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# Mechanism of Endosomal TLR Inhibition by Antimalarial Drugs and Imidazoquinolines

Alenka Kužnik,* Mojca Benčina,*,† Urban Švajger,‡ Matjaž Jeras,‡,§ Blaž Rozman,¶ and Roman Jerala*,†,‖

Endosomal TLRs play an important role in innate immune response as well as in autoimmune processes. In the therapy of systemic lupus erythematosus, antimalarial drugs chloroquine, hydroxychloroquine, and quinacrine have been used for a long time. Their suppression of endosomal TLR activation has been attributed to the inhibition of endosomal acidification, which is a prerequisite for the activation of these receptors. We discovered that chloroquine inhibits only activation of endosomal TLRs by nucleic acids, whereas it augments activation of TLR8 by a small synthetic compound, R848. We detected direct binding of antimalarials to nucleic acids by spectroscopic experiments and determined their cellular colocalization. Further analysis revealed that other nucleic acid-binding compounds, such as propidium iodide, also inhibited activation of endosomal TLRs and colocalized with nucleic acids to endosomes. We found that imidazoquinolines, which are TLR7/8 agonists, inhibit TLR9 and TLR3 even in the absence of TLR7 or TLR8, and their mechanism of inhibition is similar to the antimalarials. In contrast to bafilomycin, none of the tested antimalarials and imidazoquinolines inhibited endosomal proteolysis or increased the endosomal pH, confirming that inhibition of pH acidification is not the underlying cause of inhibition. We conclude that the direct binding of inhibitors to nucleic acids mask their TLR-binding epitope and may explain the efficiency of those compounds in the treatment of autoimmune diseases. *The Journal of Immunology*, 2011, 186: 4794–4804.

T he innate immune response is the first line of defense against pathogens and has also been implicated in autoimmune-mediated inflammatory disorders. This response is triggered by activating TLRs with molecules of pathogenic origin or in immune-mediated inflammatory disorders with molecules of host origin. Endosomal TLRs, comprising type I membrane receptors TLR3, TLR7, TLR8, and TLR9, recognize different classes of bacterial, viral, and endogenous nucleic acids. TLR3 is a receptor for dsRNA, TLR7 and -8 are activated by ssRNAs and imidazoquinoline compounds, whereas TLR9 is activated by unmethylated ssDNA (1).

The recognition of pathogen-associated molecular patterns leads to immune defense, whereas the recognition of endogenous Ags through endosomal TLRs can contribute significantly to autoimmune diseases, such as systemic lupus erythematosus (SLE) (2, 3). Patients with SLE have circulating DNA- and RNA-containing immune complexes in the blood (4), which activate plasmacytoid dendritic cells (pDCs) through TLR9 and TLR7, inducing proinflammatory cytokine production and disease development (2, 3). Inhibition of endosomal TLRs has a great therapeutic potential for the treatment of autoimmune diseases (5). The antimalarial drugs chloroquine, hydroxychloroquine, and quinacrine have been used for a long time to treat immune-mediated inflammatory disorders such as SLE, rheumatoid arthritis, and Sjogren's syndrome (5).

Chloroquine and its analog quinacrine inhibit CpG DNA-driven cellular activation (6). Both compounds are weak bases that can partition into acidic vesicles. Therefore, their inhibitory activity has been attributed to the inhibition of endosomal acidification, because acidic pH is a prerequisite of endosomal TLR activation (6–8). CpG-DNA signaling is also efficiently blocked by dominant negative Rab5 and bafilomycin A1, which interfere with endosomal trafficking or acidification, respectively (7, 9). However, although chloroquine, quinacrine, and bafilomycin A1 dose-dependently inhibited CpG-DNA–driven NF-κB activation, SPR biosensor experiments showed that only chloroquine and quinacrine inhibit binding of CpG to the TLR9 ectodomain, whereas bafilomycin A1 does not affect this interaction (10). These findings suggested that chloroquine and quinacrine may act as TLR9 antagonists.

With the aim to clarify the mechanism of endosomal TLR inhibition, we therefore investigated direct binding of antimalarials to nucleic acid TLR ligand. Our results show that interaction between chloroquine or quinacrine and nucleic acids affected their conformation and availability for TLR binding sites. Further analysis revealed that other nucleic acid-binding compounds also inhibit activation of endosomal TLRs. Because of structural similarity between antimalarials and imidazoquinolines, TLR7/8 agonists, which also have been reported to inhibit TLR9 (11), we also investigated the potential similarities in their mechanism of endosomal TLR inhibition. Tested compounds did not inhibit endosomal proteolysis in contrast to bafilomycin, confirming that

inhibition of pH acidification in the presence of antimalarials and imidazoquinolines are not the cause for their inhibitory action. Our findings provide a novel insight into the mechanism of antimalarials and imidazoquinoline's inhibition of endosomal TLR signalization and indicate new therapeutic strategies to inhibit endosomal TLR activation.

## Materials and Methods

### SLE sera collection

Sera of SLE patients with high titers of antinuclear and anti-DNA Abs were tested on the induction of cytokines. Antinuclear Abs were determined by indirect immunofluorescence on HEp-2 cell line substrate (Immuno Concepts). Anti-DNA Abs were detected by the Farr RIA (14C DNA; Amersham Biosciences). Only sera with the titer of antinuclear Abs >1:640 and with the DNA-binding activity >0.70 according to the Farr technique were selected for positive samples. The informed consent of patients to use their sera for research purposes was obtained (National Ethics Committee, grant P3-0314).

### Human cell isolation

Buffy coats from the venous blood of normal healthy volunteers were obtained from the Blood Transfusion Centre of Slovenia according to institutional guidelines. PBMCs were isolated using Lympholyte-H (Cedarlane Laboratories, Ontario, Canada). pDCs were enriched from PBMCs by positive immunoselection using anti-BDCA4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) strictly following the manufacturer's protocol. The cells were washed twice with Dulbecco's PBS, counted, and used for further experiments.

### Cell cultures and reagents

Human embryonic kidney cells (HEK) 293 and HEK293T were cultivated in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (BioWhittaker, Walkersville, MD) at 37°C in 5% $CO_2$. Primary human dermal lymphatic neonatal microvascular endothelial cells (HMVEC-dLy; Lonza) were cultivated in microvascular endothelial cell growth medium-2 (EGM2-MV BulletKit; Lonza) according to the supplier's recommendations. Mouse macrophages RAW264.7 and immortalized mouse macrophage cell line generated from wild-type C57BL/6 mouse were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (BioWhittaker).

Cells were treated with different CpG-oligodeoxynucleotides (ODN): ODN10104 (Coley), ODN2395 (Coley), ODN2006 (InvivoGen), ODN2006n (InvivoGen), polyinosinic-polycytidylic acid [poly(I:C)], and ssRNA40 (both InvivoGen), R848 (Alexis), and A-DNA1 (Operon), either alone or in combination with gardiquimod (Alexis), chloroquine, quinacrine, hydroxychloroquine (Sigma-Aldrich), bafilomycin A1 (LC Laboratories/Sigma-Aldrich), Hoechst 34580 (Invitrogen), and propidium iodide (Fluka; Sigma-Aldrich).

### Cytokine detection

PBMCs were cultured at $5 \times 10^6$ cells/well and pDCs at $1 \times 10^5$ cells/well in 100 μl X-vivo15 Medium (BioWhittaker) in 96-well plates. The cells were treated for 24 h with 20% serum of SLE patients, either negative (negative controls) or positive for anti-DNA Abs (positive samples), in the absence or presence of chloroquine and quinacrine at the concentration of 10 μg/ml. Protein level of cytokines (TNF-α, IL-8, IL-6, and IFN-α) in the supernatants was measured by sandwich ELISA according to the manufacturer's protocol (Bender MedSystems).

HMVEC-dLy cells were cultured at $1 \times 10^5$ cells/well in 100 μl EGM2-MV in 96-well plates. The cells were treated for 24 h with poly(I:C) in the absence or presence of hydroxychloroquine (10 μg/ml), gardiquimod (10 μg/ml), or bafilomycin A1 (0.5 μM). Human IL-6 protein level in the supernatants of HMVEC-dLy cultures was measured by sandwich ELISA according to the manufacturer's protocol (Bender MedSystems).

