Lipid nanoparticle formulation of niclosamide (nano NCM) effectively inhibits SARS-CoV-2 replication in vitro

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Abstract

As exemplified by the COVID-19 pandemic, highly infective respiratory viruses can spread rapidly in the population because of lack of effective approaches to control viral replication and spread. Niclosamide (NCM) is an old anthelminthic drug (World Health Organization essential medicine list) with pleiotropic pharmacological activities. Several recent publications demonstrated that NCM has broad antiviral activities and potently inhibits viral replication, including replication of SARS-CoV-2, SARS-CoV, and dengue viruses. Unfortunately, NCM is almost completely insoluble in water, which limits its clinical use. We developed a cost-effective lipid nanoparticle formulation of NCM (nano NCM) using only FDA-approved excipient and demonstrated potency against SARS-CoV-2 infection in cells (Vero E6 and ACE2-expressing lung epithelium cells).

Keywords

Lipid nanoparticle; COVID-19; SARS-CoV-2; niclosamide
INTRODUCTION

Respiratory viruses are an emerging and immediate threat to the modern world. Vaccination can offer a broad protection via adaptive immunity, but there is always a possibility of viral mutation, and there is unfortunately a resistance to vaccination in certain populations. There is an acute unmet need for antiviral drugs that can be manufactured at low cost and administered to large populations. While remdesivir has been shown to improve the course of COVID-19 in some patients, the drug is administered intravenously, and costly (5 days treatment exceeds $3,000). In the environment of a pandemic, antiviral drugs should be inexpensive, formulated with readily available GRAS (generally regarded as safe) excipients, and readily manufacturable as scale.

Niclosamide (NCM) is a generic anthelminthic drug (World Health Organization (WHO) with pleiotropic pharmacological properties. NCM was developed by the Bayer chemotherapy research laboratories in 1953 as a molluscicide and was marketed as Bayluscide. Later, NCM was found to be effective against human tapeworm (cestoda) infection, and it was marketed as Yomesan for human use in 1962. NCM was approved by the US FDA for use in humans to treat tapeworm infection in 1982 and is included in the WHO’s list of essential medicines. It has been used to treat millions of patients and its excellent safety and tolerability have been established for many species and administration routes. NCM has pleiotropic anticancer and macrophage-reprogramming/immunomodulating properties. In particular, several reports demonstrated that NCM is a potent inhibitor of STAT-3 phosphorylation, Wnt/β-catenin, mTORC1, NF-κB and Notch signaling, causing direct antitumor effects and reprogramming of macrophages from M2 to M1 type. It is also a potent mitochondrial uncoupler. The drug appears to inhibit IL-6 and TNF alpha signaling via Jak/STAT-3 inhibition.

Several recent publications demonstrated that NCM has broad antiviral activities, including against SARS-CoV-2, SARS-CoV, and dengue viruses. NCM inhibits SARS-CoV-2 infection in Vero E6 cells with nanomolar IC₅₀, without affecting cell viability. The mechanism of action does not involve inhibition of viral binding, but could be due to the inhibition of intracellular acidification, fusion, and/or direct effects on viral replication and autophagy. There are several ongoing clinical trials with oral NCM tablets in COVID-19 patients, which is the original dosage form approved in several counties for anthelmintic therapy. However, NCM is a class II drug by the biopharmaceutical classification system (BCS) with very low aqueous solubility (0.25 μg/mL) and with poor oral bioavailability, with plasma concentrations in most subjects below 0.1 μg/mL, which is below the IC₉₀ value.

There is a major effort on developing NCM formulations for COVID-19. Thus, ANA pharmaceuticals is starting Phase I trial with proprietary oral formulation. Union Pharmaceuticals is starting trials on a nasal ointment and Daewoon Pharma is going to test an inhalation product and possibly intramuscular injection. Here we developed a simple process for nano solubilization of NCM that takes minutes to prepare and can be potentially scaled up for clinical use. We demonstrated potent inhibition of SARS-CoV-2 replication.
in Vero E6 cells and human ACE2-expressing lung epithelial cells, while maintaining high selectivity index of the drug. We suggest that nano NCM can be used for testing in animal models and subsequently for development of clinical therapies.

