Artemisinin inhibits innate immune cell chemotaxis, cytokine production and NET release

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Abstract

Immune cell chemotaxis to the sites of pathogen invasion is critical for fighting infection, but in life-threatening conditions such as sepsis and Covid-19, excess activation of the innate immune system is thought to cause a damaging invasion of immune cells into tissues and a consequent excessive release of cytokines. In these circumstances, tempering excessive activation of the innate immune system may, paradoxically, promote recovery. Here we identify the antimalarial compound artemisinin as a potent and selective inhibitor of neutrophil and macrophage chemotaxis induced by many chemotactic agents. Artemisinin released calcium from intracellular stores in a similar way to thapsigargin, a known inhibitor of the Sarco/Endoplasmic Reticulum Calcium ATPase pump (SERCA), but unlike thapsigargin, artemisinin blocks only the SERCA3 isoform. Inhibition of SERCA3 by artemisinin was irreversible and was inhibited by iron chelation, suggesting iron-catalysed alkylation of a specific cysteine residue in SERCA3 as the mechanism by which artemisinin inhibits immune cell motility. In murine infection models, artemisinin potently suppressed immune cell invasion into both peritoneum and lung *in vivo* and inhibited the release of cytokines/chemokines and neutrophil extracellular traps (NETs). This work suggests that artemisinin may have value as a therapy in conditions such as sepsis and Covid-19 in which over-activation of the innate immune system causes tissue injury that can lead to death.

Introduction

An important cause of the deterioration leading to death in Covid-19 patients is thought to be an excessive release of pro-inflammatory cytokines from innate immune cells, that precipitates the severe lung condition named acute respiratory distress syndrome (ARDS)[1]. Other organs are also often affected in Covid-19, in a syndrome more akin to systemic sepsis[2]. ARDS in Covid-19 may be attributable to overactivation of the innate immune system, leading to (i) neutrophil invasion of the lung[3]; (ii) excess release of pro-inflammatory cytokines[1,2]; and (iii) release of Neutrophil Extracellular Traps (NETs), sticky aggregations of DNA, histones and other proteins released from neutrophils[4]. In more localised infections, pro-inflammatory cytokines and chemokines perform a beneficial role, as chemoattractants that recruit immune cells to join the attack on invading pathogens, while NETs play a vital role in the physical snaring and immobilisation of pathogens. In sepsis, ARDS or Covid-19, however, both excess cytokine and NET release are thought to be critical in the events leading to clinical deterioration, organ dysfunction and death[1–3, 5].

In the present study, we identified the natural compound artemisinin, which has achieved significant success as a front-line antimalarial, as a potent inhibitor of immune cell chemotaxis induced by a wide variety of chemoattractant molecules. We show that artemisinin suppresses neutrophil invasion *in vivo* and also successfully inhibits cytokine/chemokine secretion and NET release. In view of the favourable clinical profile of artemisinin and its analogues, we suggest that these compounds may be useful as therapies in conditions such as systemic sepsis, ARDS and Covid-19.

Materials And Methods

Animals

Black C57BL/6 WT mice (6–8 weeks old) were purchased from Charles River Inc. All animal work was conducted under UK Home Office personal and project licences, approved by the Animal Welfare Ethical Review Board (AWERB) of King's College London and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and in compliance with the ARRIVE guidelines.

Chemicals and reagents

Hydrogen peroxide (H_2O_2, 31642), adenosine 5'diphosphoribose (ADPR, A0752), thioglycolate (70157), lipopolysaccharide (LPS, LPS25), CXCL2 (SRP4251), complement component 5a (C5a, SRP4895A), *N*-(p-Amylcinnamoyl)anthranilic acid (ACA, A8486), lumefantrine (PHR2186), mefluoquine (PHR1705), hydroxychloroquine (PHR1782) and arteether (SML2592) were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK). Pluronic F-127 (P3000MP) and Fura-2AM (F1221) were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific Life Technologies, Waltham, Massachusetts, U.S). The RAL DIFF-QUIK kit (a modified version of the May-Grünwald-Giemsa stain) (RAL555) was purchased from RAL Diagnostics, (RAL Diagnostics, Martillac, France). Deoxyartemisinin (2-deoxy-artemisinin) (20428) and artemether (11815) were purchased from Cambridge Bioscience (Cambridge UK). Artesunate (A3731), thapsigargin (T9033), desferrioxamine (BP987), L-cysteine (168149), pyrimethamine (BP1227) and dihydroartemsinin (1200520) were purchased from Merck Life Sciences (Feltham, UK). The XTT cell viability kit (9095S) was purchased from New England Biolabs (Ipswich, Massachusetts, U.S.) The natural products: artemisinin, beta-carotene, bisabolol, capsaicin, carvacrol, citral, citronellal, curcumin, D-biotin, ergosterol, eugenol, farnesene, farnesol, ferulic acid, gallic acid, geraniol, hesperidin, isoeugenol, lanosterol, lawsone, limonene, myrcene, *N*-acetylcysteine, neomenthol, (+) – pulegone, (-) – pulegone, quercetin, rutin-hydrate, thymol, vanillin and veratrylamine were kindly donated by Dr Suaib Luqman from CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, Uttar Pradesh, India.

Isolation of mouse peritoneal neutrophils and macrophages

In vitro chemotaxis experiments

Mice were injected i.p. with 3% thioglycolate solution (10 µl/g) and, after 4 h (for neutrophils) or 4 d (for macrophages), were euthanised by cervical dislocation. The peritoneal-covering skin was removed, 5 ml PBS injected into the peritoneal cavity which was massaged gently for 60 s to dislodge cells. The peritoneal fluid was gently extracted by syringe and centrifuged for 10 min at 200 RCF. The supernatant was discarded and cells resuspended in DMEM + 10% FBS. These methods generated cell suspensions containing > 90% of either neutrophils or macrophages, identified through a fast-acting modified version of the May-Grünwald-Giemsa staining and subsequent cell type identification as shown in[8] (neutrophils) and Supplementary Fig 3 (macrophages).