### Transfection and reporter gene assay

HEK293 cells were harvested from an actively growing culture and plated into CoStar White 96-well plates (Corning) at $6 \times 10^4$ cells/well. After 24 h at 90% confluency, they were transfected with a mixture of the Lipofectamine 2000 reagent and plasmid DNA according to the manufacturer's instructions (Invitrogen). For TLR9-expressing cells, we used 50 ng pUNO-hTLR9-HA plasmid (Invivogen), 200 ng ELAM1-luc reporter plasmid expressing firefly luciferase induced with NF-κB, and 5 ng phRL-TK (Promega) transfection control plasmid expressing *Renilla* luciferase. For TLR8- and TLR7-expressing cells, we used 50 ng pUNO-

hTLR8-HA or pUNO-hTLR7-HA (Invivogen), 150 ng ELAM1-luc reporter plasmid, and 5 ng phRL-TK control plasmid. For TLR3-expressing cells, we used 30 ng pUNO-hTLR3-HA (Invivogen), 50 ng pIFN-β-Fluc reporter plasmid expressing firefly luciferase induced with IFN-β, and 5 ng phRL-TK transfection control plasmid. For TLR5-expressing cells, we used 30 ng pUNO-hTLR5 plasmid (Invivogen), 50 ng ELAM1-luc reporter plasmid, and 5 ng phRL-TK transfection control plasmid. Twenty-four hours posttransfection, the culture medium was replaced with fresh DMEM supplemented with 10% FBS, and the cells were treated with different CpG-ODN (ODN2006, ODN2006n, ODN10104), R848, ssRNA or poly(I:C), and other compounds: quinacrine, chloroquine, gardiquimod, propidium iodide, Hoechst 34580, or A-DNA1. After the cell treatment, 6 h for cell transfected with TLR3 or 18–20 h for cells transfected with TLR8 or TLR9, the cells were lysed in Passive Lysis Buffer (Promega). The expression of the firefly luciferase reporter gene was analyzed using Dual Glo Luciferase Assay System reagents (Promega) and the Mithras LB940 luminometer plate reader (Berthold Technologies). The relative luciferase activity was calculated by normalizing each sample's luciferase activity for constitutive *Renilla* luciferase activity measured within the same sample.

### Western blot analysis

Actively growing HEK293T cells were plated into 12-well plates (TPP) at $2 \times 10^5$ cells/well. After 24 h at 50% confluency, they were transfected with TLR9, TLR3, or TLR8 plasmid and with control vector pcDNA3 (1000 ng DNA/well) using Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). Twenty-four hours posttransfection, the culture medium was replaced with fresh DMEM with 10% FBS, and cells were stimulated with ODN10104 (3 μM), poly(I:C) (10 μg/ml), or with R848 (10 μg/ml) in the absence or presence of chloroquine or quinacrine (10 μg/ml). The cells were lysed 6 h after stimulation (TLR3-transected cells) or 20 h after stimulation (TLR9- and TLR8-transfected cells) using RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS, and 0.5% deoxycholate) with Complete Mini protease inhibitors (Roche) and sonicated for 20 min with 60% amplitude. Samples were centrifuged for 30 min at 13,200 rpm at 4°C. The protein-containing supernatant was harvested, and the remaining pellet was resuspended in fresh RIPA buffer and subjected to a second round of sonication and centrifugation as above. The total protein amount was quantified using the BCA Protein Assay (Pierce). Cell extracts were added sample buffer (SDS with 2-ME) and loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Hybond-ECL; Amersham Biosciences) and detected with the following Abs: rabbit anti-HA (H6908; Sigma-Aldrich), mouse monoclonal anti-TLR3 (IMG-315A; Imgenex), mouse anti–β-actin (3700; Cell Signaling Technology), goat anti-rabbit IgG-HRP (ab6721; Abcam), and goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology). The blots were developed using ECL substrate (Thermo Scientific).

### Confocal microscopy

HEK293T cells ($1 \times 10^6$ cells/well) were plated in eight-well tissue culture chambers (Ibidi; Integrated BioDiagnostics). The following day, cells were transfected with the pcDNA3-TLR9-YFP plasmid (300 ng; Addgene) using the GeneJuice transfection reagent according to the manufacturer's instructions (Novagen; Merck). Twenty-four hours posttransfection, ODN10104-Alexa Fluor 633 (2 μM), quinacrine (1 μg/ml), or propidium iodide (10 μg/ml) was added to the cells and incubated for 18 h. Lysosomes were stained with LysoTracker Red DND-99 (50 nM; Molecular Probes) for 45 min.

DQ-OVA was used to determine the activity of endosomes together with dextran-Alexa Fluor 647 as a control for endocytosis. Nontransfected HEK293T cells were treated with chloroquine (3 μM), hydroxychloroquine (3 μM), gardiquimod (3 μM), R848 (3 μM), or bafilomycin A1 (0.2 μM) for 15–30 min, then DQ-OVA (10 μg/ml; Molecular Probes) and dextran-Alexa Fluor 647 (100 μg/ml; Molecular Probes) were added. Hoechst 34580 (1 μg/ml) was used for labeling cells nuclei, with an incubation period of 15 min. Images of HEK293T or PBMC cells were acquired using a Leica TCS SP5 inverted laser-scanning microscope on a Leica DMI 6000 CS module (Leica Microsystems) equipped with a HCX Plane-Apochromat λ blue 63× oil-immersion objective with numerical aperture 1.4. A 458-nm laser line of the multi-ion argon laser was used for the excitation of quinacrine, and the emission was detected between 500 and 530 nm. The LysoTracker Red DND-99 and propidium iodide were excited with a 1-mW, 543-nm HeNe laser. The light emission was detected at 600–650 nm. TLR9-YFP and DQ-OVA were excited with the 514-nm laser line of the multi-ion argon laser, and their fluorescence was detected between 520 and 550 nm. The

ODN10104-Alexa Fluor 633 and dextran-Alexa Fluor 647 were excited with a 10-mW, 633-nm HeNe laser, and their fluorescence was detected between 650 and 700 nm. A 50-mW, 405-nm diode laser was used for Hoechst 34580 excitation, and fluorescence emission was detected at 500 to 530 nm.

## Fluorescence spectroscopy

Fluorescence measurements were performed at 20˚C in a 5 × 5 mm quartz cuvette (Hellma) with an LS55 spectrofluorimeter (PerkinElmer). Interactions of quinacrine, gardiquimod, or R848 (10 μM) with nucleic acids ODN10104, ODN2006, or A-DNA1 were expressed as a change in fluorescence emission intensity at maximum peak using excitation at 430 nm for quinacrine and 300 nm for gardiquimod and R848. The emission fluorescence spectra for quinacrine and gardiquimod or R848 were recorded between 450 and 580 nm and between 310 and 400 nm, respectively. All compounds were dissolved in 0.01 M phosphate buffers with pH 5, 5.5, 6, 6.5, or 7. A concentrated solution of nucleic acid was added to a 1-ml solution of the compounds, which increased the volume of the solution by <0.03%.

## Circular dichroism measurements

For the nonfluorescent compound chloroquine, its interaction with nucleic acid was determined using circular dichroism (CD) measurements. The CD spectra were taken between 190 and 310 nm for ODN10104, between 210 and 310 nm for poly(I:C), and between 300 and 600 nm for R848 on a Chirascan CD spectrometer (Applied Photophysics) fused with nitrogen gas and equipped with a temperature controlled cuvette holder. A cell path length of 1 mm was used with sample concentrations of 100 μg/ml ODN10104 and 50 μg/ml poly(I:C). All samples were dissolved in 0.01 M phosphate buffer (pH 7). A fixed concentration of ODN10104 and poly (I:C) was titrated with an increasing concentration of chloroquine until saturation was achieved. A cell path length of 10 mm was used for CD spectra measurements of R848 with sample concentrations of 50 μg/ml dissolved in 0.01 M phosphate buffer (pH 5). A fixed concentration of R848 was titrated with an increasing concentration of quinacrine until saturation was achieved. Results are the average of three spectra measured at 20˚C.

## Endosomal pH measurement

The changes of endosomal pH were measured using silica nanoparticles (200 nm) with covalently bound pH-sensitive Oregon Green 514 dye and pH-insensitive Alexa Fluor 633 dye. Different primary cell lines (mouse macrophages, RAW 264.7, and HMVEC) were plated in eight-well tissue culture chambers (Ibidi; Integrated BioDiagnostics). After 24 h, cells were treated with pH-sensitive nanoparticles for at least 3 h. The cells loaded with pH-sensitive nanoparticles were treated with bafilomycin (200 nM) or chloroquine (4 μM) and imaged using a sequential excitation of pH probe. For sequential excitation, a 50-mW, 514-nm line of a 25-mW argon laser and the 633-nm HeNe laser were used. Successive images excited at 514 and 633 nm were captured. Fluorescence emission was detected at 525–570 nm (excitation 514 nm) and 650–700 nm (excitation 633 nm). The ratio between fluorescence intensity of Oregon Green 514 and Alexa Fluor 633 was used to validate changes of endosomal pH. Increased ratio indicates higher endosomal pH in the cells treated with bafilomycin. A Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems) with an HCX plan apo 63× (numerical aperture 1.4) oil immersion objective was used for imaging.

