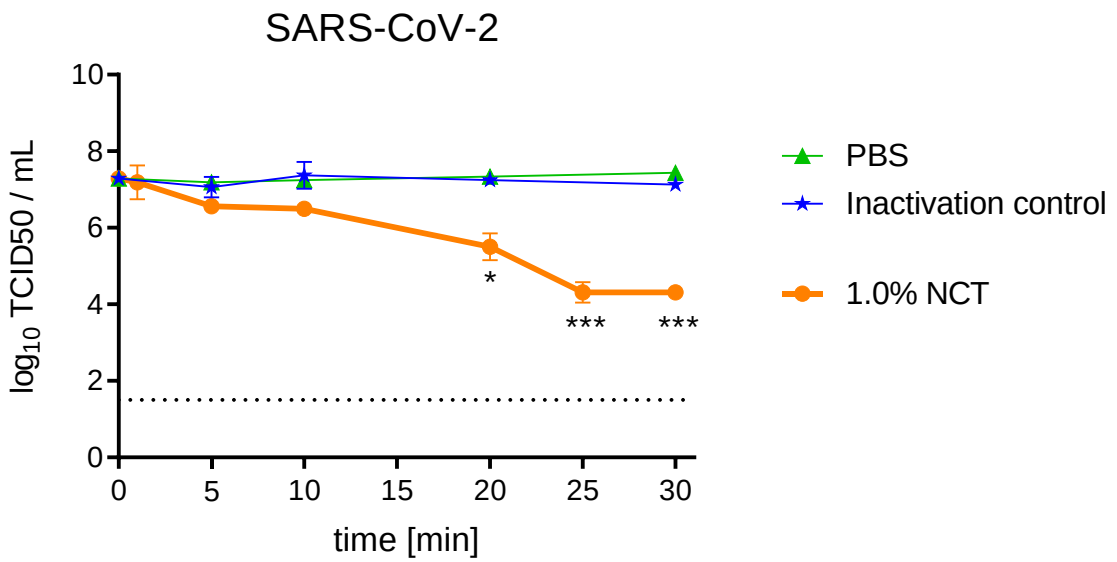


Fig. 2e

Fig. 2: Inactivation of SARS-CoV-2 by NCT.

e, Virus titration by $TCID_{50}$. Mean values ± SD of two independent experiments. Detection limit 1.50 log_{10} (dotted line).

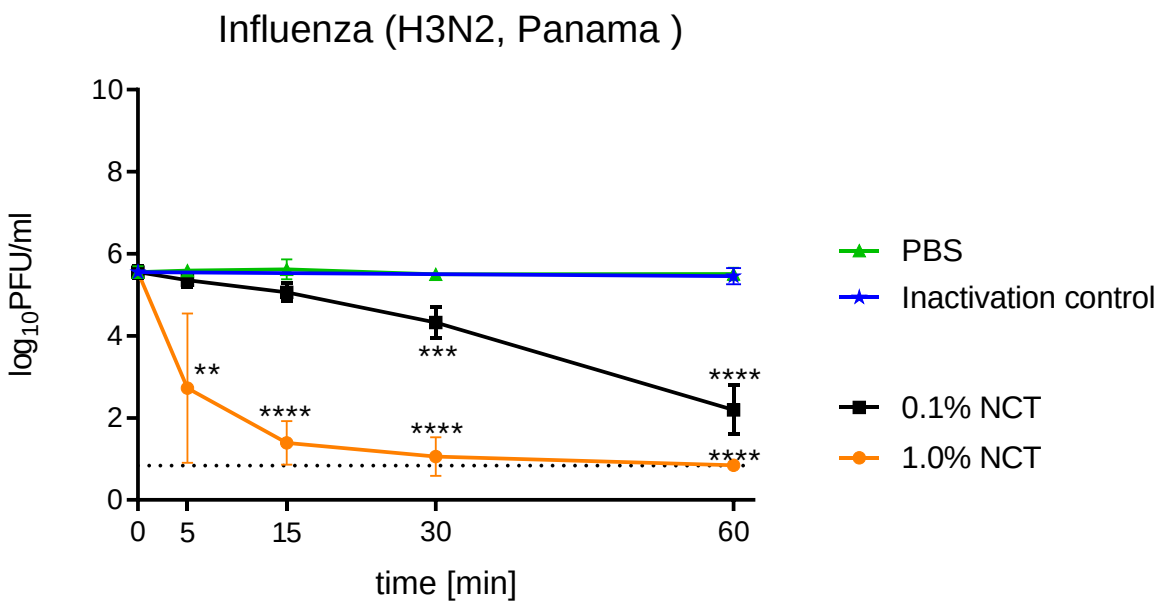
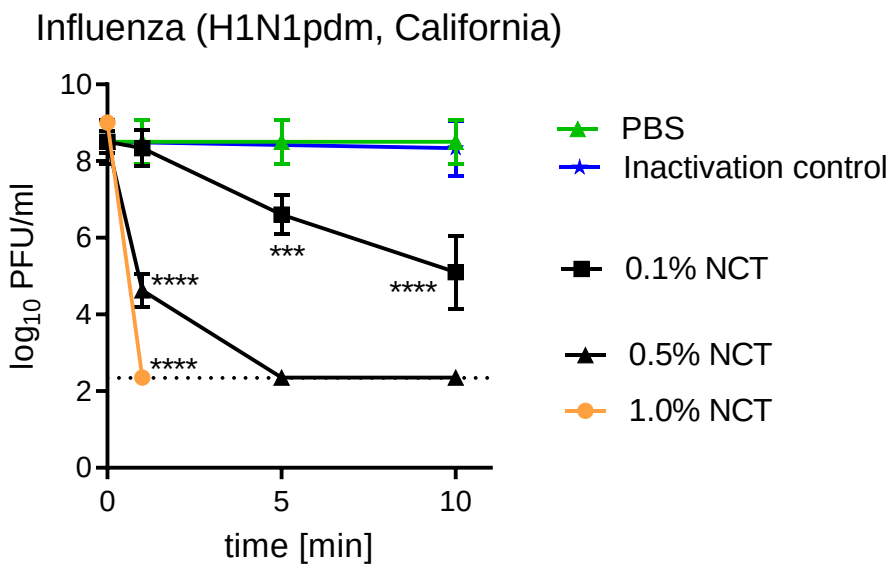


