DEVELOPMENT OF A NOVEL RATIONALLY-DESIGNED ANTIBIOTIC TO INHIBIT A NON-TRADITIONAL BACTERIAL TARGET

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DEVELOPMENT OF A NOVEL RATIONALLY-DESIGNED ANTIBIOTIC TO INHIBIT A NON-TRADITIONAL BACTERIAL TARGET

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Running Head: Primary Na⁺ pump as a novel antibiotic target

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ABSTRACT

The search for new non-traditional targets is a high priority in antibiotic design today. Bacterial membrane energetics based on sodium ion circulation offers potential alternative targets. The present work identifies the Na⁺-translocating NADH:ubiquinone oxidoreductase (Na⁺-NQR), a key respiratory enzyme in many microbial pathogens, as indispensable for the *Chlamydia trachomatis* infectious process. Infection by *Chlamydia trachomatis* significantly increased first H⁺ and then Na⁺ levels within the host mammalian cell. A newly designed furanone Na⁺-NQR inhibitor, PEG-2S, blocked the changes in both H⁺ and Na⁺ levels induced by *Chlamydia trachomatis* infection. It also inhibited intracellular proliferation of *Chlamydia trachomatis* with a half-minimal inhibitory concentration in the submicromolar range but did not affect the viability of mammalian cells or bacterial species representing benign intestinal microflora. At low nanomolar concentrations (IC₅₀ value = 1.76 nM), PEG-2S inhibited the Na⁺-NQR activity in sub-bacterial membrane vesicles isolated from *Vibrio cholerae*. Taken together, these results show, for the first time, that Na⁺-NQR is critical for the bacterial infectious process and is susceptible to a precisely targeted bactericidal compound *in situ*. The obtained data have immediate relevance for many different diseases caused by pathogenic bacteria that rely on Na⁺-NQR activity for growth, including sexually transmitted, pulmonary, oral/gum, and ocular infections.

Keywords: Na⁺-translocating NADH:ubiquinone oxidoreductase, Na⁺-NQR, antibiotic design, *Chlamydia*; bacteria; infection

Despite the increasing incidence of antibiotic resistance today, the number of new antibiotics grows at a distressingly slow rate (Russell 2002). The essential part of the problem is that the vast majority of antibiotics target the same three general prokaryotic pathways: protein synthesis, DNA replication and bacterial cell wall assembly. The identification of new targets outside of these traditional “over-targeted” pathways is, therefore, critical if the problem of antibiotic resistance is to be solved.

Bacterial energetics represents an attractive alternative target. Although the majority of modern bacteria exploit the transmembrane circulation of protons (H^+ cycle) in their membrane energetics, a number of species rely on the sodium ion circulation (Na^+ cycle) rather than the H^+ cycle (Skulachev 1985). In these bacteria, primary Na^+ pumps generate the transmembrane electrochemical gradient of Na^+ which can serve as a direct source of energy for vital functions including substrate accumulation, motility and ATP synthesis (Skulachev 1985). Importantly, the Na^+ cycle is widespread among bacterial pathogens (Hase et al 2001).

Chlamydiae are a good example of such pathogenic bacteria. All chlamydial genomes encode a complete Na^+ cycle including the Na^+ -translocating NADH:ubiquinone oxidoreductase (Na^+ -NQR) as a major primary Na^+ pump (Dibrov et al 2004; Dzioba et al 2003; Hase et al 2001; Reyes-Prieto et al 2014). The Na^+ -NQR is the major respiratory Na^+ pump in aerobic pathogens (Hase et al 2001; Reyes-Prieto et al 2014). Although not homologous to the respiratory Complex I (Dibrov et al 2004; Dzioba et al 2003; Reyes-Prieto et al 2014; Steuber et al 2014), it occupies the same position in the electron transport chain, using the oxidation of NADH by ubiquinone to drive translocation of Na^+ across the membrane. Reduction of quinone by the NQR complex is tightly coupled with Na^+ translocation (Juarez et al 2009; Steuber et al 2014) and is inhibited by the quinone analogs HQNO and korormicin (Hayashi et al 2002; Nakayama et al 1999; Yoshikawa et al 1997). The Na^+ cycle may be