## Flow cytometry

HEK293T cells (2,5 × 10⁵ cells/well) were seeded in a six-well plate. The following day, cells were transfected with the pcDNA3-TLR9-YFP plasmid (2 μg; Addgene) using the GeneJuice transfection reagent according to the manufacturer's instructions (Novagen; Merck). After 48 h, the cells were treated with ODN10104 (3 μM) in the absence or presence of chloroquine (10 μg/ml). After 20 h, the cells were harvested, washed twice with PBS, and resuspended in 500 μl PBS. Flow cytometry analysis was performed on a PICS ALTRA flow cytometer (Beckman Coulter). In each sample, 10,000 cells were analyzed. Collected data were analyzed using a WinMDI flow cytometry application.

## Statistical analysis

Statistical significance was determined using the Student $t$ test (*$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$).
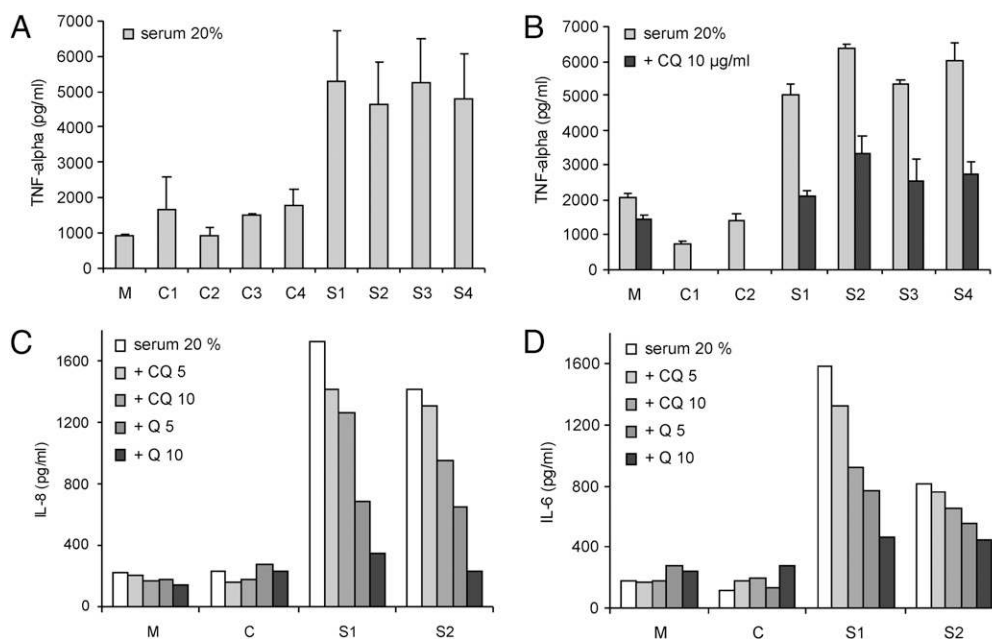


**FIGURE 1.** Chloroquine and quinacrine reduce the production of proinflammatory cytokines in PBMCs treated with anti-DNA Ab-positive sera of SLE patients. *A*, PBMCs were treated for 24 h with 20% serum of SLE patients, negative (negative controls C1, C2, C3, C4) or positive for anti-DNA Abs (positive samples S1, S2, S3, S4), for which binding activities were >0.70 according to the Farr technique. Human TNF-α protein levels in the PBMC supernatants were measured by ELISA. Graphs show mean ± SD (*n* = 3). *B*, PBMCs were treated for 24 h with 20% serum of SLE patients, negative (negative controls C1, C2) or positive for anti-DNA Abs (positive samples S1, S2, S3, S4), for which binding activities were >0.70 according to the Farr technique. Chloroquine (CQ) was added to anti-DNA Ab positive samples at a concentration 10 μg/ml. Human TNF-α protein level in the supernatants of PBMC cultures was measured by ELISA. Graphs show mean ± SD (*n* = 3). *C* and *D*, PBMCs were treated for 24 h with 20% serum of SLE patients, negative (negative control C) or positive for anti-DNA Abs (positive samples S1, S2), for which binding activities were >0.70 according to the Farr technique. CQ or quinacrine (Q) was added at final concentration of 5 or 10 μg/ml. Human IL-8 and IL-6 protein level in the supernatants of PBMC cultures was measured by ELISA. M, X-vivo15 medium.

## Results

### Antimalarials reduce cytokine synthesis in PBMCs exposed to sera of SLE patients

Self-recognition through endosomal TLRs can cause autoimmune diseases, such as SLE (3). Circulating immune complexes consisting of Abs and endogenous nucleic acids activate pDCs and B cells through activation of TLR7 or TLR9 (12–14). Stimulation with nucleic acids leads to high levels of IFN-α production by pDCs (3, 15) and causes proliferation and differentiation of autoreactive B cells into plasma cells, producing pathogenic autoantibodies against nuclear Ags (16). Anti-DNA Abs isolated from sera of SLE patients, when combined with DNA from apoptotic cells to form immune complexes, were shown to induce IFN-α as well as TNF-α production in PBMCs (16, 17). Normal PBMCs were stimulated with 20% serum from SLE patients, either negative (negative controls) or positive for anti-DNA Abs (positive samples). Only SLE sera with very high DNA-binding activity (>0.70 according to the Farr technique) induced TNF-α expression in human PBMCs (Fig. 1A; $p < 0.0001$). Chloroquine inhibited the production of TNF-α (Fig. 1B; $p < 0.001$), whereas the serum from the SLE patient undergoing hydroxychloroquine therapy induced TNF-α expression in human PBMCs in the similar range as sera obtained from healthy volunteers (data not shown). In our experiments, we also detected a dose-dependent decrease in IL-8 (Fig. 1C) and IL-6 (Fig. 1D) production in the presence of chloroquine and quinacrine. Additional experiments on pDCs also revealed inhibitory effect of chloroquine and quinacrine on IFN-α production, which was induced by some SLE sera (Supplemental Fig. 1A). Our results showed that the immune complexes of SLE sera were able to induce the production of different types of proinflammatory cytokines in PBMC containing B cells and pDCs (Supplemental Fig. 1C), which was in all cases inhibited with antimalarial agents.
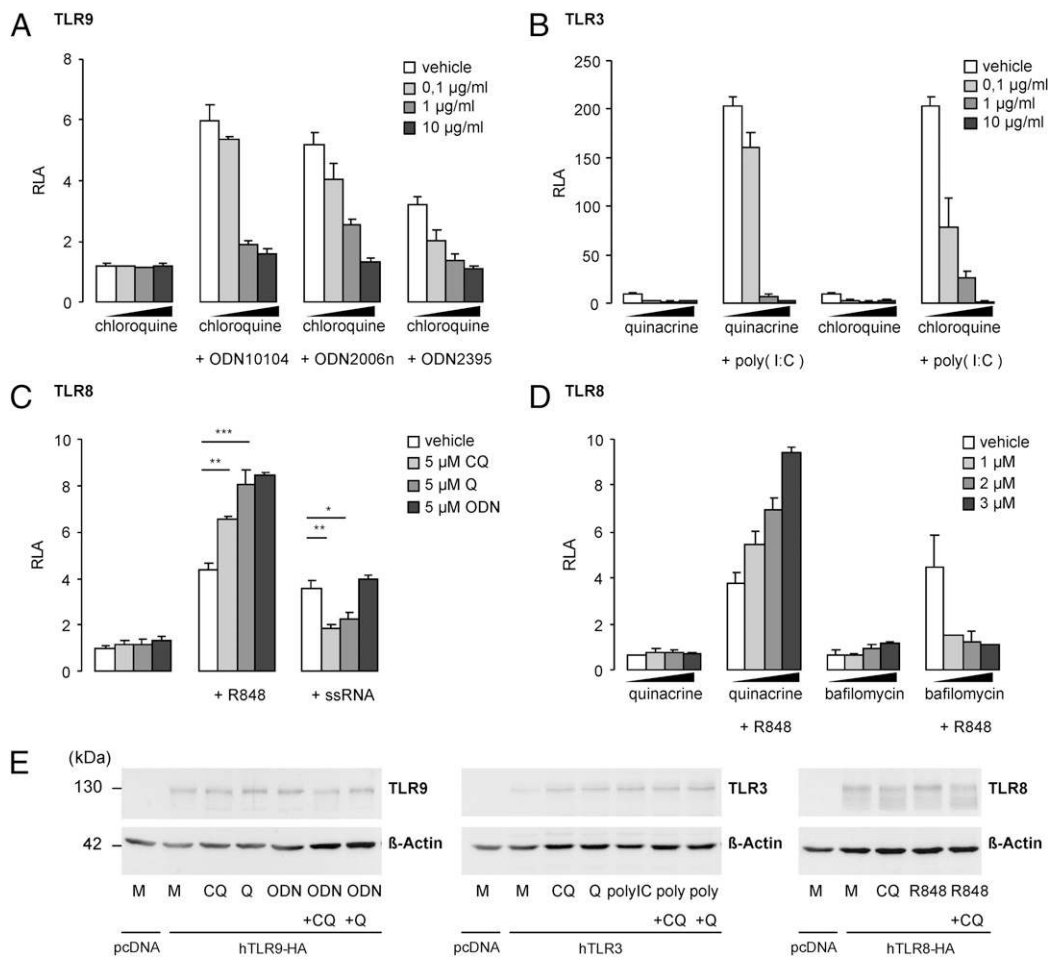