MATERIALS AND METHODS

Materials –

Niclosamide (N3510-50G) was from Sigma-Aldrich (St. Louis, MO, USA). Egg phosphatidylcholine (Egg PC), cholesterol, distearoyl phosphatidylethanolamine (DSPE)-PEG1000 and DSPE-PEG750 were from Avanti Polar Lipids (Alabaster, AL, USA). DSPE-PEG2000 was from Avanti or NOF America Corporation (White Plains, NY, USA) (880120 and DSPE-020CN, respectively, both in powder). Lipids were dissolved in ethanol at 10 mM and kept in glass vials (224752) at −20 °C before use. DiD (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine, 4-chlorobenzenesulfonate salt, 60014) was from Biotium (Hayward, CA, USA) and was stored as 10 mM sock in ethanol at −20 °C. Glass vials (224752 and 224881) were from Duran Wheaton Kimble (DWK) Life Sciences, LLC (Milliville, NJ, USA). Sodium hydroxide (NaOH, S318-1) and hydrochloric acid (HCl, SA812-4), dextrose anhydrous (BP350-1), and sodium chloride (NaCl, S671-3) was from Thermo-Fisher Scientific (Hampton, NH, USA). Methyl-beta cyclodextrin (33261-5) was from Sigma-Aldrich. Fetal bovine serum (FBS, 26140-079) was from Thermo-Fisher Scientific. Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with glucose and L-glutamine (10-013-CV), Trypsin (25-053-CI) and Penicillin-Streptomycin (30-002-CI) were from Corning Inc. (New York, NY, USA). Ethyl alcohol (pure) was from Sigma-Aldrich (E7023-1L). VETRANAL™ Niclosamide-(2-chloro-4-nitrophenyl-13C6) hydrate (11-101-2945) was from Thermo-Fisher Scientific. LC/MS grade acetonitrile and Niclosamide-13C6 was obtained from Fisher Scientific (Fairlawn, NJ, USA). HPLC grade water was obtained from Burdick and Jackson (Morristown, NJ, USA). Acetic acid (695092) was obtained from Sigma-Aldrich. Pierce™ BCA Protein Assay Kit (23225) was purchased from Thermo-Fisher Scientific. Nuclear staining reagent Hoechst 33342 trihydrochloride trihydrate (H3570) was purchased from Life Technologies (Carlsbad, CA, USA).

Nanoparticle preparation and characterization –

Formulations are described in detail in the Results section. Briefly, 1 mg NCM aliquots were lyophilized from a 20 mg/mL DSMO solution in 2mL glass vials to form a cake. Ethanol, 0.1N NaOH and the excipient in ethanol were mixed by gentle shaking, until the drug was dissolved. The mix was diluted within 5 min with aqueous vehicle and then neutralized with 0.1N HCl. In some cases, the excipient was added in the vehicle during the last dilutions step. The resulting neutral pH was verified with pH strips. Transmission electron microscopy (TEM) imaging was conducted on uranyl acetate counterstained samples using FEI Tecnai G2 transmission electron microscope (Hillsboro, OR, USA) with an AMT digital camera (Woburn, MA, USA) at a 100 kV working voltage. Size and zeta potential measurements of NPs were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). The intensity weighted size distribution peak value was used to report hydrodynamic diameters.
Cell culture, viral infection and cell viability - -

