Electronic Supplementary Material (ESI) for Nanoscale.

Scalable nanoprecipitation of niclosamide and in vivo demonstration of long-acting delivery after intramuscular injection

James Hobson,[a,b] Alison Savage,[a,b] Andrew Dwyer,[a,b] Catherine Unsworth,[a,b] Jonthan Massam,[a,b] Usman Arshad,[b,c] Henry Pertinez,[b,c] Helen Box,[b,c] Lee Tatham,[b,c] Rajith KR Rajoli,[b,c] Megan Neary,[b,c] Joanne Sharp,[b,c] Anthony Valentijn,[b,c] Christopher David,[b,c] Paul Curley,[b,c] Neill J Liptrott,[b,c] Tom Mcdonald,[a,b] Andrew Owen,*[,b,c,d] Steve P Rannard,*[,a,b,d]

[a]Department of Chemistry, University of Liverpool, Crown Street, L69 7ZD, UK

[b]Centre of Excellence in Long-acting Therapeutics (CELT), University of Liverpool, Liverpool, L7 3NY, UK

[c]Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, L7 3NY, UK

[d]Materials Innovation Factory, University of Liverpool, Crown Street, L69 7ZD, UK

Email: srannard@liverpool.ac.uk

Supplementary Information

Materials

All materials were used without further purification or preparation. Niclosamide (>98% purity) was purchased from Carbosynth LTD (Compton, UK). Analytical grade acetone, methanol, and ethanol were obtained from Fisher Scientific UK LTD (Loughborough, UK), as was tetrahydrofuran (THF), chloroform, dichloromethane (DCM), ethyl acetate, 2-butanone, and Tween 20. Ultra-pure Lipopolysaccharide (LPS) was purchased from Invivogen (Toulouse, France), and Luminex suspension array cytokine/chemokine panel from R&D systems (Abingdon, UK). Niclosamide for bioanalysis and the internal standard (IS) tizoxanide were purchased from Stratech Scientific Ltd (Cambridge, UK). Drug free rat plasma was purchased from VWR International (PA, USA). All other reagents and chemicals were purchased from Merck Life Science LTD (Dorset, UK).

Experimental Methods

Solubility studies

Table S1. Maximum soluble concentrations of NCL in various organic solvents and binary solvent mixtures, all of which were water-miscible and therefore suitable for the nanoprecipitation investigations

Solvent A	Solvent B	Temperature (ºC)	Concentration achieved (mg/mL)
Acetone		20	10
Acetone		50	20
Acetone		65	50
DMSO 85	Acetone 15	60	20
Ethanol 85	Acetone 15	60	20
Ethanol 80	Acetone 20	20	20
Ethanol 80	Acetone 20	60	40
Acetone 80	Ethanol 20	20	30
Acetone 80	Ethanol 20	60	65-70
Butanone		40	20
Butanone 80	Ethanol 20	60	75

Synthesis of 60 wt% loaded niclosamide SDN- HPMC (50 mL) and Sucrose (50 mL) were added to a 250 mL sample jar. Niclosamide (4.5 g) was added to a 175 ml sample jar and dissolved in acetone/ethanol (80/20; 75 mL) at 60 °C. The niclosamide solution was then added dropwise to the excipient solution at a flow-rate of 10 mL min^{-1} using a peristaltic pump. The subsequent dispersion was then sonicated for in three 30s bursts, with agitation in between each run to draw down any solid which had deposited onto the walls of the sample jar (Hieschler UP400s probe sonicator with H14 probe, cycle 1, amplitude 100%). The solution was immediately passed through the spray dryer at a flow-rate of 5 ml min^{-1} (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar) Q-flow gauge 45, Outlet temperature 65 ^{o}C). The powder was collected and gave a yield of 78%.

Physical Analysis of Particle Dispersion by Dynamic Light Scattering- Spray dried powder uniformity was confirmed by measurement of 1 mg mL^{-1} dispersion in saline (0.9% w/v) using a Malvern Panalytical ZetaSizer Ultra Photon Correlation Spectroscope. The Z-average hydrodynamic diameter of 3 measurements was recorded and an average value was reported; the instrument being set to record the intensity using backscattering detector at a temperature of 25ºC, with fluorescent sample filter in place. Data analysis was conducted using the general-purpose model within the ZS Xplorer software and data plotted using Microsoft Excel (Office 365 Edition) and SigmaPlot (version 14).