**FIGURE 2.** Chloroquine and quinacrine inhibit activation of endosomal TLRs only after receptor stimulation by nucleic acid ligands without influencing the TLRs expression. *A*, HEK293 cells expressing human TLR9 were stimulated with either ODN10104 (CpG B), ODN2006n (CpG B, fosfodiester bound), or ODN2395 (CpG C) (all 5 μM) in the presence of chloroquine at concentrations of 0.1, 1, or 10 μg/ml. *B*, HEK293 cells expressing human TLR3 stimulated with 10 μg/ml poly(I:C) in the presence of quinacrine or chloroquine at concentrations of 0.1, 1, or 10 μg/ml. *C*, HEK293 cells expressing human TLR8 were stimulated with R848 or ssRNA40 (both 10 μg/ml) in the presence of either chloroquine (CQ), quinacrine (Q), or ODN10104 all at a concentration of 5 μM (i.e., 1.6 μg/ml chloroquine, 2 μg/ml quinacrine, and 37 μg/ml ODN). *D*, HEK293 cells expressing human TLR8 stimulated with R848 at a concentration of 10 μg/ml in the presence of quinacrine or bafilomycin A1 at concentrations of 1, 2, or 3 μM. Concentration of 1 μM represents 0.4 μg/ml quinacrine and 0.62 μg/ml bafilomycin A1. Cells were cotransfected with a reporter plasmid expressing a firefly luciferase with a suitable promoter and with a transfection control plasmid expressing *Renilla* luciferase under a constitutive promoter. Data are represented as mean ± SD (*n* = 3). A representative experiment of three independent replicates is shown. *E*, Western blot analysis of HEK293T cells transfected with either plasmid encoding hTLR9-HA or hTLR3 or hTLR8-HA and plasmid pcDNA3 to prove expression of TLR9, TLR3 and TLR8 after stimulation with their ligands [ODN10104, poly(I:C) or R848] in the absence or presence of chloroquine and quinacrine (10 μg/ml). The blots were probed for β-actin using anti–β-actin Abs and either anti-HA Abs for TLR9 and TLR8 or anti-TLR3 Abs for TLR3. Data shown are representative of repeat experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

*Effect of antimalarials on endosomal TLR inhibition depend on the type of TLR ligand*

Activation of endosomal TLRs plays an important role in the autoimmune process in SLE patients (14, 18–20). To determine the effect of quinacrine and chloroquine on separate endosomal TLRs, we used HEK293 cells transfected with each of the endosomal TLR receptors and stimulated them with different TLR agonists in the presence or absence of quinacrine and chloroquine. TLR9 activation with agonists ODN10104 (B-type CpG, phosphorotioate backbone), ODN2006n (B-type CpG, phosphodiester backbone), and ODN2395 (C-type CpG) was inhibited in a dose-dependent manner with chloroquine (Fig. 2*A*). The presence of 1 µg/ml chloroquine inhibited TLR9 activation by >50%. Flow cytometry confirmed that chloroquine had no effect on the level of TLR9 expression (Supplemental Fig. 2). In a similar experiment, quinacrine and chloroquine inhibited TLR3 activation with poly(I:C) (Fig. 2*B*). However, when we analyzed the activation of TLR8 by two different TLR8 ligands, ssRNA and R848, we demon-

strated that chloroquine and quinacrine inhibited TLR8 activation by ssRNA, but unexpectedly observed a costimulatory effect in combination with R848 (Fig. 2*C*). In contrast, bafilomycin A1, which inhibits vacuolar ATPase and consequently causes a decrease in endosomal pH (21), had an inhibitory effect on TLR8 activation by R848 (Fig. 2*D*). These results showed that chloroquine and quinacrine most likely did not inhibit endosomal TLR activation due to its effect on endosomal pH. Additionally, Western blot analysis showed that chloroquine and quinacrine had no influence on the expression of the receptors TLR9, TLR3, and TLR8 (Fig. 2*E*). Together, these data demonstrated that the effect of the antimalarials is dependent on the type of TLR ligand used for stimulation of the receptor and suggest that antimalarials do not influence the endosomal pH and expression of TLRs.

*Direct interaction of antimalarials with endosomal TLR ligands*

Antimalarial compounds inhibited TLR activation only when nucleic acid ligands were used for TLR stimulation. Therefore, we
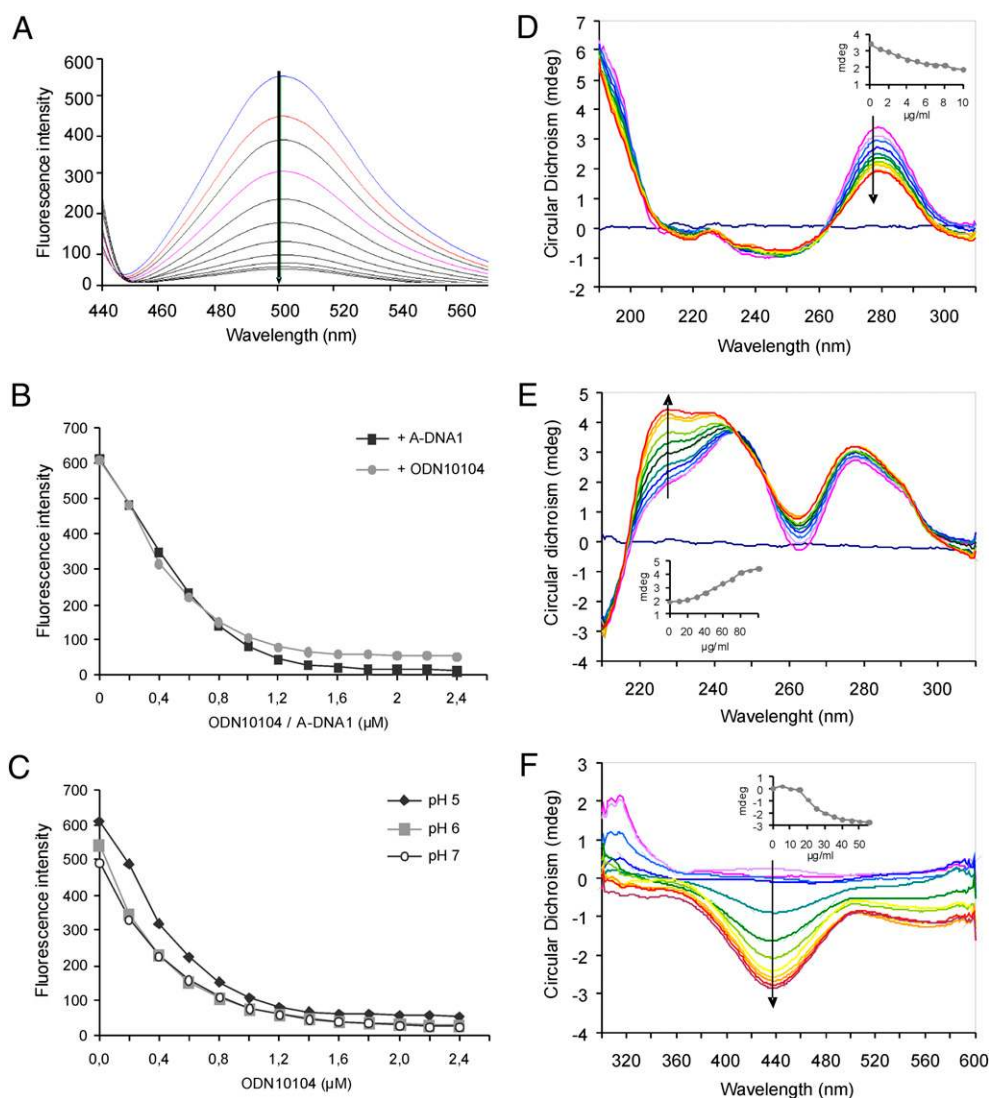