In vivo peritoneal chemotaxis experiments

Mice were injected i.p. with H_2O_2 or LPS and at experimental time points (see methods below), mice were euthanised by cervical dislocation. The peritoneal lavage was recovered as above, and samples of the suspensions were immediately spun down onto glass slides using a cytocentrifuge (Sigma 2-7 Cyto, Shandon^{TM}, Germany as described below) and leukocytes (neutrophils, macrophages) identified through a fast-acting modified version of the May-Grünwald-Giemsa staining and subsequent cell type identification as shown in Morad *et al.* 2021. The remaining cell suspension was then centrifuged for 10 min at 200 RCF and supernatants were collected and frozen at -20^{o}C for cyto/chemokine analysis by ELISA and cf-DNA(NET) quantification using Quant-iT™ PicoGreen kit (Thermo Fisher).

Isolation of mouse BALF neutrophils

The nostrils of mice were infused with H_2O_2 or LPS and at experimental time points (see lung methods below), mice were euthanised by destruction of the brain. The mice were placed in the supine position, limbs were secured and the skin around the neck was removed. Salivary glands were separated to reveal the sternal hyoid muscle and forceps used to incise the muscle around the trachea. A cotton suture was then threaded under the tracheal tissue. A needle was then used to puncture the middle of the trachea between two cartilage rings and a pre-made plastic catheter was inserted ~0.5 cm into the tracheal lumen and stabilised with the suture. A syringe, loaded with 1 ml PBS was then attached to the catheter and PBS slowly injected. The thorax was massaged gently for 60 s, before BAL fluid was aspirated. This was repeated 3 times to maximise the BAL fluid recovery.

Samples of the BAL fluid were immediately spun down onto glass slides using a cytocentrifuge (as described below) and neutrophils identified through a fast-acting modified version of the May-Grünwald-Giemsa staining and subsequent cell type identification as shown in Morad et al. 2021. The remaining cell suspensions was then centrifuged for 10 min at 200 RCF and supernatants were collected and frozen at -20°C for cyto/chemokine analysis by ELISA and cf-DNA (NET) quantification using Quant-iT™ PicoGreen kit (Thermo Fisher).

Cell identification in peritoneal and BALF extracts

Cell suspension was isolated from peritonea/lungs of WT mice as above, spun down onto glass slides using a cytocentrifuge at 400 RPM for 5 min and left to air-dry overnight. A modified version of the May-Grünwald-Giemsa staining was used to identify cell types (RAL DIFF-QUIK kit, RAL diagnostics). Slides were suspended in RAL Diff-Quick fixative solution (methanol based solution to stabilize cellular components) for 1 min, in RAL Diff-Quik solution I (Xanthene solution; a buffered solution of Eosin Y) for 1 min and in RAL Diff-Quik solution II (a buffered solution of thiazine dyes, consisting of methylene blue and Azure A) for 1 min. Nuclei were meta-chromatically stained red/purple and cytoplasm pink/yellow (see ref[8] and Supplementary Fig 4).

Neutrophil and macrophage chemotaxis assays

Ibidi μ-slide chemotaxis assay chambers, precoated with collagen IV along the central migration strip, were purchased from Thistle Scientific Ltd (Uddingston, Glasgow, UK). Neutrophils or macrophages, isolated as above from peritonea of WT mice, were re-suspended within 30 min of collection in DMEM + 10% FBS at a concentration of 5×10^5 cells per ml and 6 μl was seeded along the central migration strip of an ibidi μ-slide chamber as per the manufacturer's instructions. Slides were incubated for 1 h at 37 °C in humidified 95% air/5% CO_2, to allow neutrophil/macrophage adherence to the central migration strip. DMEM (without added FBS) with and without added chemoattractant was then added to the wells on opposite sides of the central migration strip. DMEM was from Thermo Fisher Scientific Cat. No. 41966-029. For experiments in which effects of compounds were to be tested, equal concentrations were added to both DMEM + chemoattractant and DMEM wells. Slides were pre-incubated at 37 °C in 95% air/5% CO_2 for 20 min to allow the generation of a gradient of chemoattractant across the 1 mm wide × 70 μm deep central cell migration strip. Live-cell time-lapse microscopy was then conducted using a 10 × lens and dark-field illumination on a Nikon Eclipse Ti-E inverted microscope equipped with the Nikon Perfect Focus System (PFS). The microscope was housed in a temperature-controlled Perspex box (Solent Scientific) at 37 °C, with slides housed in a stage-mounted block in humidified 95% air/5% CO_2. A maximum of 12 individual chambers (4 individual slides, 3 chambers per slide) could be imaged per experiment by using a motorized stage. Stage movement, lens focus and image acquisition were controlled by Nikon NIS Elements software. Experiments were conducted over 2 h for neutrophils and 1 h for macrophages, with images of each assay compartment taken every 2 min. The ImageJ Fiji TrackMate plug-in was employed to track individual neutrophils/macrophages. A chemotaxis and migration plug-in, provided by ibidi, was used to calculate speed and forward migration index (FMI) data from the neutrophil/macrophage tracks.

Calcium imaging of neutrophils

Neutrophils isolated as above from the peritonea of WT mice, were re-suspended in DMEM + 10% FBS at a concentration of 5×10^5 per ml. Neutrophils were plated onto a collagen-coated 13 mm round glass coverslip and incubated at 37 °C in 95%air/5% CO_2 for 1 h to allow neutrophils to adhere. Fura2-AM (5 μM in DMEM) was then added to the cells on the coverslip for 30 min at 37 °C in 95% air/5% CO_2. Solutions were changed as shown in the figures and fluorescence was measured during alternating illumination at 340 nm and 380 nm (OptoScan; Cairn Research Inc, Kent, UK) every 2 s using a Nikon Eclipse Ti inverted microscope with a 40 × lens and iXon 897 EM-CCD camera controlled by WinFluor 3.2 software. $F_{340/380}$ ratios were obtained using FIJI (ImageJ) and converted to calcium concentrations using the equation given by Grynkiewicz et al. with values $R_{max} = 2.501$, $R_{min} = 0.103$, both determined experimentally, full details in Vilar *et al.* and Morad *et al.*

For experiments when calcium signals during chemotaxis up a gradient of chemoattractant were to be recorded (as in Supplementary Video 1), 1 μl of Fura-2 AM solution (50 μg Fura-2 AM + 10 μl pluronic F-127 + 10 μl DMSO) was added to 500 μl of peritoneal neutrophil suspension and incubated for 1 h at 37 °C in 95%air/5% CO_2. Fura-2 loaded cells in suspension were seeded into ibidi chambers as described above and imaged in a Nikon Ti-E microscope with a 40 × phase contrast lens. Fast-moving neutrophils

located in the middle of the central cell migration strip were selected, with typically only one cell imaged per field. Calcium ratio images were obtained with alternating 340 nm and 380 nm epi-illumination supplied by stable LED light sources (Fura-LED, Cairn Research), at 500ms intervals. All images were filtered by a broad-band 510 nm filter and captured with a Photometrics Prime 95B sCMOS camera. Stage movement, focus and image acquisition were controlled by Nikon NIS Elements software. The ImageJ Fiji RatioPlus plug-in was used to generate $F_{340/380}$ ratio images and a rainbow look-up table (LUT) was applied to the ratio images to indicate the level of calcium.