Vero E6 cells were obtained from American Type Culture Collection (ATCC). Human ACE2 (angiotensin-converting enzyme 2) stably transfected A549 cells (hACE2-A549) were obtained from Dr. Mario Santiago Laboratory at CU Anschutz. Cells were grown in 5% CO₂ atmosphere at 37 °C in DMEM containing 10% FBS, 100 U/mL penicillin and 100 ng/mL streptomycin for Vero E6 cells or puromycin (0.5 μg/mL) for hACE2-A549 cells. For infectivity assay, cells in 96-well plates were infected with SARS-CoV-2 USA-WA1/2020 (BEI Resources) at two different MOIs (0.5 or 1 FFU/cell). Following a 1 h adsorption, cells were washed with 1x PBS and media containing a 10-point 2-fold dilution series (10-0.02 μM) of NCM formulation or NCM in DMSO. At 24 h post-infection (hpi), infectious virus in cell culture supernatants was quantified by a high-throughput focus formation assay. Briefly, 10-fold dilutions of cell culture supernatants were added to Vero E6 cells. After 1 h of incubation at 37 °C, the supernatants were removed and cells were overlaid with 1% methylcellulose in DMEM/5% FBS and incubated for 30 h at 37 °C. Cells were fixed with 4% paraformaldehyde and probed with 1,000 ng/mL of an anti-SARS-CoV-2 spike monoclonal antibody (CR3022, Absolute Antibody) in Perm Wash (1X PBS/0.1% saponin). After washing, cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG for 2 h at room temperature (RT) and SARS-CoV-2-positive foci were visualized with TrueBlue substrate and counted using a CTL Biospot analyzer and Biospot software. Cytotoxicity (CC50) to the non-infected Vero E6 and hACE2-A549 cells was measured with MTT assay. Cells were incubated with different concentrations of NCM in DMEM, or lipid NCM in a 96 well plate for 24 h. Cells were washed gently twice, and the cell viability was determined by MTT assay (M6494, Thermo-Fisher Scientific). The IC50 and CC50 were determined by fitting normalized data to variable inhibition slope using Prism 8.0 software. IC90 value was calculated from the Hill slope determined by the software.

Drug assay with HPLC -

Agilent 1100 series equipped with Kinetex® 2.6 μm C18 100 Å, LC column 100 x 3 mm (Phenomenex Corporation, USA) was used. The mobile phase consisted of Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile). The 10-min gradient was as follows: from 0 to 1 min, Buffer B was maintained at 20%; from 1 to 4.5 min, the Buffer B linearly increased from 20% to 95%, and was maintained at 95% for 1 min, then returned to 20% in 0.5 min and was maintained at 20% for 3 min. The flow rate was set at 0.6 mL/min, temperature 25°C. Injection volume was 20 μL. Standard solution of NCM (1.019 mg/mL, 3 mM) was prepared in a 1:9 volume ratio of ethanol: acetonitrile. For free drug and encapsulated drug, 100 μL of formulation was centrifuged at 80,000rpm for 15 min using Beckman Optima ultracentrifuge (TLLA-100.3 rotor). The supernatant was collected, and the pellet was resuspended in 100 μL HPLC water. For stability in basic ethanol solution, NCM was dissolved in 30 μL of 0.1N NaOH, 500 μL of ethanol and 489 μL of water to prepare 1.019 mg/mL. After 15 min, 1 h, 3 h, and 24 h, 100 μL of the degradation sample was mixed with 889 μL of acetonitrile and 30μL of 0.1N HCl. The mixture was vortexed for 30 seconds. The standard solution was diluted the same way but without addition of HCl. For stability, the formulation of 1 mg/mL NCM stored at 4°C was
mixed at different time points at 1:10 ratio with acetonitrile. Integrated area under the NCM peak (retention time 5.78min) was plotted versus time.

**Drug uptake quantification by cells with LC-MS/MS -**

An internal standard stock containing 1mg/mL of niclosamide-\textsuperscript{13}C6 was prepared in 9:1 acetonitrile: DMSO. This internal standard stock was diluted to 1.56 ng/mL in 1% formic acid in acetonitrile to use as a protein precipitation solution. A stock solution containing 1mg/mL of niclosamide was prepared in 9:1 acetonitrile: DMSO. Calibration standards (10 concentrations total) were prepared with 1:2 serial dilutions with protein precipitation solution. The concentration range was from 0.0244 ng/mL up to 12.5 ng/mL. All stock solutions, calibration standards and protein precipitation solutions were stored at −20°C until use. Immediately before use, 100 μL of the calibration standards were combined with 25 μL of LC/MS water in an autosampler vial with a glass insert and vortexed for 5 seconds. The resulting standard concentrations were 0.0195 ng/mL up to 10 ng/mL, with the internal standards being 1.25 ng/mL. These standards were stable for up to 48 hours after preparation.