**FIGURE 3.** Quinacrine and chloroquine interact with nucleic acids and influence their structure. *A*, Decrease in fluorescence intensity of quinacrine (10 µM) after titration with ODN10104. The excitation wavelength was 430 nm. *B*, Decrease in fluorescence intensity of quinacrine (10 µM) in the presence of ssDNA (ODN10104) and dsDNA (A-DNA1). *C*, Decrease in fluorescence intensity of quinacrine (10 µM) in phosphate buffer after titration with ODN10104 at the different pH values. The excitation wavelength was 430 ± 2.5 nm, and the emission was recorded at 501 ± 2.5 nm. *D*, CD spectral changes in ODN10104 at a concentration of 100 µg/ml in phosphate buffer (pH 7) following the addition of chloroquine. *E*, CD spectral changes of poly(I:C) at a concentration of 50 µg/ml in phosphate buffer (pH 7) following the addition of chloroquine. *F*, CD spectral changes of R848 at a concentration of 50 µg/ml in phosphate buffer (pH 5) following the addition of quinacrine. Data are representative of two independent experiments.

proposed that the inhibition of endosomal TLR activation could be a result of interactions between nucleic acids and quinacrine or chloroquine. Molecular interactions were determined using either fluorescence spectroscopy for fluorescent quinacrine or CD in the case of chloroquine. Quinacrine emits strong fluorescence by excitation at 430 nm. A titration of quinacrine with ODN10104 from a 0–1.5 μM final concentration resulted in a gradual fall in fluorescent intensity (Fig. 3A). The drop in fluorescence intensity is observed both in ssDNA and dsDNA (Fig. 3B) and is independent of pH (Fig. 3C). This fluorescence quenching indicates the formation of interactions between quinacrine and nucleic acids.

The interactions of the tested antimalarials with nucleic acids cause a change in the chemical environment of the nucleic acid chain and can affect their secondary structure. We used CD spectroscopy to provide evidence of structural changes in DNA and RNA upon titration with chloroquine. A positive-induced CD band with significant ellipticity was developed around 280 nm by the addition of chloroquine to the ODN10104 solution, whereas a small negative-induced CD band appeared around 245 nm (Fig. 3D). Addition of bafilomycin A1 to ODN10104 resulted in no change in the CD spectra, confirming that bafilomycin A1 does not interact with ssDNA (data not shown). Titration of poly(I:C) with chloroquine initiated a large enhancement in the positive (225 nm) and small enhancement in the negative (310 nm) CD bands of poly (I:C) in phosphate buffer at pH 7 (Fig. 3E). Those modifications

show the effect of antimalarials on the conformation of nucleic acids, which could make nucleic acid ligands unavailable to TLR receptor binding sites.

The unexpected costimulatory activity of quinacrine and R848 is also likely to be caused by direct interactions between both compounds and TLR8. Indeed, we observed an induced CD effect for the interaction between R848 and quinacrine in solution (Fig. 3F), providing a new insight into the mechanism of TLR8 activation by small nucleoside analogs. It is likely that quinacrine forms a complex with R848, stabilized by stacking of aromatic rings, which bind cooperatively to the neighboring binding sites of TLR8, thus triggering dimerization of its ectodomains and increasing the TLR8 activation.

*Inhibition of endosomal TLRs by other nucleic acid-binding compounds*

The inhibitory effect of chloroquine on TLR signaling was shown to be due to its perturbation of nucleic acid ligand structure, suggesting that the same effect may be observed in other nucleic acid-binding compounds. We tested two well-known nucleic acid-binding compounds, propidium iodide and Hoechst 34580, which are frequently used for labeling DNA in fluorescent microscopy. Both of them showed inhibitory activity on TLR9 signaling (Fig. 4A), similar to that caused by chloroquine and bafilomycin A1. There was almost no difference in the inhibition
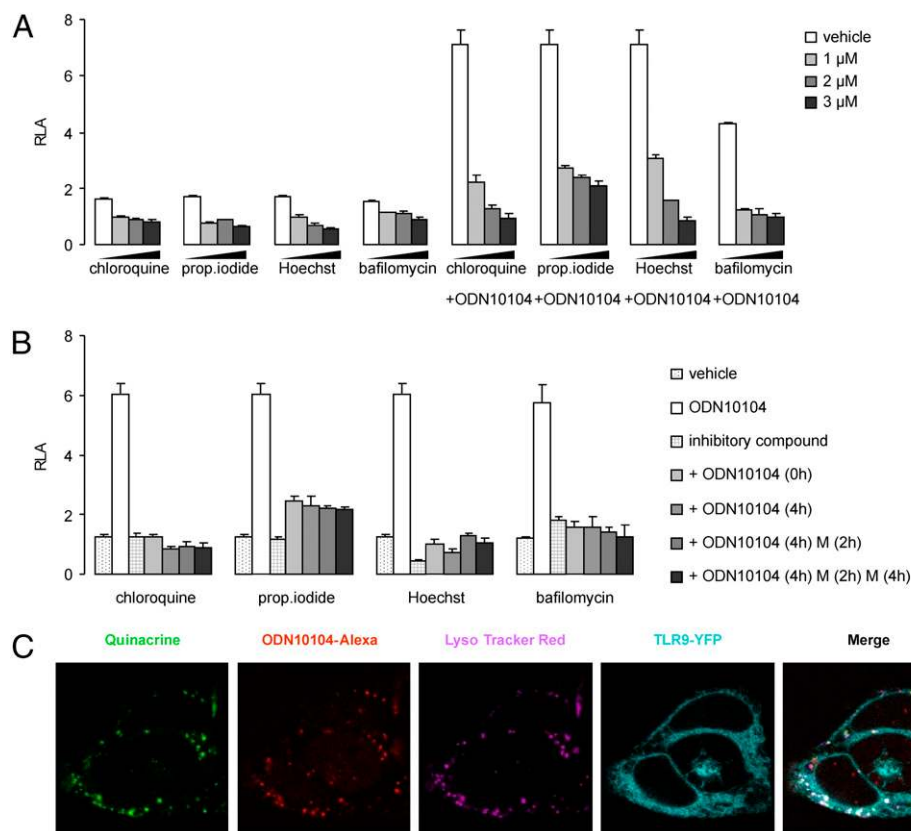


**FIGURE 4.** Nucleic acid-binding compounds inhibit TLR9 activation by ODN10104 in endosomes. *A*, Inhibition of TLR9 by different nucleic acid-binding compounds: chloroquine, Hoechst 34580, propidium iodide, and bafilomycin A1 at concentrations of 1, 2, or 3 μM. Stimulation of HEK293 cells transfected with TLR9 was achieved by ODN10104 at a concentration of 3 μM for 20 h. *B*, Inhibition of TLR9 by different nucleic acid-binding compounds at a concentration of 3 μM. Stimulation of HEK293 cells transfected with TLR9 was achieved by ODN10104 at a concentration of 3 μM, which was added: 1) together with an inhibitory compound [+ ODN10104 (0h)]; 2) 4 h after addition of an inhibitory compound [+ ODN10104 (4 h)]; 3) 4 h after addition of an inhibitory compound, for which the medium was changed 2 h after the inhibitor's addition [+ ODN10104 (4 h) M (2 h)]; and 4) 4 h after addition of an inhibitory compound, for which the medium was changed 2 and 4 h after inhibitor's addition [+ ODN10104 (4 h)] M (2 h) M (4 h)]. Data in *A* and *B* are represented as mean ± SD (*n* = 3). *C*, HEK293T cells transfected with TLR9-YFP (blue) 18 h after stimulation with quinacrine (1 μg/ml) (green) and ODN10104-Alexa Fluor 633 (2 μM) (red). Lysosomes were dyed with LysoTracker Red DND-99 (50 nM) (violet) for 45 min. The colocalization of quinacrine and ODN10104-Alexa Fluor 633 and LysoTracker Red DND-99 is shown as white in overlay image. Data are representative of repeat experiments.

when the inhibitor was added before ODN10104 or if the medium was changed before the addition of ODN (Fig. 4*B*). The DNA-binding compound probably accumulates in endosomes and can there form a complex either before endocytosis or afterward.

### Antimalarials colocalize in the endosomes with nucleic acids

To confirm the interactions between quinacrine and TLR ligands inside living cells, we used confocal fluorescent microscopy. Quinacrine enters the cells very rapidly, because it can be detected in the endosomal vesicles after only 10 s, which was confirmed by Lyso Tracker Red costaining (not shown). After 18 h of incubation of TLR9-YFP–transfected HEK293T cells with ODN10104-Alexa Fluor 633 and quinacrine, we found colocalization of ODNs and quinacrine inside lysosomes (Fig. 4*C*, Supplemental Fig. 3*A*). We also detected colocalization of ODN10104-Alexa Fluor 633 and quinacrine in human PBMCs (Supplemental Fig. 3*B*). A similar experiment was performed using propidium iodide, which we could detect inside cells only in the presence of ODN10104, thus demonstrating the formation of a complex within the endosomes, because propidium iodide exhibits fluorescence only when it is intercalated with nucleic acids (Supplemental Fig. 3*C*). We determined colocalization of ODN10104-Alexa Fluor 633 and TLR9-YFP. Therefore, we can conclude that in endosomes, TLR9 coexists together with ODN and quinacrine.