Fig. 3a

Fig. 3: Inactivation of influenza viruses by NCT.

a, Inactivation of Influenza A/Panama/2007/1999 (H3N2) by 1.0% (55 mM) and 0.1% (5.5 mM) NCT. Virus suspension was incubated with NCT or PBS for 5 min, 15 min, 30 min, or 60 min at 37°C, after which samples were diluted 1:1 in met/his solution for inactivation of NCT. Remaining infectious virus particles were determined using plaque titration. To control for inactivation of NCT by met/his, virus was added after dilution of 1.0% NCT in met/his in the inactivation control. Mean values ± SD of five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus PBS control. Detection limit 0.84 $\log_{10}$ (dotted line).



816

817

818 Fig. 3b

819

820 **Fig. 3: Inactivation of influenza viruses by NCT.**

821 **b**, Inactivation of Influenza A/California/ Swine Origin Virus/2009 (H1N1pdm) by 0.1%, 0.5%

822 and 1.0% NCT.

823 Virus suspension was incubated with NCT in RPMI or plain RPMI for 1 min, 5 min, and 10

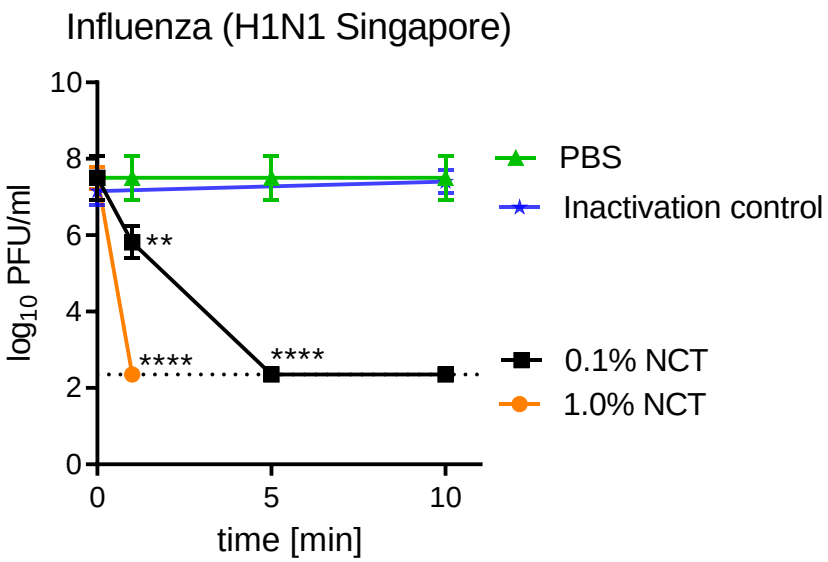
824 min at 22°C, after which samples were diluted 1:1 in met/his solution for inactivation of NCT.

825 Remaining infectious virus particles were determined using plaque titration. Mean values ±

826 SD of four independent experiments. Detection limit 2.35 $\log_{10}$ (dotted line).

827

828



829

830

831 Fig. 3c

832

833 **Fig. 3: Inactivation of influenza viruses by NCT.**

834 **c**, Inactivation of Influenza A/Singapore/Hongkong/2339/2000 (H1N1) by 0.1% and 1.0%

835 NCT and (**d**) by 0.1% NCT and 0.1% (5.5 mM) NCT plus 0.1% (18.7 mM) ammonium

836 chloride compared. Test procedure and number of independent experiments as in Fig. 3b.