Transfection of HEK293 cells

Human embryonic kidney HEK293 cells were split at a confluency of 80%, resuspended in media to a concentration of 7×10^4 cells per ml and 0.5 ml was plated into a four-well plate containing 13 mm glass coverslips pre-coated with poly-d-lysine (1 mg/ml), ready for transfection the following day. Cells were transfected with 0.5 µg of a plasmid containing cDNA for SERCA1, 2 or 3 using a modified calcium-phosphate protocol, as previously described[47]. Cells were used for calcium imaging 2d post-transfection.

Rat SERCA1a (pMT2) was a gift from Jonathan Lytton (Addgene plasmid # 75182 ; http://n2t.net/addgene:75182 ; RRID:Addgene_75182)[48]. Human SERCA2a (pcDNA3.1+) was a gift from Jonathan Lytton & David MacLennan (Addgene plasmid # 75187 ; http://n2t.net/addgene:75187 ; RRID:Addgene_75187)[49]. Human SERCA3 (pMT2) was a gift from Jonathan Lytton & David MacLennan (Addgene plasmid # 75189; http://n2t.net/addgene:75189 ; RRID:Addgene_75189)[50]

Patch Clamp

Transfection of HEK293 cells with TRPM2, a kind gift from Prof Y. Mori, University of Kyoto, Japan, was carried out as described above. Manual whole-cell patch clamp recording was carried out as previously described[51].

XTT cell viability assay

Peritoneal neutrophils, isolated as above, were seeded into four individual 96 well plates (2×10^5/well) and incubated for 1 hour at 37^{o}C in 95% air/5% CO_2 to allow adherence. Artemisinin was then added to half of the wells on all plates at a 10 µM concentration. Following incubation for: 0 hrs, 12 hrs, 24 hrs and 48 hrs, respectively, 50 µL of XTT/PMS solution was added to all wells, and plates were incubated for a further 2 hrs, before absorbance was analysed on a FLUOstar Omega microplate reader (BMG LABTECH, Buckinghamshire, UK) at 450 nm.

In vivo peritoneal H_2O_2 chemotaxis experiments

WT mice were injected s.c. with either sham or artemisinin/artesunate (either 28mg/kg or 6 mg/kg for both) 30 mins prior to being injected i.p. with H_2O_2 (10 µM in PBS, 10 µl/g body weight) or PBS alone for the control baseline group. Mice were then euthanised over 10-210 mins and peritoneal lavage was

extracted and cell types identified as described above, before supernatants were analysed for cytokines/chemokines by ELISA and for NETs by cf-DNA quantification.

In vivo peritoneal LPS chemotaxis experiments

WT mice were injected s.c. with either sham or artemisinin (28mg/kg) 30 mins prior to being injected i.p. with LPS (30 ng/cavity) or PBS alone for control group. Further sham/artemisinin s.c. injections were administered at 90 and 210 mins, before mice were euthanised at 300 mins and peritoneal lavage was extracted and cell types identified as described above, before supernatants were analysed for cytokines/chemokines by ELISA and for NETs by cf-DNA quantification.

Lung BAL fluid H_2O_2 chemotaxis experiments

WT mice were injected s.c. with either sham or artesunate (28mg/kg or 6 mg/kg) 30 mins prior to having H_2O_2 (10 μM in PBS) or PBS alone for control group infused into both nostrils. Mice were then euthanised after 60 mins and BALF lavage was extracted and cell types identified as described above, before supernatants were analysed were analysed for cytokines/chemokines by ELISA and for NETs by cf-DNA quantification.

Lung BAL fluid LPS chemotaxis experiments

WT mice were injected s.c. with either sham or artesunate (28mg/kg or 6mg/kg) 30 mins prior to having LPS (300 ng in PBS) or PBS alone for control group infused into both nostrils. Further sham/artemisinin s.c. injections were administered at 90 and 210 mins, before mice were euthanised at 300 mins and BALF lavage was extracted and cell types identified as described above, before supernatants were analysed were analysed for cytokines/chemokines by ELISA and for NETs by cf-DNA quantification.

Analysis of cytokines and chemokines in peritoneal and lung fluid

At the indicated times after injection of the stimuli (H_2O_2 or LPS), animals were terminally anesthetized and the peritoneal lavage or BALF was collected in PBS. IL-6, IL-1β, CXCL1 and CXCL2 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (DuoSet; R&D Systems) as previously described[52]. The results are expressed as pg/mL of each cytokine/chemokine. As a control, concentrations of these cytokines/chemokines were measured in mice injected with vehicle (PBS).

Quantification of cf-DNA (NETs) in peritoneal and lung fluid

Peritoneal lavage or BALF were collected at different time points after injection of stimuli (H_2O_2 or LPS) and the amount of cell free-DNA (cf-DNA) was quantified using the Quant-IT™ PicoGreen® kit (Thermo Fisher) according to the manufacturer's instructions. The fluorescence intensity (excitation at 488 nm and emission at 525 nm wavelength), a measure of the amount of DNA, was quantified by a fluorescence

reader (FlexStation 3 Microplate Reader, Molecular Devices, CA, USA) as previously described[53]. The results are expressed as ng/mL of cf-DNA.