Immediately after treatment, hACE2-A549 cells were washed with 1X PBS 3 times, and after completely removing the PBS, 200 μL of a 4:1 mix of protein precipitation solution: water containing 1.25 ng/mL of internal standard was added. The scraped cells/extraction solution was vortexed for 10 sec and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was removed and placed into a 1.8 mL screw cap amber autosampler vial with a 250 μL insert. The prepared sample was stored at −20 °C until analysis.

High performance liquid chromatography was performed using a 1260 series HPLC from Agilent (Santa Clara, CA) using an Agilent Eclipse Plus C18 2.1X50mm 1.8um column. Buffer A consisted of water with 10 mM ammonium acetate, and buffer B consisted of 50:50 acetonitrile: isopropanol. Two microliters of the extracted sample was analyzed using the following gradient at a flow rate of 0.3 mL/min: starting composition=10% B, linear gradient from 10-100% B from 0-5 min, hold at 100% B from 5-7 min followed by re-equilibration at 10% B for 5 minutes. The column temperature was held at 60°C for the entire gradient. Tandem mass spectrometry was performed on an Agilent 6490 triple quadrupole mass spectrometer in negative ionization mode. The drying gas temperature and flow rate was 230°C and 15 L/min, respectively. The nebulizer pressure was 35 psi. Sheath gas temperature and flow rate was 400°C and 11 L/min, respectively. The capillary voltage was 4000V. Fragmentor voltage was 380V. The iFunnel RF parameters were 90 for the high pressure funnel and 60 for the low pressure funnel. Cell accelerator voltage was set to 4. Multiple Reaction Monitoring (MRM) transitions and collision energies (CE) were determined by injecting authentic standards individually. NCM was monitored for m/z=325>171, CE=29 (quantifier) and 325>289, CE=17 (qualifier). NCM-\textsuperscript{13}C6 was monitored for m/z=331>177, CE=29 (quantifier) and 331>295, CE=17 (qualifier). Calibration curves for NCM were constructed using Agilent Masshunter Quantitative Analysis software. The uptake was normalized by the levels of cell protein measured with BCA assay per manufacturer’s instructions.
Microscopy –

Nano NCM labeled with DiD (1μM NCM) was added to cells grown on a slide for 24h and then washed away 3 times with PBS. Cells were fixed with 10% formalin solution for 30 min, stained with Hoechst, mounted and imaged with Nikon Eclipse AR1HD inverted confocal microscope using 405 nm and 640 nm excitation lasers.

RESULTS

1. Development and characterization of nano NCM formulation

NCM is a class II drug with poor bioavailability, limiting its potential use. Its LogD at pH7 is 4.48 and it is essentially insoluble in water. Several publications described nanoformulations for enhanced solubility of NCM, but they use complicated and expensive manufacturing processes, require non-FDA approved excipients, or have low loading capacity. A simple, cost effective, and safe NCM formulation could be very valuable for anti-viral and other indications. To make a colloidally stable solid lipid nanoparticle, the first common step is to dissolve the payload and the excipient in an organic phase, followed by fast dilution in the water phase. We first attempted to solubilize NCM in an organic solvent for subsequent mixing with other excipients. Since NCM has a weakly ionizable aromatic alcohol group in the position 2 of benzamide, we hypothesized that NCM and the excipient can be dissolved in a common organic solvent in basic conditions. Due to regulatory safety and the preference to avoid solvent evaporation/dialysis steps, we selected ethanol as the organic solvent. Solubility of NCM in ethanol is negligible but can be slightly improved by addition of a base (0.25 mg/mL for ethanolamine salt of NCM). Addition of equinormal amount of NaOH in 50% ethanol/water to lyophilized NCM cake resulted in partial dispersion of NCM (Fig. 1A). For the lipid excipient, we selected distearoyl phosphatidylethanolamine (DSPE)-PEG2000, which is the FDA-approved lipid commonly used in stealth micelles, lipid NPs and liposomes, including Onivyde® and Doxil®. Addition of DSPE-PEG2000 (10 mM in ethanol) to the basic ethanol afforded a fully clear dispersion at 11.1 mg/mL NCM and 3.33 mM DSPE-PEG2000 (Fig. 1A, step 1, and Fig. 1B). Upon subsequent 10-fold dilution step with water (Fig. 1A, step 2), the formulation presented as clear, colloidally stable dispersion (Fig. 1B). In the final step done immediately after the dilution, the dispersion was neutralized with equinormal HCl (Fig. 1A, step 3, and Fig. 1B). The resulting formulation contained NCM: DSPE-PEG2000 weight ratio of 1.19:1, which corresponds to 54.3% loading capacity (Fig. 1A). The other excipients’ concentration was 30 mM NaCl and 6% ethanol. The particles had a diameter below 200 nm (Fig. 1C and Table 2) and negative zeta potential due to the phosphate moiety of DSPE-PEG2000 (Fig. 1C). Negative contrast TEM showed rounded NPs less than 200 nm diameter (Fig. 1D).