837

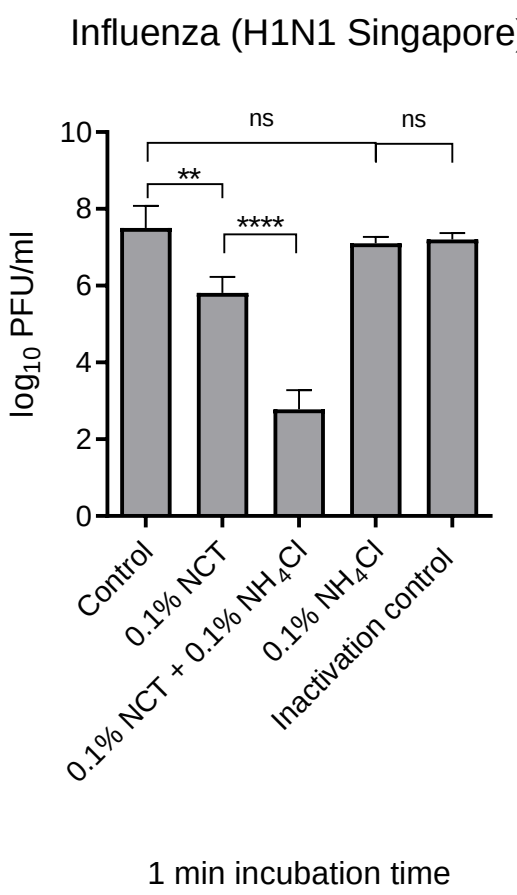


Fig. 3d

Fig. 3: Inactivation of influenza viruses by NCT.

c, Inactivation of Influenza A/Singapore/Hongkong/2339/2000 (H1N1) by 0.1% and 1.0% NCT and (**d**) by 0.1% NCT and 0.1% (5.5 mM) NCT plus 0.1% (18.7 mM) ammonium chloride compared. Test procedure and number of independent experiments as in Fig. 3b. Inactivation control in (**d**) consisting of 0.1% NCT plus 0.1% NH_4Cl plus inactivator.

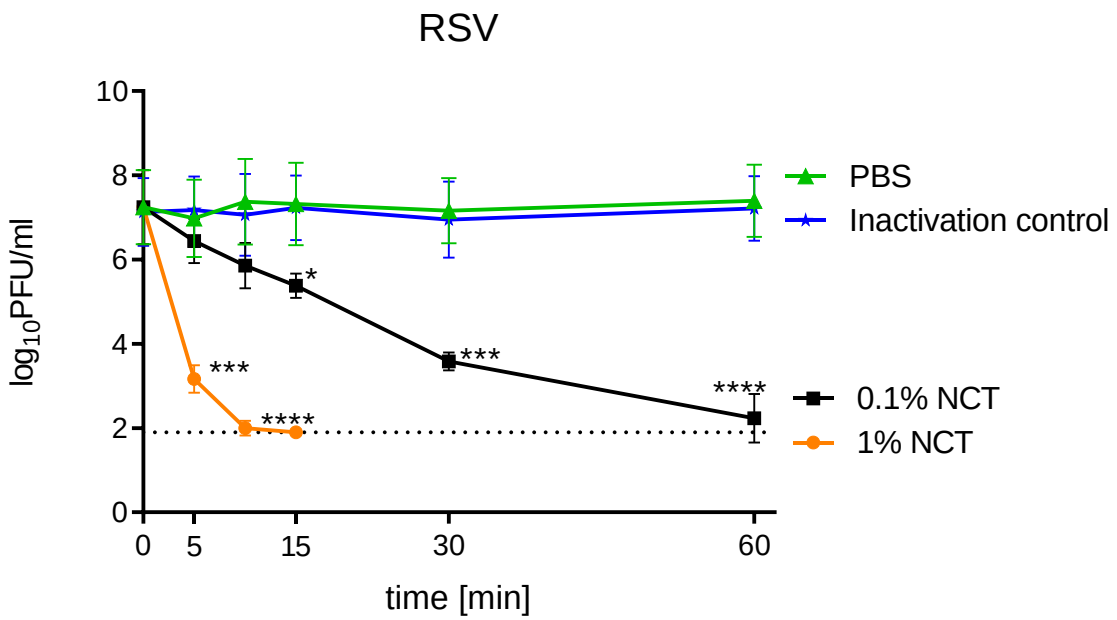


Fig. 4

Fig. 4: Inactivation of respiratory syncytial virus by NCT.

Virus suspension was incubated with 1.0% or 0.1% NCT or PBS for 5 min, 10 min, 15 min, 30 min, or 60 min at 37°C, after which samples were diluted 1:1 in met/his solution for inactivation of NCT. Remaining infectious virus particles were determined using plaque titration. Mean values ± SD of three independent experiments. Detection limit 1.90 $\log_{10}$ (dotted line).