Results

Artemisinin is a potent inhibitor of neutrophil and macrophage chemotaxis

Hydrogen peroxide (H_2O_2) is known to act as a potent immune cell chemoattractant[6,7]. In previous work we have shown that the TRPM2 ion channel, which is activated by H_2O_2, mediates the chemotactic action of H_2O_2 by preferentially inducing a calcium influx at the neutrophil leading edge[8]. We initially searched for inhibitors of neutrophil chemotaxis by screening a natural compound library, using neutrophil chemotaxis towards H_2O_2 as the assay. Figure 1A shows the forward migration index (FMI), the ratio of linear distance travelled in the direction of the H_2O_2 gradient to the total distance travelled, which gives an index of the directionality of cell movement. Interestingly, capsaicin, a TRPV1 agonist[9] and eugenol, a TRPV3 agonist[10] both significantly *potentiated* directional chemotaxis, perhaps because they have a weak agonist action at TRPM2. Of the compounds that caused an inhibition of chemotaxis, five were identified as interesting for further investigation (red boxes in Fig. 1A), based on a significant reduction in FMI together with a significant reduction in average speed of migration (Supplementary Fig. 1A). The dose-response relations of four of these compounds, beta-carotene, curcumin, ferulic acid and N-acetylcysteine, were similar, suggesting a common action, while artemisinin was more potent (Supplementary Fig. 1B). The four compounds showing a similar potency are antioxidants, so we investigated the possibility that they may act indirectly by dissipating the gradient of H_2O_2. To test this idea, we used a gradient of adenosine diphosphate ribose (ADPR), which like H_2O_2 is a potent neutrophil chemoattractant that acts via TRPM2[8,11]. ADPR directly activates TRPM2 at an intracellular location[12–14], while H_2O_2 does not directly activate TRPM2 but acts by increasing intracellular levels of ADPR[13,14]. When a gradient of ADPR was used to activate neutrophil chemotaxis (Fig. 1B) none of the four antioxidant compounds was able to inhibit chemotaxis, showing that their action was indeed to dissipate the H_2O_2 gradient rather than to directly inhibit chemotaxis. Artemisinin, on the other hand, inhibited neutrophil chemotaxis towards both ADPR and H_2O_2 (Fig. 1B and 1C, respectively), demonstrating that its action in abolishing chemotaxis is independent of any effect on the gradient of H_2O_2. We next compared the ability of artemisinin to inhibit neutrophil chemotaxis with that of other well-established antimalarial compounds. Artemisinin was the only antimalarial that inhibited immune cell migration (Fig. 1C) and therefore has a unique mechanism of action. We found that artemisinin has no effect on neutrophil viability, ruling out the possibility of a toxic action of artemisinin as a basis for its inhibition of neutrophil chemotaxis (Supplementary Fig. 2).

Figure 1D shows that artemisinin and artesunate (an artemisinin analogue) are highly potent inhibitors of neutrophil chemotaxis driven by H_2O_2, with $IC_{50} \approx 0.3$ nM. Artemisinin and artesunate also strongly

inhibit chemotaxis towards a diverse range of other chemotactic signals, including the chemokine CXCL2 (Fig. 1E), the complement factor C5a and the bacterial cell wall component lipopolysaccharide (LPS) (Supplementary Fig. 3). In each case, the values of IC_{50} for artemisinin and artesunate were close to 0.3 nM, with none being significantly different from the value obtained for chemotaxis towards H_2O_2. Supplementary Fig. 4 shows that macrophage chemotaxis was also potently inhibited by artemisinin and artesunate in a similar way to the effects of these compounds on neutrophils. This work identifies artemisinin as a potent inhibitor of innate immune cell chemotaxis driven by a wide variety of chemoattractant agents.

Mechanism of inhibition of chemotaxis by artemisinin

In previous work we showed that neutrophil chemotaxis depends on the ability of chemoattractants to generate leading-edge calcium “pulses” that determine the direction of cell migration[8]. Supplementary Video 1 shows the generation of calcium pulses in a neutrophil migrating up a gradient of H_2O_2 (left-hand video), and the complete suppression of calcium pulses, together with chemotaxis, in the presence of artemisinin (second-left video). In the presence of a gradient of ADPR, calcium pulses drive chemotaxis in a similar way, and artemisinin also inhibits both calcium pulses and chemotaxis (pair of videos on right). These experiments suggest that artemisinin prevents chemotaxis by inhibiting the generation of leading-edge calcium pulses.

A number of active analogues of artemisinin have been developed for use as antimalarials, including arteether, artemether and artesunate (structures shown in Supplementary Fig. 5). All are rapidly metabolised *in vivo* to dihydroartemisinin (DHA), a more metabolically stable analogue with a longer *in vivo* half-life (~1.3h) than any of its precursors[15,16]. All of these analogues, including the stable metabolite DHA, showed an equally high potency in inhibiting neutrophil chemotaxis towards a range of chemotactic signals (Fig. 1D, E and Supplementary Fig. 4; $IC_{50} \approx 0.3$nM for all analogues). These experiments show that none of the chemical modifications in these artemisinin analogues impacts on a site critical for the inhibitory action of artemisinin on chemotaxis.

An unusual feature of artemisinin is the endoperoxide 1,2,4-trioxane ring (top left in Supplementary Fig. 5). We found that deoxyartemisinin, which lacks the peroxide bridge but is otherwise identical to artemisinin, is completely inactive in inhibiting neutrophil chemotaxis (Fig. 2A), showing that the presence of the peroxide bridge is essential for the action of artemisinin on chemotaxis. The critical role of the peroxide bridge suggests that artemisinin may inhibit its protein target by oxidation. It has been known for many years that hydrogen peroxide can oxidise the sulfhydryl group in cysteine, and that this reaction depends on free ferrous iron[17]. We therefore investigated whether the action of artemisinin on neutrophil chemotaxis also depends on iron. Removing ferrous iron with the specific chelator desferrioxamine completely abrogated the ability of both artemisinin and artesunate to inhibit neutrophil chemotaxis at all concentrations (Fig. 1D, E). Antagonism by desferrioxamine of the inhibition of chemotaxis by artemisinin was also independent of the chemoattractant (Supplementary Fig. 6), even though chemotaxis is controlled by at least two independent intracellular calcium-signalling pathways (see below). These

observations suggest that artemisinin and its active derivatives may inhibit their protein target not by reversible antagonist binding, as has previously been supposed[18,19], but instead by covalent modification of a cysteine residue, catalysed by Fe^{2+}. Artemisinin and its analogues have been shown to be capable of alkylating both cysteine itself[20] and the central cysteine residue in a cysteine-containing tripeptide, glutathione[21], by oxidising and combining with the cysteine sulfhydryl (Supplementary Fig. 5B, C).