### Inhibition of TLR9 and TLR3 activation by TLR7/8 agonistic imidazoquinolines

Because of reports that imidazoquinolines, the TLR7/8 ligands, also cause the inhibition of TLR9 receptors, we were interested in determining if their mechanism of action was similar to that of the antimalarial compounds. It has been proposed that the coexpression of TLR7 and TLR9 in B cells and pDCs creates the potential for intracellular physical interactions of these receptors, resulting in a tight control of TLR7 signaling by TLR9 (11). It has also been assumed that TLR7-dependent inhibition of TLR9-induced IFN-γ production may occur by direct interaction of the TLR7 receptor with TLR9 ligands (22). These studies involved experiments on cells that express both TLR9 and TLR7 receptors. To decouple the potential interaction between the two receptors, we used HEK293 cells expressing only the TLR9 receptor and investigated whether both receptors are indeed necessary for the inhibition of TLR9 signaling by TLR7/8 agonists.

When TLR9-transfected HEK293 cells were treated with TLR9 ligand (ODN2006) in the presence of imidazoquinolines, we observed a decrease in TLR9 activity (Fig. 5*A*). These experiments revealed that TLR7/8 ligands R848 and gardiquimod inhibited TLR9 signalization directly, without any indirect effect through TLR7 costimulation. Inhibition by imidazoquinolines was also observed for TLR3-transfected HEK cells activated by poly(I:C) (Fig. 5*B*). The inhibition of TLR9 and TLR3 with gardiquimod was dose dependent and comparable to that caused by quinacrine and chloroquine (not shown). To further confirm the relevance of these findings, we made experiments with two different ODNs (ODN10104 and ODN2006) (Fig. 5*C*) and showed a decrease in IL-8 synthesis using TLR9-transfected HEK293 cells treated with ODN2006 in the presence of gardiquimod (Supplemental Fig. 4*A*). We also looked at the inhibition of IL-6 synthesis by using primary HMVEC-dLy cells, which do not express TLR7 (23), treated
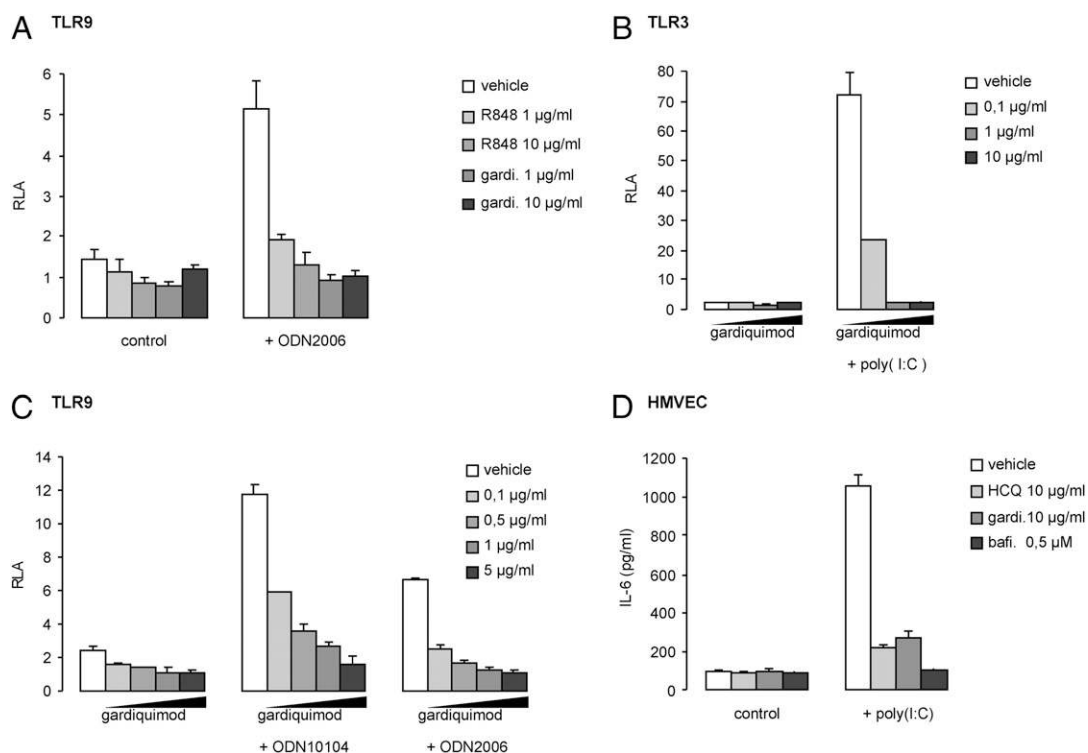
**FIGURE 5.** Imidazoquinolines R848 and gardiquimod inhibit TLR9 and TLR3 activation in a similar manner as chloroquine and quinacrine. *A*, Inhibition of TLR9 with gardiquimod and R848. HEK293 cells expressing TLR9 were treated with 3 μM ODN10104 in the absence or presence of R848 and gardiquimod, both at concentrations of 1 and 10 μg/ml. *B*, Inhibition of TLR3 with gardiquimod. HEK293 cells expressing TLR3 were treated with 10 μg/ml poly(I:C) in the absence or presence of gardiquimod (0.1, 1, and 10 μg/ml). *C*, Inhibition of TLR9, stimulated by ODN10104 (3 μM) or ODN2006 (3 μM), with gardiquimod. HEK293 cells expressing TLR9 were treated with ODNs in the absence or presence of gardiquimod (0.1, 0.5, 1, and 5 μg/ml). *D*, Inhibition of IL-6 synthesis. HMVEC-dLy were incubated with poly(I:C) (10 μg/ml) in the absence or presence of hydroxychloroquine (10 μg/ml), gardiquimod (10 μg/ml), and bafilomycin A1 (0.5 μM). After 24, human IL-6 protein level in the supernatants was measured by ELISA. Data are represented as mean ± SD (*n* = 3).

with poly(I:C) in the presence of hydroxychloroquine, gardiquimod, and bafilomycin A1 (Fig. 5D). Additionally, we showed that gardiquimod and resiquimod inhibited TLR9 similarly to chloroquine and propidium iodide and did not require preincubation to exhibit their activity (Supplemental Fig. 4B). None of inhibitory compounds, including chloroquine, propidium iodide, gardiquimod, and resiquimod, inhibited TLR5 activation by flagellin used as a control (Supplemental Fig. 4C), thereby confirming that the inhibition is limited to ligand-mediated endosomal TLR ectodomain dimerization.

*Direct interactions of imidazoquinolines with nucleic acids*

We presumed that the underlying mechanism for the inhibition of TLR9 activation by imidazoquinolines could be the interaction between ODN and TLR7/8 ligands, much like the inhibition by antimalarial drugs, which share the structure of condensed heteroaromatic rings. To check our hypothesis, we investigated the binding of R848 and gardiquimod to ODN. Fluorescence spectroscopic experiments showed fluorescence quenching of the intrinsic fluorescence of gardiquimod after the addition of ssDNA (ODN10104) (Fig. 6A), which was even enhanced in the case of dsDNA. The chemical structure of the nucleotide backbone in ODNs did not have much influence on these interactions (Fig. 6B). In contrast to the experiments with quinacrine, pH affected the interactions of gardiquimod with ODN (Fig. 6C). Fluorescence quenching was more efficient at a lower pH, which indicates that interactions between gardiquimod and ODN are stronger in endosomes than outside the cells. Quinacrine bound more strongly to ODNs compared with gardiquimod (Fig. 6D), whereas interactions with R848 were the weakest, which also explains why R848 had less inhibitory effect on TLR9 activation within the cells than gardiquimod.