Having identified the excipient leading to formation of lipid NPs, we further investigated the parameters and critical steps that determine the size and colloidal stability. The study is summarized in Table 2 with some formulations shown in Fig. 2. Neutralization step 3 was critical for colloidal stability and polydispersity, however, neutralization with two fold molar excess of acid resulted in smaller and more monodisperse particles (113 nm, PDI 0.155). Neutralization before to the dilution step resulted in large, unstable particles (682.7 nm, PDI 0.639). Reducing the amount of DSPE-PEG by 33% or 67% (DSPE-PEG2000: NCM weight...
ratio of 0.56 and 0.28, respectively) led to increase in particle size and eventual aggregation. Also, addition of DSPE-PEG2000 at the step 3 rather than at the step 1 resulted in much larger, polydisperse particles, suggesting that the lipid needs to be co-dissolved with the drug prior to the dilution step. NCM without the lipid was colloidally unstable and crashed out of solution shortly after the dilution step, with or without neutralization. We prepared 4 mg/mL NCM by reducing the volumes in steps 1 and 3 (but keeping the NCM: DSPE-PEG ratio the same. This resulted in somewhat larger particles (265 nm, PDI 0.183).

We next explored the effect of different aqueous vehicles as well as the size of PEGylated lipids on the colloidal stability. As shown in Table 2 and Fig. 2A-B (picture and size graph), saline and dextrose did not affect dramatically the size and PDI of the NPs. The molecular weight of PEG was important, with DSPE-PEG2000 producing monodisperse NPs, whereas DSPE-PEG1000 and especially DSPE-PEG750 led to increased size and polydispersity in all vehicles tested.

We also explored other FDA-approved GRAS excipients, either in combination with or instead of DSPE-PEG2000 (Table 2). Substitution of 90 mol % and 50 mol % of DSPE-PEG2000 with equimolar amount of egg phosphatidylcholine (Egg PC) did not result in stable monodisperse particles and the formulation quickly aggregated. Methyl-beta-cyclodextrin (MCD) alone used at MCD: NCM weight ratio of 10:1 did not result in a stable formulation, but addition of NCM to the DSPE-PEG formulation improved the polydispersity for all DSPE-PEG types (Table 2 and Fig. 2B), probably due to synergism with DSPE-PEG. Also, partial substitution of DSPE-PEG2000 with cholesterol was well tolerated (up to 67 mol % cholesterol), and the formulation was smaller and more monodisperse than without cholesterol (152 nm, PDI 0.162).

Finally, for the water based formulation, we tested reproducibility, size stability, drug release and chemical (NCM) stability. The formulations prepared on 5 different days showed ≤ 10% coefficient of variation in diameter and PDI (8.5% and 10%, respectively, Fig. 3A). There was no increase in diameter after 7 days of storage at 4°C but an increase in PDI was observed after 6 weeks (Fig. 3B). The HPLC stability study of NCM in basic ethanol solution showed stability at 24h (Fig. 3C). Furthermore, the NCM in the final formulation stored at 4°C was stable for at least 2 weeks (Fig. 3D). Lastly, the HPLC drug assay after ultracentrifugation of nanoparticles showed about 7% of free drug in the supernatant at 24h.