Chemical Composition Analysis by ^{1}H-NMR Spectroscopy- ^{1}H-NMR spectra were obtained using Bruker Avance spectrometer operating at 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm). The drug composition of the formulations was determined using a known concentration of benzyl methacrylate (BzMA) as an internal standard. Comparison of integrations between the resonances of the internal standard and known resonances of the drug enabled calculation of the moles of drug and therefore the mass of drug within the sample. Niclosamide formulations were run in DMF-d_7 with a 5 mg mL^{-1} concentration of BzMA (figure S1)

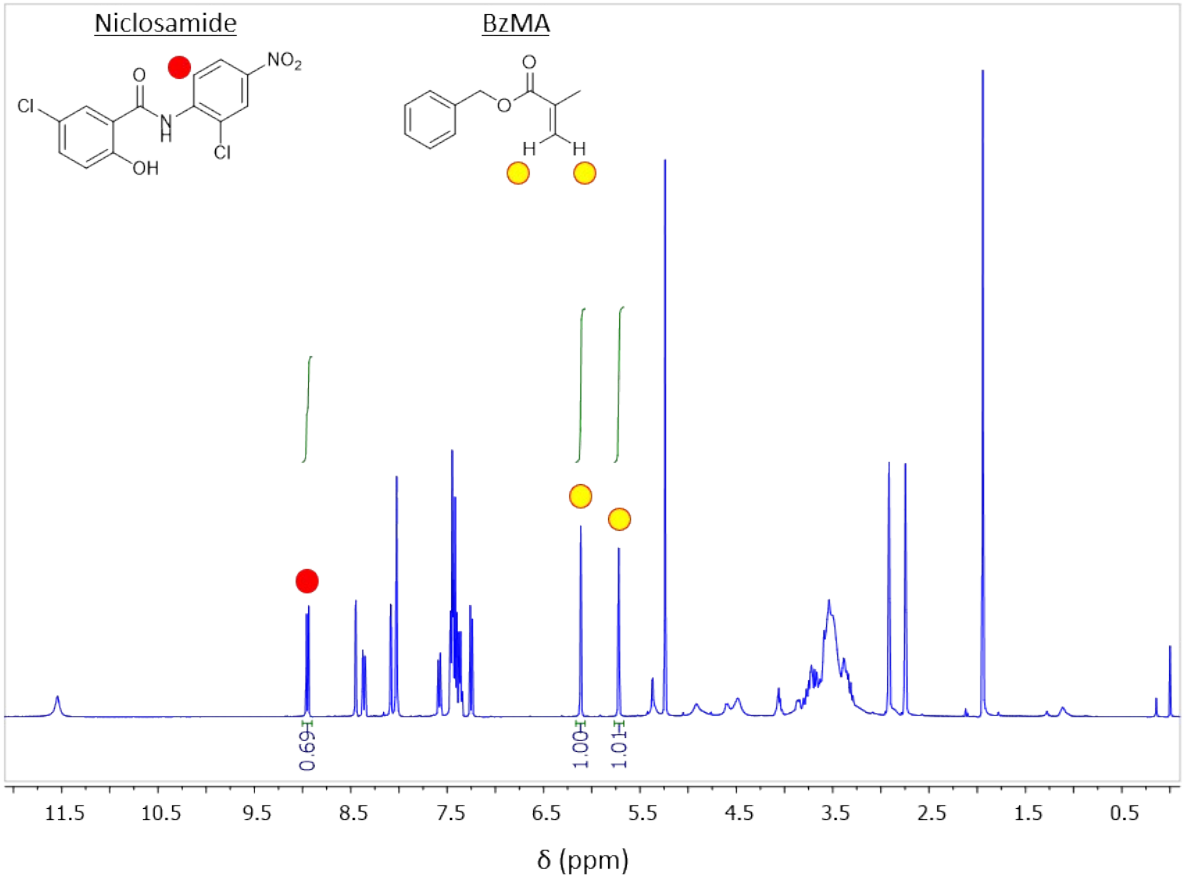

Figure S1. ^{1}H-NMR (DMF-d_7) analysis of niclosamide/HPMC/sucrose (60/20/20 wt.%) large-scale formulation. Sample mass of 10.6 mg. Benzyl methacrylate (BzMA, 5 mg mL^{-1}) as internal standard. Moles of BzMA within the NMR sample = 2.837 x10^{-5} mol. Therefore, 1.958 x10^{-5} mol and 6.4 mg of niclosamide within the NMR sample. Calculated drug composition of 60 wt.%.