SERCA is the cellular target of artemisinin

In previous work we showed that chemotaxis towards H_2O_2 depends on activation of the TRPM2 ion channel[8], and we therefore tested whether artemisinin might inhibit chemotaxis by blocking TRPM2. In patch-clamp experiments with heterologously expressed TRPM2, we activated TRPM2 by alternate positive and negative pulses (Fig. 2B). Artemisinin had no significant effect on the current carried by TRPM2, by contrast with the known TRPM2 blocker ACA, which caused prompt and near-complete current inhibition.

A second reason for discarding TRPM2 as a target is that artemisinin inhibits, with equal potency, chemotaxis towards H_2O_2 or ADPR (Fig. 1), both of which depend on activation of TRPM2 for their chemotactic action[8], and also blocks chemotaxis to a range of cytokines/chemokines (Fig. 1E, Supplementary Fig. 3), whose ability to activate chemotaxis does not depend on TRPM2[8]. The schematic diagram in Supplementary Fig. 10 (steps 1–4) shows H_2O_2 generating leading-edge calcium “pulses” that steer chemotaxis[8]. Leading-edge calcium pulses also steer chemotaxis in response to cyto/chemokines, but they do so via an independent pathway that does not depend on TRPM2[8] (Supplementary Fig. 10, steps 6, 7). The ability of artemisinin to inhibit chemotaxis activated by these two distinct pathways implies that the action of artemisinin must be at a point downstream of the generation of calcium pulses, such as the sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA) that is responsible for refilling subcellular calcium stores, or the store-operated calcium entry mechanism (SOCE) that mediates calcium entry following discharge of subcellular stores[22]. Both SERCA and SOCE are present in neutrophils[23].

Thapsigargin, a potent and selective SERCA blocker[24], evoked an increase in internal calcium concentration in neutrophils, due to release from internal stores (Fig. 2C, black), followed by a return to baseline levels as cytoplasmic calcium was extruded by surface membrane calcium pumps. Readmission of external calcium caused a sustained store-operated calcium entry (SOCE), attributable to store-operated activation of calcium-selective Orai channels in the surface membrane[25]. Artemisinin evoked a similar dose-dependent increase in neutrophil intracellular calcium (Fig. 2C), showing that artemisinin, like thapsigargin, acts to release calcium from intracellular stores of neutrophils and is therefore likely to be a SERCA inhibitor. Artemisinin was effective down to a concentration of 1nM in releasing calcium from intracellular stores, consistent with the high potency of artemisinin in inhibiting neutrophil chemotaxis ($IC_{50} \approx 0.3$nM, Fig. 1D, E). The intracellular calcium release evoked by thapsigargin

was unaffected by the Fe^{2+} chelator desferrioxamine, while calcium release by artemisinin was completely suppressed (Fig. 2C), results that echo the effect of Fe^{2+} chelation on chemotaxis (Fig. 1D, E). At all concentrations of artemisinin, the profile of SOCE following readmission of calcium was similar to that caused by thapsigargin, showing that artemisinin does not interact with SOCE. These experiments identify SERCA as the downstream target of artemisinin in neutrophils.

Artemisinin irreversibly inhibits SERCA3

Thapsigargin is toxic to mammals[26], while artemisinin has an excellent clinical safety record as an antimalarial, a difference that could arise from selective inhibition by artemisinin of a non-critical mammalian SERCA isoform, in contrast to the known ability of thapsigargin to inhibit all three SERCA isoforms equally[24]. SERCA1 is essential for muscle contraction, while SERCA2 is widely expressed in many essential organs. Inhibition of either isoform would therefore be likely to cause significant toxicity. SERCA3, on the other hand, has a more limited expression pattern, which includes expression in immune cells[27]. These considerations suggest that SERCA3 may be the target of artemisinin in neutrophils.

In Fig. 2D-F we overexpressed mammalian SERCA1, 2 or 3 in HEK cells and used the protocol shown in Fig. 2C to test for SERCA inhibition by thapsigargin or artemisinin. In all cases, thapsigargin released calcium from intracellular stores with a similar time course, consistent with its ability to inhibit all SERCA isoforms[24]. Artemisinin, on the other hand, was inactive on cells transfected with SERCA1 and 2 but released calcium with a similar time course to thapsigargin in cells transfected with SERCA3. Removal of Fe^{2+} with desferrioxamine did not affect SERCA3 inhibition by thapsigargin, but completely prevented inhibition of SERCA3 by artemisinin (Fig. 2F). These experiments show that SERCA3 is the mammalian target of artemisinin.

Thapsigargin inhibits SERCA by binding reversibly to a location between membrane-spanning helices 3 and 7, deep within the motile machinery of the calcium pump[28]. The experiments above show that inhibition of SERCA3 by artemisinin depends, on the other hand, on an unusual peroxide bond, not present in thapsigargin, together with the presence of Fe^{2+} as a probable catalyst, suggesting a different mechanism involving irreversible covalent binding to a cysteine residue. In Fig. 2G we used the protocol used in Fig. 2C to test the reversibility of SERCA inhibition by thapsigargin. Following exhaustion of calcium stores by thapsigargin, and consequent calcium influx via SOCE on readmission of external calcium, the intracellular calcium level returned to its normal level over 20min following removal of thapsigargin, showing that SERCA had reactivated and intracellular stores had refilled, thus switching off SOCE. Readmission of thapsigargin again released calcium from intracellular stores, confirming the reversibility of thapsigargin binding to SERCA. However, when the same experiment was repeated using artemisinin, calcium levels remained high, showing that SERCA inhibition was maintained, and on reapplying artemisinin in zero calcium, very little calcium release was observed (Fig. 2G). This experiment confirms that inhibition of SERCA by artemisinin is irreversible on the time scale used.