2. Efficacy and selectivity index for SARS-CoV-2 inhibition and cell uptake

To evaluate the efficacy of our NCM formulation against SARS-CoV-2 infection, we used Vero E6 cells, which are derived from monkey kidney and are commonly used for testing antiviral agents in virus infected cells. First, we confirmed activity against SARS-CoV-2 infection of non-formulated NCM (in DMSO), with 0.042μM IC50, 0.251μM IC90 (Fig 4A), and a selectivity index (CC50/IC50 ratio) 464. The nano NCM formulation demonstrated activity with 0.595 nM IC50 and 3.38 μM IC90 (Fig 4B), while achieving the selectivity index of 52. Cell lines from airway epithelium have been proposed as more relevant to SARS-CoV-2 infection due to dependence of TMPRSS2 protease for entry, which is similar to the human infection (preprint 19). We confirmed that the nano NCM formulation showed antiviral efficacy against SARS-CoV-2 infection in human lung
epithelial cell line A549 cells expressing human ACE2 (to enable SARS-CoV2 infection) with 0.154 μM IC50 and 1.38 μM IC90 (Fig. 4C), while achieving the selectivity index of 137.

To test the uptake efficiency, we performed LC-MS/MS analysis of intracellular NCM following 24h incubation of hACE2-A549 cells with 1 μM NCM (DMSO) or 1μM nano NCM (F23). The analysis showed 56.5 ng/mg protein for NCM and 35.2 ng/mg for nano NCM (Table 3). The uptake efficiency was 21.4% for NCM and 15.1% for nano NCM%. Confocal microscopy imaging of cells after incubation with DiD-labeled F23 (Table 2) detected accumulation of intracellular DiD, on punctuated pattern resembling endosomal uptake (Fig. 5).

**DISCUSSION AND CONCLUSIONS**

Motivated by the pandemic, we set out to develop a fast and cost-efficient formulation of NCM. While additional R&D is required to scale up and optimize the stability and size uniformity, our formulation shows several advantages over previously published approaches. Thus, it includes only low cost FDA-approved excipients, achieves high loading capacity (55% w/w), and requires much shorter time to prepare. The formulation is sufficiently stable and can be prepared in various isotonic vehicles at neutral pH.

Given the potent antiviral effects *in vitro*, the next step is to evaluate the *in vivo* efficacy of our NCM formulation against SARS-CoV-2 infection in an immunocompetent animal model. For example, human ACE2 transgenic mouse mice and Syrian golden hamsters are suitable models for SARS-CoV-2 and other coronaviruses. Upon viral challenge, these mice progressively develop high viral burden in the lungs. Various administration routes for the nano NCM could be tested. Intravenous administration is acceptable in moderate to severe COVID-19, however intravenous nano drugs may face additional hurdles to translation, including anaphylactic reactions towards PEGylated component. On the other hand, several studies demonstrated that nasal epithelium is the primary target for SARS-CoV-2 infection and other SARS viruses due to higher expression of ACE2 and other entry factors in the nasal cavity. There was a recent report on activity of lysozyme coated NCM microparticles *via* nasal route. Inhalation therapy also makes sense and can be administered *via* nebulizer. While there are no inhalational products for COVID-19 yet, some other FDA approved drugs, for example, liposomal Amikacin (Arikace®) for treatment of refractory *Mycobacterium avium*, is administered *via* a dedicated nebulizer.

In conclusion, we developed a simple process for nano solubilization of NCM that can be tested in animal models and potentially scaled up for clinical use.

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RATIONALE AND PURPOSE

Niclosamide as a generic anthelmintic drug with anti-viral and anti-SARS-CoV-2 properties. Due to its lack of solubility, it will benefit from nanoformulation. There is a need in simple and cost effective formulation approaches, which is addressed in this work. The resulting formulation can be scaled up and tested in preclinical models and in COVID-19 patients.
Fig. 1. Nano NCM Preparation and Characterization.
A-B) description and appearance of formulation at each step; C) size and zeta potential of a representative formulation in water; D) negative contrast (uranyl acetate) TEM. Size bar 200nm.
**Fig. 2. Effect of PEG size and excipient.**