*Antimalarials and imidazoquinolines do not inhibit endosomal proteolytic processing*

The importance of proteolytic processing of endosomal TLRs has been demonstrated for TLR9 (24, 25). This process could, in principle, be affected by antimalarial compounds at concentrations that do not affect acidification. We wanted to exclude the influence of antimalarials and imidazoquinolines on the proteolytic processing of endosomal TLRs that was shown to be required for TLR9 activation (24, 25). As a positive control, we used bafilomycin, which is a specific inhibitor of the vacuolar $H^+$-ATPase responsible for the acidification of vacuolar compartments. Endosomal acidification is required for the activity of proteases that function optimally at low pH and also for the ordered progression of cargo along the endocytic pathway. HEK293T cells were therefore loaded with fluorescent dyes DQ-OVA and Alexa 647-dextran following their preincubation with DNA-binding compounds to compare the influence of hydroxychloroquine and bafilomycin A1 on proteolysis and endocytosis. The confocal microscopy imaging showed that bafilomycin A1 inhibited proteolytic degradation of DQ-OVA at a concentration of 0.2 μM (Fig. 7A, 7B). The presence of red-colored Alexa 467-dextran showed that endocytosis was not affected. In contrast, the presence of 3 μM chloroquine did not decrease proteolytic degradation of DQ-OVA (Fig. 7C). Moreover, when we investigated other antimalarials and imidazoquinolines, the results indicated that hydroxychloroquine, gardiquimod, and R848 (Fig. 7D–F) did not inhibit proteolysis of fluorescently-tagged OVA inside endosomes either. Further experiments applying 10-fold higher concentration (30 μM) also showed no inhibition of proteolysis. Results of pH assessment in endosomes showed that bafilomycin increased pH in endosomes of RAW cells, whereas chloroquine at a concentration that inhibited TLR signaling did not influence the pH value. Ex-
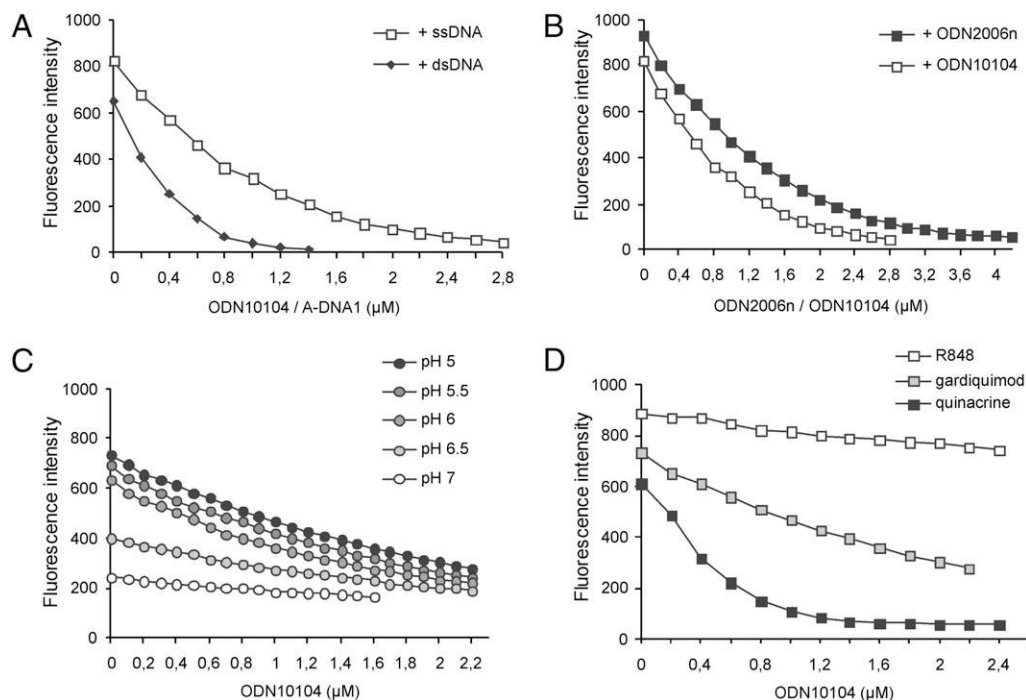
**FIGURE 6.** Imidazoquinolines interact with nucleic acids in a similar manner as antimalarials. *A*, Decrease in the fluorescence intensity of gardiquimod (10 μM) after titration with ssDNA (ODN10104) and dsDNA (A-DNA1). *B*, Effect of the phosphodiester (ODN2006n) and phosphotioate (ODN10104) nucleic acid backbone on the decrease in fluorescence values after titration of gardiquimod (10 μM). *C*, Decrease in fluorescence intensity of gardiquimod (10 μM) in phosphate buffer after titration with ODN10104 at the different pH values. *D*, Decrease in fluorescence intensity of gardiquimod, R848, and quinacrine (all 10 μM) in phosphate buffer (pH 5) after titration with ODN10104.
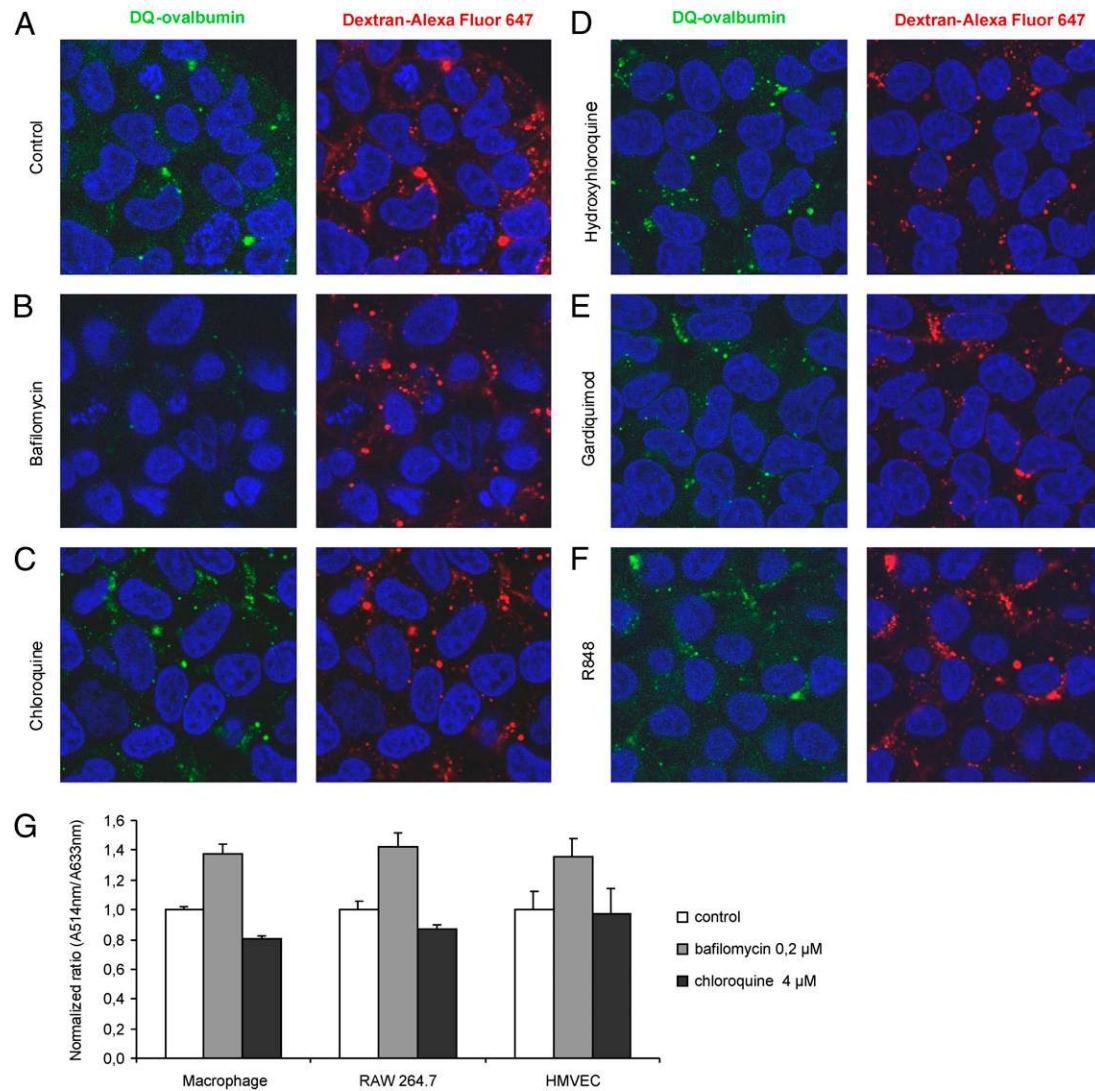
**FIGURE 7.** Antimalarials and imidazoquinolines have a different mechanism of endosomal TLR inhibition than bafilomycin. *A*, Control HEK293T cells 3 h after the addition of DQ-OVA (10 μg/ml) and dextran-Alexa Fluor 647 (100 μg/ml). *B*, HEK293T cells preincubated with bafilomycin A1 (0.2 μM) for 30 min before the addition of DQ-OVA (10 μg/ml) and Alexa Fluor 647-dextran (100 μg/ml). HEK 293T cells preincubated with chloroquine (*C*; 3 μM), hydroxychloroquine (*D*; 3 μM), gardiquimod (*E*; 3 μM), or R848 (*F*; 3 μM) for 30 min before the addition of DQ-OVA (10 μg/ml) and Alexa Fluor 647-Dextran (100 μg/ml). Hoechst 34580 (1 μg/ml) was used to detect nuclei. Images are representative of 10 random fields of view from two separate experiments (1 cm on the figure means 25 μm). *G*, Changes of endosomal pH in primary cells treated with bafilomycin or chloroquine. Primary cell lines were loaded with pH sensitive nanoparticles and treated with bafilomycin (0.2 μM) or chloroquine (4 μM). The change in pH was determined from ratios between emission intensities of pH-dependent Oregon Green 514 and pH-independent Alexa Fluor 633, covalently bound to the same nanoparticles. Higher ratio represents more alkaline pH, and lower ratio represents more acidic pH.

periments on C57BL/6 mouse macrophages and human lung microvascular endothelial cells showed quite similar results (Fig. 7*G*). The evidence that antimalarials do not affect TLR via acting on endosomal pH additionally supports our proposed mechanism of endosomal TLR inhibition (Fig. 8).