Preparation of highly concentrated NCL SDNs for *in vivo* studies- Spray dried power was generated as detailed above and collected in a glass sample jar. *In vivo* samples were weighed out into individual 4 mL glass vials such that upon addition of sterile water for injection, concentrations of up to 200 mg mL^{-1} (relative to NCL mass) could be achieved. In order to homogeneously disperse samples were agitated for around 30-60 seconds using a benchtop vortex mixer, with the resultant viscous dispersion being able to be taken up into a 25G hypodermic syringe. The 60 wt% loading of NCL in the SDN formulations was taken into account to ensure that reported concentrations (both in mg mL^{-1} and mg kg^{-1}) correspond to pure API, rather than total formulation mass (API+HPMC+Sucrose)

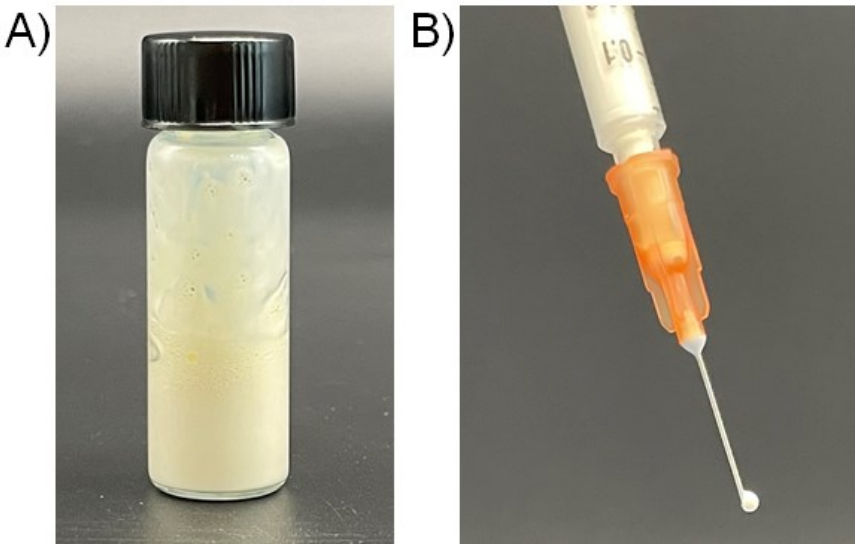

Figure S2. NCL formulation at 175 mg mL^{-1}. A) Formulation after spray drying and redispersion of the powder into water) B) Formulation within a syringe fitted with 25 gauge needle

Physical Analysis of NCL formulations by Scanning Electron Microscopy

Samples of the spray-dried powders and aqueous dispersions of NCL (dried) were analysed using a Tescan S8000G FIB/SEM fitted with a thermal Field Emission Gun capable of producing 400 nA of beam current. Powders were dispersed at 0.5 mg/mL into deionised water before drying onto SEM sample stubs and coated with gold.

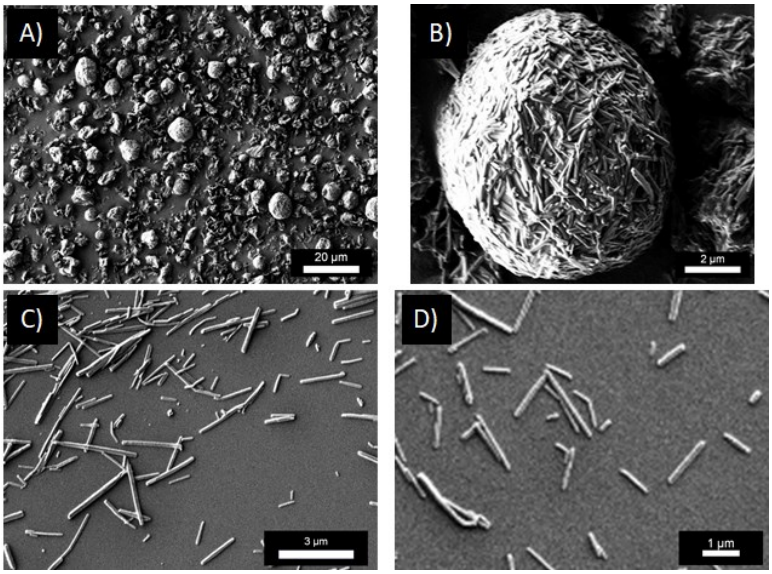

Figure S3. SEM microscopy of NCL formulations – A) spray dried powder at low magnification; B) individual spray dried powder particle at increased magnification; C) dried aqueous dispersion dried (0.5 mg mL^{-1}) at low magnification; D) dried aqueous dispersion dried (0.5 mg mL^{-1}) at increased