A) Images of selective formulations taken ~1h after preparation. Water-based formulations are shown. From left to right: F0: no lipid; F23: DSPE-PEG2000; F24: DSPE-PEG1000; F25: DSPE-PEG750; F26: DSPE-PEG2000 without neutralization; F27: DSPE-PEG2000 added with water during the dilution step; B) size as a function of aqueous vehicle and excipient. Note that shorter DSPE-PEG (1000 Da and 750 Da) result in unstable heterogeneous formulations, whereas methyl-beta-cyclodextrin stabilizes formulations. See Tables 1-2 for full details.
Fig. 3. Stability of water-based DSPE-PEG2000 formulation (F23).
A) Size reproducibility across different batches. Black bars refer to left axis, right bars refer to right axis; B) size stability of the formulation upon storage. Black bars refer to left axis, right bars refer to right axis; C) HPLC stability of the API (NCM) in basic ethanol conditions (step 1); D) HPLC stability of NCM in the final formulation at 4°C and room temperature (RT); E) HPLC assay of NCM release in the supernatant after ultracentrifugation of formulation (stored at 4°C).
Fig. 4. Efficacy and selectivity towards SARS-CoV-2 virus.
A) NCM (in DMSO) effect on infection (left) and cell growth (right) in Vero E6 cells; B) nano NCM effect on infection (left) and cell growth (right) in Vero E6 cells; C) nano NCM effect on infection (left) and cell growth (right) in hACE2-A549 lung epithelial cells. N=3 technical replicates for each data point.
Fig. 5. Uptake by hACE2-549 cells.
DiD labeled nano NCM formulation (Table 2) was incubated with cells for 24h at 1μM NCM concentration. Punctate pattern suggest endosomal localization of DiD.
Table 1.

Effect of DSPE-PEG2000 and formulation parameters on the stability of nano NCM. Some formulations include the code (in parathesis) corresponding to Fig. 2A.

| Excipient; experimental variables | Size, intensity weighted | PDI |
|-----------------------------------|--------------------------|-----|
| DSPE PEG2000; 1mg/mL NCM (F23)    | Peak 1: 159.1 ± 73.61 nm, 97.2%  
                                 | Peak 2: 4854 ± 699.9 nm, 2.8%  | 0.238 |
| DSPE PEG2000, no neutralization (F26) | Peak 1: 70.04 ± 7.232 nm, 45.8%  
|                                    | Peak 2: 13.95 ± 1.695 nm, 41.1%  
|                                    | Peak 3: 1.274 ± 0.1315 nm, 13.1% | N/A |
| DSPE PEG2000; double HCl neutralization | Peak 1: 113.3 ± 41.31 nm, 100% | 0.155 |
| DSPE- PEG2000; neutralization before dilution step | Peak 1: 682.7 ± 89.02 nm, 100% | 0.639 |
| DSPE PEG2000; added in water in step 3 (F27) | Peak 1: 265.8 ± 42.98 nm, 70.9%  
|                                    | Peak 2: 72.26 ± 10.4 nm, 29.1%  | 0.692 |
| DSPE PEG2000 67%                   | Peak 1: 224.1 ± 115.4 nm, 100% | 0.242 |
| DSPE PEG2000 33%                   | Peak 1: 226 ± 47.28 nm, 93.4%  
|                                    | Peak 2: 44.67± 5.719 nm, 6.6%  | 0.476 |
| no excipient (F0)                  | aggregation               | N/A |
| no excipient; no neutralization    | aggregation               | N/A |
| no excipient; diluted to 0.1mg/mL and neutralized | aggregation | N/A |
| DSPE-PEG2000; 4mg/mL NCM           | Peak 1: 265.2 ± 85.34 nm, 100% | 0.183 |
### Table 2.

Effect of dilution vehicle and excipients on formulation stability. Some formulations include the code (in parathesis) corresponding to Fig. 2A