Artemisinin and analogues suppress in vivo **neutrophil invasion in response to H_2O_2**

The potent action of artemisinin and its analogues in suppressing neutrophil chemotaxis *in vitro* suggests that these compounds may have a similar action *in vivo*, and therefore may potentially be useful as therapeutics in conditions where excess immune cell invasion contributes to the pathology. We tested neutrophil invasion into mouse peritoneum following intraperitoneal injection of 10μM H_2O_2, a concentration that has a maximal effect in activating neutrophil chemotaxis *in vitro*[8]. Supplementary Fig. 7 shows that 10μM H_2O_2 i.p. strongly activated an influx of neutrophils, and that neutrophil invasion was largely suppressed by injections of either artemisinin or artesunate at 28mg/kg s.c., 30min prior to injection of H_2O_2, with a slightly lesser effect at 6mg/kg, a dose close to a typical clinically-used dose for artesunate of 2.4mg/kg i.v. The similar *in vivo* responses to artemisinin and artesunate mirror the similar actions of these two analogues in inhibiting neutrophil chemotaxis *in vitro* (Fig. 1D, E).

The time course of neutrophil invasion in response to i.p. H_2O_2 is shown in Fig. 3A. In this experiment total cell counts are shown; the background level of c. $2x10^6$ cells (lower dotted line) is attributable to the presence of tissue-resident macrophages. Following injection of H_2O_2, neutrophil invasion causes the cell count to rise rapidly, reaching a peak of $6.5x10^6$ cells at 60 min and maintaining that level until 120 min, followed by a return to baseline over the following 90min. Injection of artesunate s.c. 30 min prior to injection of H_2O_2 largely suppresses the neutrophil invasion up to 120min, at which time the effect diminishes owing to the short *in vivo* lifetime of artesunate and its active metabolite dihydroartemisinin[15,16].

Excess release of cytokines/chemokines is thought to be critical in the pathology of conditions such as Covid-19 in which immune cell invasion plays an important role[1,2]. In Fig. 3B – E we used ELISA to measure the concentration of two pro-inflammatory cytokines, IL-1β and IL-6, and two chemokines, CXCL1 and CXCL2. In each case, the profile of increase following injection of H_2O_2 is similar to the profile of neutrophil invasion, rising from a low level to a broad peak at 60-120min, followed by a return to undetectable levels by 210min, a time at which the level of invading neutrophils had declined back to baseline. The suppression caused by prior injection of artesunate is striking, with the cytokine/chemokine increase near-completely abolished in all cases up to 120min. An increase is seen at 150min, in line with the recovery of neutrophil chemotaxis as the effect of artesunate wears off (Fig. 3A).

The release of NETs from neutrophils may augment the damaging effect of cytokines[3,5,29]. In Fig. 3F we examined the release of NETs by assaying cell-free DNA release. The profile is broadly similar to the release of cytokines; NET release shows a broad peak at 30–150 min, followed by a decline to low levels by 210 min as neutrophil invasion reverses. Artesunate completely inhibits NET release at times earlier than 150min.

A similar experiment carried out with infusion of H_2O_2 into the lung (Supplementary Fig. 8) shows that neutrophil invasion, cytokine and chemokine release and NET release are all strongly suppressed by artesunate, as was found in the peritoneum. In summary, the release of cyto/chemokines and NETs in

response to H_2O_2 parallels neutrophil invasion, and the ability of artesunate to inhibit neutrophil invasion has a striking effect in preventing the release of proinflammatory cytokines/chemokines and NETs.

Artemisinin and analogues suppress in vivo **neutrophil invasion in response to LPS**

Lipopolysaccharide (LPS), a constituent of the cell wall of gram-negative bacteria, plays a critical role in the interactions of many bacterial pathogens with the innate immune system[30]. LPS is a potent neutrophil chemoattractant *in vitro*, inducing chemotaxis that is steered by leading-edge calcium pulses[8]. The pathway by which LPS induces neutrophil chemotaxis is different from that activated by H_2O_2, however, because genetic deletion or pharmacological block of TRPM2 does not suppress chemotaxis towards LPS[8] (see Supplementary Fig. 10). In the experiment shown in Supplementary Fig. 9, we tested the ability of LPS to induce peritoneal invasion of neutrophils and the effect of artemisinin on this invasion. Neutrophil invasion in response to LPS was activated more slowly than that induced by H_2O_2, so we sampled invasion at 5h, and gave three doses of artemisinin s.c. at intervals of 2h to maintain systemic levels throughout this time. LPS activated neutrophil invasion similar in magnitude to that induced by H_2O_2, and the invasion was also largely suppressed by artemisinin (Supplementary Fig. 9A). The production of cytokines IL1-β and IL-6 and chemokines CXCL1 and CXCL2 was also strongly suppressed by artemisinin (Supplementary Fig. 9B-E), as was NET release (Supplementary Fig. 9F).

The invasion of neutrophils into the lung has been proposed to be critical for the pathogenesis of Covid-19[1–3,5]. We therefore tested whether artemisinin and its analogues are effective in suppressing neutrophil invasion into the lung, and what effect these treatments have on cytokine/chemokine and NET release. Figure 4A shows that lung neutrophil invasion in response to LPS was strongly suppressed by artesunate at both 28mg/kg and 6mg/kg, the latter dose being close to the clinically used dose of 2.4mg/kg. Production of the pro-inflammatory cytokines IL1-β and IL-6, and chemokines CXCL1 and CXCL2, was strongly suppressed (Fig. 4B-E), and in addition the release of NETs, as assayed from DNA release, was also inhibited (Fig. 4F). In summary, these experiments show that artemisinin and its analogues potently suppress neutrophil invasion into both peritoneum and lung, and almost totally inhibit release of cytokines, chemokines and NETs.

Discussion

The work described here shows that artemisinin and its active analogues are potent inhibitors of mammalian immune cell chemotaxis. We find that artemisinin inhibits chemotaxis by blocking the generation of the leading-edge calcium signals that are required for innate immune cell chemotaxis. Artemisinin inhibits only one isoform, SERCA3, of the three mammalian SERCA calcium pump isoforms that are responsible for filling intracellular stores with calcium. We also find that artemisinin and its analogues are highly effective at reducing neutrophil chemotaxis and inhibiting cytokine/chemokine and NET release *in vivo*, in both peritoneum and lung.