## Discussion

The antimalarial drugs hydroxychloroquine, chloroquine, and quinacrine are widely used in the therapy of autoimmune diseases such as rheumatoid arthritis and SLE (26). They can promote a remission in non-major organ SLE, especially in variants with skin and joint affliction (27). However, despite the common use of antimalarials as therapeutic drugs, very little is known about their mechanism of action.

We described that antimalarial agents efficiently inhibited production of inflammatory cytokines in pDC and PBMCs incubated with immune complexes of sera from SLE patients. The inhibitory activity of quinacrine on the activation of endosomal TLRs has been generally ascribed to be a consequence of blocking of endosomal acidification and maturation (7) as strong interactions of nucleic acids with TLR9 occur only under acidic conditions (pH 4.5–6.5) (28). However, we showed that the effect of antimalarials on vesicular pH at concentrations required to suppress the signal transduction was negligible, as also reported before (6, 29). We propose that antimalarials affect endosomal TLR activation by their direct interaction with TLR ligands. Further results confirmed the inhibitory effect of chloroquine and quinacrine on TLR9, TLR3, and TLR8 signaling, when these receptors were stimulated with nucleic acids. In contrast, we observed that antimalarials increased the activation of TLR8 by imidazoquinoline R848. The costimulatory effect of CpG-ODN has been previously shown for TLR8 activation by imidazoquinolines (30).

Additional experiments proved direct interactions between antimalarials and nucleic acid TLR ligands. We detected the inter-
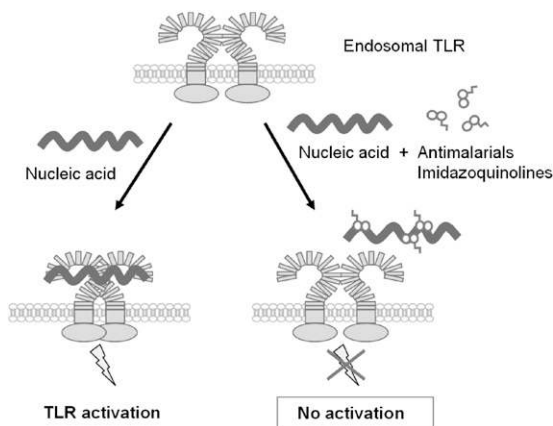
**FIGURE 8.** Mechanistic model of endosomal TLR inhibition by nucleic acid-binding compounds (i.e., antimalarial drugs and imidazoquinolines). Nucleic acid-binding to endosomal TLR induces activation of the signaling pathway. When a nucleic acid-binding compound (inhibitor) is present, there is no activation of the endosomal TLR by the nucleic acid ligand. Antimalaric drugs and imidazoquinolines interact with nucleic acids, and the interactions consequently cause structural modifications of the TLR ligand that prevent their binding to TLR.

actions by fluorescence quenching of quinacrine after the addition of ODN, whereas CD was applied to detect conformational changes in ODN10104 and poly(I:C) caused by the presence of chloroquine. Changes in the conformation of nucleic acids as a consequence of interactions with chloroquine can explain the inability of ODN or poly(I:C) to trigger activation of TLR3 or TLR9. Additionally, the observed interaction between R848 and quinacrine may cause cooperative binding of their complex to the binding sites of TLR8, causing increased activation of the receptor instead of inhibition as with ssRNA. This sheds additional light on the enigmatic activation of TLR7/8 by small molecules (31, 32). It seems likely that imidazoquinolines self-aggregate at the receptor binding sites, mimicking binding of polynucleotide and bridging the two TLR8/7 ectodomains.

We confirmed our hypothesis on the mechanism of endosomal TLR inhibition with other nucleic acid-binding compounds, such as propidium iodide and Hoechst 34580. Both compounds inhibited TLR9 activation much like chloroquine. The inhibition was not dependent on the preincubation of nucleic acids with the inhibitor as the complex formed within the endosomes, thus supporting our hypothesis that quinacrine–nucleic acid interactions are indeed relevant inside the living cells. We detected propidium iodide fluorescence in living cells when ODN was added, indicating that propidium iodide forms a fluorescent complex with ODN within endosomes.

We also investigated the effect of TLR7/8 ligands imidazoquinolines, which had been shown in our experiments to prevent TLR9 signaling. Their inhibitory effect on TLR9 has already been reported, but the mechanism of inhibition was not clear. It had been proposed that physical interactions between TLR7 and TLR9 underlay this effect (11). However, our experiments with TLR9-transfected HEK293 cells showed that the presence of TLR7 was not necessary for the inhibition of TLR9 signaling caused by resiquimod or gardiquimod. Moreover, we observed inhibition of IL-6 synthesis by gardiquimod on TLR3-triggered stimulation of primary human lung microvascular endothelial cells, similar to the inhibition by hydroxychloroquine and bafilomycin A1.

Again, we detected direct interactions between ODN and imidazoquinolines, suggesting that the mechanism of TLR9 and TLR3 inhibition by those compounds is similar to that of an-

timalarials. We observed that the interactions between ODN and gardiquimod were stronger than those between ODN and R848 but weaker than in the case of quinacrine. The differences in the strength of interaction could be a consequence of intercalation properties of compounds guided by properties such as planarity and the number and position of nitrogen atoms. NMR studies have previously demonstrated a physical association between DNA oligomers and imidazoquinolines (30). Preferential binding of quinacrine to adenine- and thymine-rich DNA regions has also been reported (33), which is in agreement with our results (data not shown). Aminoacridine molecules, such as quinacrine, intercalate between base pairs and cause a slight unfolding and elongation of nucleic acid chains (34). Therefore, such nucleic acids with a changed conformation may not be able to bind to the TLR binding sites. We already demonstrated the effect of the nucleic acid conformation on activation of TLR3, which, due to the distance between the binding sites, binds nucleic acid duplex in an A- rather than in a B-type conformation (35).

The role of proteolysis and endosomal acidification connected to it has been reported for TLR9 activation (24, 25). We were able to demonstrate that antimalarials and imidazoquinolines do not inhibit endosomal proteolytic processing (Fig. 6). As expected, bafilomycin A1 with its selective inhibition of $H^+$-ATPase prevented the acidification of endosomes (21, 36, 37) and inhibited the activity of lysosomal proteases requiring acidic pH. In contrast, quinacrine, chloroquine, hydroxychloroquine, gardiquimod, and R848 did not inhibit proteolysis of DQ-OVA, thus demonstrating that those compounds did not affect endosomal and lysosomal acidification. Additionally, we measured the pH in endosomes and confirmed that chloroquine did not influence the endosomal pH in different types of primary cells, whereas bafilomycin increased the value of pH in endosomes.

Our proposed mechanistic model of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines is illustrated in Fig. 8. Although TLR9 is activated by binding of stimulatory DNA resulting in rearrangement of the receptor (38), the addition of nucleic acid-binding compounds leads to the masking of TLR binding sites on nucleic acids and consequently preventing TLR ligand binding. Conformational modification of nucleic acid could retain binding to single TLR binding site but would prevent the productive rearrangement of the ectodomains. Therefore, interactions between antimalarial drugs or imidazoquinolines and nucleic acids do not rule out the possibility of antagonistic effect caused by the inhibitors on endosomal TLRs.

Altogether, our results suggest that the molecular mechanism of action of antimalarial drugs in the therapy of autoimmune diseases, such as SLE, involves binding to nucleic acids, which changes the chemical environment and masks TLR ligand-binding epitopes. Moreover, the direct interactions between ODNs or poly(I:C) and imidazoquinolines cause inhibition of TLR9 and TLR3 by a mechanism, similar to the mechanism by antimalarials. Understanding the mechanism of endosomal TLR inhibition by antimalarials and imidazoquinolines represents a promising potential for the design of improved drugs for the therapy of autoimmune diseases.

## Disclosures

The authors have no financial conflicts of interest.

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