***In vivo* analysis of long acting injectable niclosamide-** Following 7 days acclimatisation, adult male Sprague Dawley rats (300–350 g) (Charles River, UK) were dosed with 50, 100, or 200 mg kg^{-1} doses of niclosamide SDN or vehicle alone, delivered as intramuscular injections. Doses were made up within a vehicle of 20% HPMC, 20% sucrose and 60% water and administered as a 0.2 mL volume through a 25-gauge needle. Each animal received two injections, one in each thigh, of a dose of either 25, 50 or 100 mg kg^{-1} niclosamide SDN or vehicle alone. The rodents were housed with environmental enrichment and a 12 h light/dark cycle at 21 °C ± 2 °C, with access to food and water provided at all times. Blood samples were taken via tail vein bleed 1, 3, 6 and 24 hours post dose followed by bleeds at day 3, 4, 7 and 14. At day 28, animals were sacrificed via cardiac puncture under terminal anaesthesia (isoflurane/ oxygen), followed by exsanguination of blood from the heart. An overdose of sodium pentobarbitone (Animalcare, UK) was then administered using the same in situ needle. Blood samples were collected in heparinised eppendorf tubes and centrifuged at 3000 rpm for 5 minutes. Plasma was then collected and stored at −80 °C. For cytokine assessments whole blood was used, whereas plasma was used for drug quantification. All animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) implemented by the UK Home Office and reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Liverpool. This work was conducted under project licence PP9284915.

Niclosamide bioanalysis in rat plasma- Niclosamide was quantified from rat plasma using a previously validated LC-MS/MS assay[1,*]. In brief, niclosamide was extracted via protein precipitation using 500uL of ACN. Detection was performed using a SCIEX 6500+ QTRAP mass spectrometer coupled with an Exion AD liquid chromatography system. Chromatographic separation was achieved using a multi-step gradient with H2O with 0.1% formic acid and ACN with 0.1% formic acid run on a kinetex C18 column (100x2.1mm, 2.6uM, Phenomenex, UK).

* all original data are available from the authors on request.

Pharmacokinetic modelling and analysis- A PK model with biexponential elimination and first order input was fitted to the Niclosamide plasma concentration timecourse data in the R programming environment (v 4.0.3)[2] making use of the Pracma library[3] and lsqnonlin function for nonlinear regression with a weighted least-squares objective function. The PK model parameterisation was kept in a basic, sums of exponentials form to avoid any implicit inferences that fitting a standard compartmental mammillary PK model parameterisation to data following this dose formulation might make regarding Niclosamide disposition in rats. Data following IV dose will be required to confirm, but it is very likely that the observed PK profile here is flip-flop in nature, reflecting the slow release of niclosamide in this formulation from the injection site; parameters from a compartmental PK model fitting to this data could therefore show a mismatch with genuine disposition parameters for niclosamide in the rat. The model fitting is therefore acting solely as a convenience function to provide a summary of the PK profiles as a function of dose and in terms of half-lives of the exponential phases, AUC and apparent clearance.

Determining cytokine concentrations, and response to inflammatory stimuli, in rats exposed to niclosamide- Following termination of the study, blood samples were collected via cardiac bleed from rats exposed to excipients only (hereafter referred to as controls), 50 mg kg^{-1} or 100 mg kg^{-1} of niclosamide. Whole blood was separated into two sample sets for either direct quantification of cytokines or treated with lipopolysaccharide (LPS, 20ng mL^{-1}) and phytohaemagglutinin (PHA, 10 µg mL^{-1}) for 24 hours. Plasma was isolated from whole blood samples by centrifugation (2000 rpm, 5 minutes). Subsequent plasma samples were then assayed for cytokine concentrations using the Luminex Rat Discovery 17-plex panel, including analytes for CXCL2, CXCL3, GM-CSF, ICAM-1, IFNγ, IL-1α and β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, L-selecting, TIMP-1, TNFα and VEGF according to the manufacturer's instructions.