There are several reasons for thinking that the mechanism of action of artemisinin is the same for inhibition of immune cell chemotaxis and killing of malaria parasites: in both cases the potency is high ($IC_{50} \approx 5$ nM for malarial killing[31] vs. $IC_{50} \approx 0.3$ nM for inhibition of neutrophil chemotaxis, see Fig. 1); efficacy is completely abolished in both cases by replacing the unusual peroxide bridge with a single oxygen (ref.[18] and Fig. 2A); and the action depends in both cases on low micromolar concentrations of free ferrous iron as a catalyst (refs[18,32] and Fig. 1D, E). Thus, discovering the mechanism of action of artemisinin in inhibiting neutrophil chemotaxis is likely also to give clues to the mechanism of action in killing malaria parasites. The lack of toxicity of artemisinin in mammals, which express three SERCA isoforms, is explained because the critical isoforms SERCA1 and 2 are insensitive to artemisinin (Fig. 2D-F). Malaria parasites, on the other hand, express a single SERCA isoform (also known as PfATP6)[18]. Malarial SERCA was proposed some years ago to be the target of artemisinin[18], but subsequent studies did not confirm this work[33,34] and the idea has not gained general acceptance in the field. The work in this paper suggests that malarial SERCA is indeed likely to be the target of artemisinin, as originally proposed[18].

How can artemisinin achieve selective inhibition of SERCA3 but not the closely-related isoforms SERCA1 and SERCA2? Alkylation of a specific cysteine residue in SERCA3 could be achieved if a high-affinity binding pocket for artemisinin was located adjacent to the target cysteine residue in SERCA3 but not in other isoforms. The SERCA pump undergoes large structural rearrangements during its active cycle[35], and it is therefore plausible that the addition of a bulky residue such as artemisinin, coupled irreversibly to a cysteine residue in a critical location, could be responsible for inhibiting transporter function.

Here we also show that artemisinin and its analogues are potent inhibitors of neutrophil invasion into peritoneum and lung *in vivo* in response to chemoattractants such as H_2O_2 and LPS. The knowledge that the target of artemisinin is SERCA3 gives a molecular basis for past empirical studies using artemisinin in rodent models of lung inflammation and sepsis *in vivo*. These studies have shown that artemisinin and its analogues inhibit cytokine release, reduce lung pathology and significantly enhance survival in response to insults such as lung infusion of lipopolysaccharide or bleomycin, exposure to cigarette smoke, and inflammation caused by systemic sepsis[36–43]. In these studies, artemisinin appeared to have no adverse effects, even at large doses. Our results complement these previous studies, by showing that artemisinin and its analogues inhibit cytokine/chemokine release following injection of LPS, that mimics bacterial infection, and they also show a striking effect in inhibiting NET release. Together with previous work, the results presented here suggest that artemisinin may have value in enhancing survival in conditions such as sepsis, ARDS and Covid-19. Much of the work presented in this paper formed the basis of a proposal to the World Health Organisation (WHO) for the use of artesunate as a therapy for patients seriously ill with Covid-19. This idea is now in clinical trials as part of the ‘SOLIDARITY’ initiative[44–46].

Declarations

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Figures

Figure 1

Screening a natural compound library to identify inhibitors of neutrophil chemotaxis

A. Neutrophils migrated up a gradient of 10nM H_2O_2 over 1mm (see Methods). Forward migration index (FMI, vertical axis), the mean ratio of distance travelled in the direction of the chemoattractant gradient to total distance travelled, is a measure of directional motion. Thirty-one compounds from a natural compound library were tested at 10μM. Five compounds (red boxes) were

selected on the basis that they caused the greatest inhibition of FMI, together with the greatest reduction in speed of movement (Supplementary Fig. 1A). Each bar shows mean ± SEM from n = 3 mice. Statistical analysis: For comparison with Control: **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$ (One-way ANOVA and Tukey–Kramer post-hoc test).

B. Only artemisinin inhibited migration up a gradient of ADPR, a direct activator of TRPM2. All neutrophils in each experiment from same batch; FMI in absence of inhibitor is consistent within batches but maximum value varies somewhat between batches. Each bar shows mean ± SEM from n = 3 mice. Statistical analysis: For comparison with ADPR: ***, p<0.001, ns = not significant. (One-way ANOVA and Tukey–Kramer post-hoc test).

C. The antimalarials pyrimethamine, hydroxychloroquine, mefluoquine and lumefantrine (all 10mM) have no inhibitory effect on neutrophil migration up a 10nM gradient of H_2O_2, by comparison with artemisinin (10mM) in which the FMI was not significantly different from that in the absence of the H_2O_2 gradient. Each bar shows mean ± SEM from n = 3 mice. Statistical analysis: For comparison with H_2O_2: ***, $p < 0.001$, ns = not significant. (One-way ANOVA and Tukey–Kramer post-hoc test).

D. Dose-response relation for inhibition of neutrophil forward migration index (FMI) up a gradient of 10nM H_2O_2 by artemisinin and its analogue artesunate. Dotted lines show mean value of FMI in gradient of H_2O_2 (upper) or in no chemoattractant (DMEM, lower). IC_{50} values: artemisinin, 0.36nM; artesunate, 0.37nM. No inhibition observed in presence of Fe^{2+} chelator desferrioxamine (DesF, 50mM). Each point shows mean ± SEM from n = 3 mice. FMI in artemisinin+DesF/artesunate+DesF is significantly lower than artemisinin/artesunate values at all concentrations >0.1nM (p<0.001).

E. Similar experiment to A, carried out with neutrophils in gradient of chemokine CXCL2 (10nM). IC_{50} values: artesunate, 0.34nM; artemisinin, 0.31nM. Effect of artemisinin and artesunate completely abolished by desferrioxamine (50mM). Each point shows mean ± SEM from n = 3 mice. FMI in artemisinin+DesF/artesunate+DesF is significantly lower than artemisinin/artesunate values at all concentrations >0.1nM (p<0.001).