| Vehicle       | Excipient; experimental variables (formulation code) | Size, intensity weighted                                                                 | PDI  |
|---------------|------------------------------------------------------|----------------------------------------------------------------------------------------|------|
| water         | DSPE-PEG1000 (F24)                                   | Peak 1: 193.7 ± 105.4 nm, 96.7%  
Peak 2: 5278 ± 420.6 nm, 3.3%                                                | 0.363 |
| water         | DSPE-PEG750 (F25)                                    | Peak 1: 145 ± 38.95 nm, 90.9%  
Peak 2: 46.72 ± 10.42 nm, 9.1%                                                | 0.36  |
| saline        | DSPE-PEG2000                                         | Peak 1: 199.5 ± 76.59 nm, 100%                                                     | 0.13  |
| saline        | DSPE-PEG1000                                         | Peak 1: 213.8 ± 77.69 nm, 98.7%  
Peak 2: 5396 ± 311.9 nm, 1.3%                                                | 0.252 |
| saline        | DSPE-PEG750                                          | Peak 1: 162.9 ± 68.28 nm, 96.8%  
Peak 2: 4698 ± 780.4 nm, 3.2%                                                | 0.230 |
| 5%dextrose    | DSPE-PEG2000                                         | Peak 1: 189.6 ± 60.19 nm, 88.5%  
Peak 2: 56.32 ± 12.9 nm, 11.5%                                               | 0.417 |
| 5%dextrose    | DSPE-PEG1000                                         | Peak 1: 325.5 ± 74 nm, 74%  
Peak 2: 76.85 ± 15.98 nm, 26%                                                | 0.552 |
| water         | 17.6 mg MCD/mg NCM                                   | aggregation                                                                          | N/A  |
| water         | 17.6 mg MCD/mg NCM; DSPE-PEG2000                     | Peak 1: 178.4 ± 76.93 nm, 100%                                                   | 0.157 |
| water         | 17.6 mg MCD/mg NCM; DSPE-PEG1000                     | Peak 1: 157.8 ± 70.21 nm, 100%                                                   | 0.211 |
| water         | 17.6 mg MCD/mg NCM; DSPE-PEG750                      | Peak 1: 160 ± 71.48 nm, 100%                                                     | 0.507 |
| water         | 90% EggPC; 10% DSPE-PEG                              | aggregation                                                                          | N/A  |
| water         | 50% EggPC; 50% DSPE-PEG                              | Peak 1: 330.1 ± 154.6 nm, 92.2%  
Peak 2: 65.52 ± 15.57 nm, 6.7%  
Peak 3: 5205 ± 471.8 nm, 1.1%                                            | 0.277 |
| water         | 67% Cholesterol; 33% DSPE-PEG2000                    | Peak 1: 152.1 ± 66.88 nm, 100%                                                   | 0.162 |
| water         | 50% Cholesterol; 50% DSPE-PEG2000                    | Peak 1: 153.6 ± 73.34 nm, 100%                                                   | 0.188 |
| water         | 33% Cholesterol; 67% DSPE-PEG2000                    | Peak 1: 164.9 ± 89.38 nm, 100%                                                   | 0.216 |
| water         | 16.7% DiD; 83.3% DSPE-PEG2000                        | Peak 1: 145.8 ± 54.20 nm, 99.0%  
Peak 2: 5560 ± 0 nm, 1.0%                                                 | 0.3   |

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### Table 3.

LC-MS/MS quantification of NCM uptake 24 post-incubation (in triplicates)

| Sample                   | NCM, ng/ml | protein, mg/ml | NCM, ng/mg | Percent uptake |
|--------------------------|------------|----------------|-------------|----------------|
| Cells only               | <0.0781    | 1647.0         | –           | –              |
| Cells only               | <0.0781    | 1479.3         | –           | –              |
| Cells only               | <0.0781    | 1625.8         | –           | –              |
| nano NCM 1μM            | 51.312     | 1275.5         | 40.2        | 15.7           |
| nano NCM 1μM            | 45.964     | 1353.6         | 34.0        | 14.1           |
| nano NCM 1μM            | 51.227     | 1631.6         | 31.4        | 15.7           |
| DSPE-PEG2000 only       | <0.0781    | 1423.0         | –           | –              |
| DSPE-PEG2000 only       | <0.0781    | 1323.7         | –           | –              |
| DSPE-PEG2000 only       | <0.0781    | 1485.2         | –           | –              |
| NCM                     | 69.224     | 1211.4         | 57.1        | 21.2           |
| NCM                     | 71.720     | 1220.1         | 58.8        | 21.9           |
| NCM                     | 69.025     | 1286.4         | 53.7        | 21.1           |