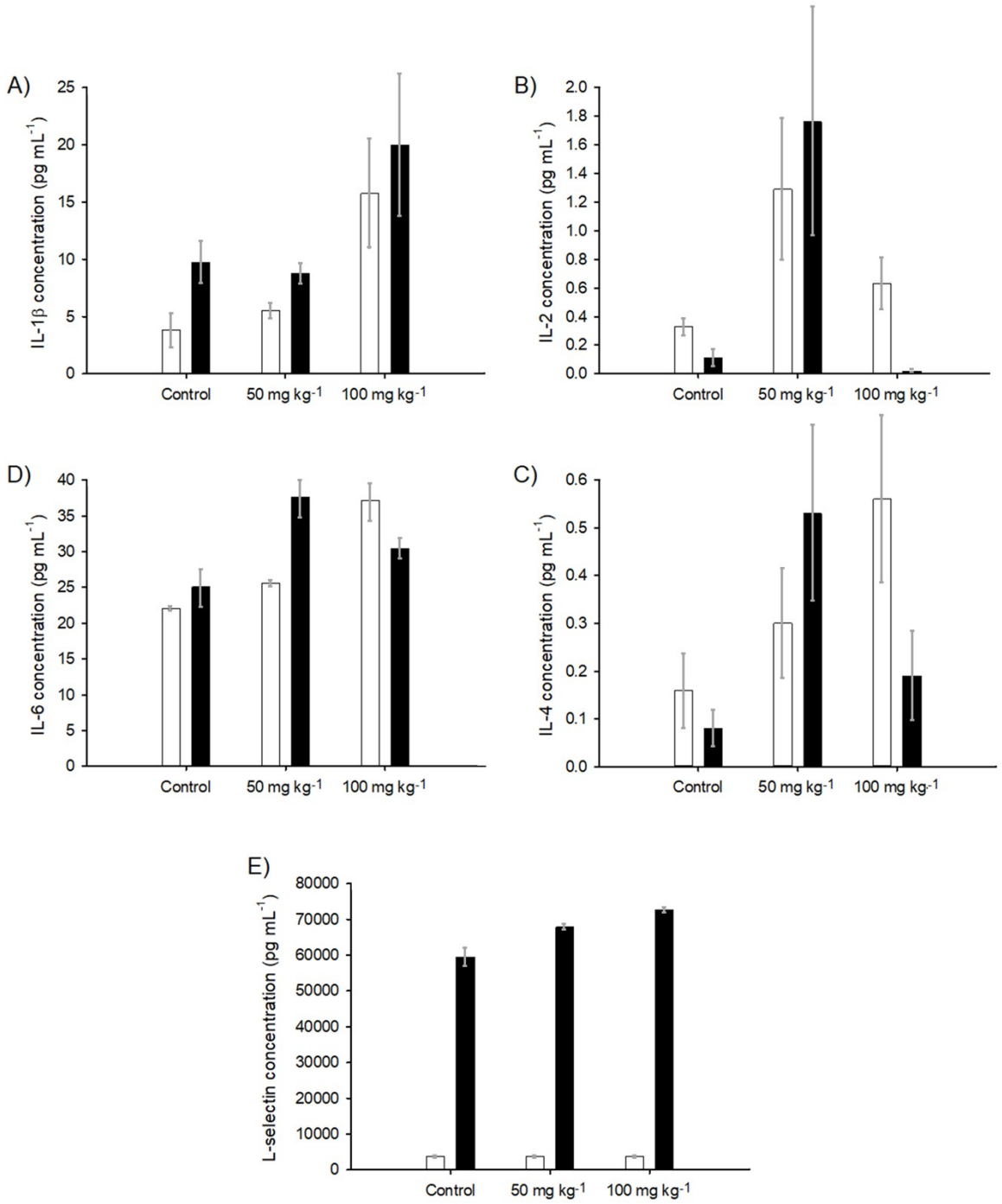

Figure S4. Summary of cytokine concentrations, and responses, in the peripheral blood of rats exposed to niclosamide long-acting formulations. Rats were administered either excipients, as controls or niclosamide at a dose of 50 mg kg^{-1} or 100 mg kg^{-1} for 28 days. Cytokine and chemokine concentrations were determined via multiplex analysis either directly (white bars; unstimulated) or following stimulation with LPS and PHA (black bars; stimulated), for 24 hours. Concentrations of IL-1 (a), IL-2 (b), IL-4 (c), IL-6 (d) and L-selectin (e) were altered following exposure to niclosamide Data expressed as mean values from each experimental group (N=4 animals per group);

References

1. U. Arshad, H. Pertinez, H. Box, L. Tatham, R. Rajoli, M. Neary, J. Sharp, A. Valentijn, J. Hobson, C. Unsworth, A. Dwyer, A. Savage, T. O. McDonald, S. Rannard, P. Curley, A.Owen, bioRxiv 2021.01.13.426426; DOI: https://doi.org/10.1101/2021.01.13.426426
2. R. C. Team, *R Foundation for Statistical Computing, Vienna, Austria*, 2020.
3. H. W. Borchers, *R package version 2.2.9*, 2019.