Figure 2

Mechanism of inhibition of immune cell chemotaxis by artemisinin and analogues

A. Inhibition of neutrophil chemotaxis by artemisinin (10mM, second column) is completely abolished by removal of the peroxide bond in artemisinin (deoxyartemisinin, 10mM, third column). Final column shows FMI in absence of gradient of H_2O_2. Each bar shows mean ± SEM from n = 3 mice. Statistical analysis: For comparison with H_2O_2: **, $p < 0.01$; ns = not significant. (One-way ANOVA and Tukey–Kramer post-hoc test).

B. Artemisinin does not block TRPM2 ion channels. Patch clamp recording of membrane current from TRPM2-transfected HEK293 cell at +80mV (orange) and -80mV (blue). Moment of breaking through to whole-cell mode shown by arrow. Artemisinin (10mM) has no effect on membrane current (fractional current change 0.99 ± 0.04 at -80mV, 0.97 ± 0.03 at +80mV, neither significantly different from 1.0, n = 6), while the known TRPM2 inhibitor N-(p-amylcinnamoyl) anthranilic acid (ACA, 20mM) suppresses membrane current at both membrane voltages (fractional current change 0.02 ± 0.01 at -80mV, 0.06 ± 0.01 at +80mV, both significantly different from 1.0, $p < 0.0001$, n = 6).

C. Application of selective SERCA inhibitor thapsigargin (black trace, 50nM) releases calcium from neutrophil intracellular stores. Calcium influx from external medium prevented with $0Ca^{2+}$/2mM EGTA (application time shown by bar at top). Similar dose-dependent release of intracellular store calcium seen with artemisinin (pink, 10mM; green, 100nM; blue, 1nM) showing that artemisinin is a SERCA inhibitor. Increase of calcium on readmission of external Ca is due to activation of store-operated calcium entry (SOCE) following store discharge, and is similar in all cases showing that artemisinin does not affect SOCE. Calcium release by artemisinin (10mM) is inhibited by Fe^{2+} chelator desferrioxamine (DesF, light pink, 50mM), but DesF has no effect on calcium release by thapsigargin (brown, 50nM). Grey trace: no application of thapsigargin or artemisinin. Left axis: fura2 fluorescence ratio; right axis: $[Ca^{2+}]_i$ determined as in Methods. Example traces shown here and in D,E,F are typical of n = 7-12 cells imaged per cover slip, each condition repeated on 3 cover slips.

D. Similar experiment to E performed on HEK293 cell transfected with rSERCA1a. Calcium is released by thapsigargin (black, 1

M) but not by artemisinin (pink, 10mM). Other conditions as in C.

E. Similar experiment with overexpressed hSERCA2a. Similar results obtained with untransfected HEK cells (SERCA2 is the main isoform expressed in kidney, from which HEK cells are derived).

F. Similar experiment with overexpressed hSERCA3. In contrast to SERCA1 and 2, artemisinin (pink, 10mM) gives a similar calcium release to thapsigargin (black, 1

M). Effect of artemisinin (10mM) is completely inhibited by chelation of Fe^{2+} with desferrioxamine (light pink, 50mM), while calcium release by thapsigargin is unaffected by desferrioxamine (brown, 50mM).

G. Irreversibility of SERCA inhibition by artemisinin. Experimental protocol shown in C – F repeated twice with 21 min gap between applications. SOCE following 1nM thapsigargin recovered and on readmission of 1nM thapsigargin a second release of calcium from subcellular stores was observed. By contrast, SOCE following application of artemisinin (1nM) diminished little and only a very small increase of calcium was observed on reapplication, showing that artemisinin is largely irreversible on the time scale tested here.

Figure 3

Artesunate suppresses neutrophil invasion and release of cytokines and NETs in response to intraperitoneal infusion of H_2O_2.

A. Number of cells recovered from intraperitoneal lavage following i.p. injection of H_2O_2 (10μM in PBS, 10ml/gm body weight). H_2O_2 causes a large influx of neutrophils that peaks at 60min and reverses by 210min (black points). Cells present in peritoneum before injection of H_2O_2 are tissue-resident macrophages, while cells entering the peritoneum following injection of H_2O_2 are neutrophils (see Methods for cell identification). Artesunate (6mg/kg s.c., delivered 30min before injection of H_2O_2) suppresses neutrophil influx for >120min (red points). Each point shows mean ± SEM from n = 4 mice.

B. Concentration of IL-1β in peritoneal lavage measured by ELISA, using i.p. lavage samples obtained as in A. Black bars show increase as function of time (mins) in vehicle-injected mice; open bars are corresponding data for mice injected with artesunate 6mg/kg s.c., 30mins before injection of H_2O_2 as in A. Each bar shows mean ± SEM from n = 3 mice.

C., D., E. Similar data for IL-6, CXCL1 and CXCL2, obtained from same samples.

F. Similar data for release of NETs, quantified using Pico-Green kit.

Statistical analysis: BLQ, below limit of quantitation; *, p<0.05; **, P<0.01; ***, p<0.001, ****, p<0.0001 compared with negative control (no H_2O_2); #, p<0.05; ##, p<0.01; ###, p<0.001; ####p<0.0001 artesunate group compared with no-artesunate group at same time point. ANOVA with Bonferroni post-hoc correction.

Figure 4

Artesunate suppresses neutrophil invasion and release of cytokines and NETs in response to infusion of LPS into lung.

A. Number of neutrophils recovered from broncho-alveolar lavage fluid (BALF), 5h after infusion of LPS (300ng each lung). LPS causes an influx of neutrophils that is suppressed by artesunate, delivered s.c. at 30 min before and at 90 and 210min after injection of LPS. Each bar shows mean ± SEM from n = 4 mice.

B-F. Concentrations of IL-1β, IL-6, CXCL1, CXCL2 and NETs in BALF measured by ELISA and Pico-Green quantification as in Fig. 3, using same experimental protocol as in A. Increase in cytokine concentration

and NET release induced by LPS was suppressed by artemisinin. Each bar shows mean ± SEM from n = 3 mice.

Statistical analysis: BLQ, below limit of quantitation; *, p<0.05; **, P<0.01; ***, p<0.001, ****, p<0.0001, LPS group compared with control group; #, p<0.05; ##, p<0.01; ###, p<0.001; ####p<0.0001, LPS group compared with LPS plus artesunate group at same time point. ANOVA with Bonferroni post-hoc correction.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.pptx
- Supplementaryvideo1.pptx