critical for maintaining bacterial energetics operative during chlamydial infection (Dibrov et al 2004). It has been hypothesized that the rapid consumption of ATP and glucose by Chlamydiae at the onset of infection would lead to acidification of the host cytoplasm which will in turn activate Na^+/H^+ exchange in the host cells to remove H^+ and increase Na^+ influx simultaneously with the inhibition of Na^+ export via Na^+/K^+ ATPase (Dibrov et al 2004). The resulting rise in $[\text{Na}^+]_i$ in the infected cell would support chlamydial accumulation of amino acids and other substrates by sodium-dependent symporters (Dibrov et al 2004). Thus, the Na^+ cycle may be instrumental in the infectious process and, in the framework of this hypothesis, the Na^+ -NQR is a key element in the energization of nutrient uptake by the invading pathogen.

If the preceding line of reasoning is correct, inhibition of Na^+ -NQR should arrest bacterial energization and proliferation, and ultimately disrupt the infectious process. Korormicin is a naturally occurring antibiotic and the most specific and potent Na^+ -NQR inhibitor known (Nakayama et al 1999). In this work, korormicin and a novel synthetic furanone inhibitor of Na^+ -NQR were used to test the hypothesis that Na^+ -NQR is a valid therapeutic target in the infection caused by *Chlamydia trachomatis*. The objectives of the present study were, therefore, two-fold: (i) to assess Na^+ -NQR as a novel antibiotic target and (ii) to test the efficacy of a newly synthesized furanone Na^+ -NQR inhibitor to arrest *Chlamydia trachomatis* infection.

MATERIALS AND METHODS

Materials. Non-stereospecific synthesis of the furanone inhibitor, named PEG-2 here, was done by Enamine Ltd (Kiev, Ukraine). According to the manufacturer, racemic PEG-2 mixture contained approximately 5% to 10% of a desired enantiomer, PEG-2S. Stereospecific synthesis of PEG-2S was accomplished by Canam Bioresearch (Winnipeg, Canada).

Bacterial strains and cultivation. *Vibrio cholerae* O395N1 strain (O1 classical biotype; Sm^R, Δ ctxA1) (Mekalanos et al 1983), and its isogenic Δ nqrA-F mutant derivative (Dibrov et al 2002), were used for isolation of sub-bacterial vesicles. To maximize the expression of Na⁺-NQR, *V. cholerae* cells were grown with vigorous aeration at 30°C in FM medium (Fadeeva et al 2007), containing 0.4% succinate.

As representatives of benign gastrointestinal microflora, *Escherichia coli* DH5 α (*supE44 hsdR17 recA1 endA1, gyrA96 thi-1 relA1*) (U.S. Biochemical Corp.), *Lactococcus lactis* (Boreal Northwest-VWR, Cat. # 851150), and *Enterococcus faecalis* (NCTC 775) were used. Cells were maintained/propagated at 37°C in standard tryptic soy broth, TSB (Difco) and on agar plates prepared on the same broth.

C. trachomatis serovar L₂ was propagated as described previously (Hirono et al 2003). The purified organism was stored in SPG buffer (0.22 M sucrose, 8.6 mM Na₂HPO₄, 3.8 mM KH₂PO₄, 5 mM glutamic acid, 0.2 μ m-filtered, pH 7.4) at –80°C until use. The titer of *C. trachomatis* was determined in HeLa cells in the presence of cycloheximide (1.0 μ g/ml), and concentrations used were expressed as inclusion-forming units (IFU)/ml.

Mammalian cells. HeLa 229 cells (ATCC, CCL-2.1), HEK-293 cells (ATCC, CRL-1573) and primary rabbit vascular smooth muscle cells (VSMCs) were used. VSMCs were harvested as previously described (Massaelli et al 1999). Cells were maintained in Dulbecco's modified

Eagle's medium (DMEM, Invitrogen) with 5% fetal bovine serum (FBS, Invitrogen) at 37°C, 5% CO₂.

Assays of the growth of free-living bacteria. Overnight starter cultures of *E. coli*, *L. lactis*, and *E. faecalis* were grown aerobically in TSB and used to inoculate 200 µl TSB medium in 96-deep-well plates (Whatman) at an initial OD₆₀₀ of 0.05. The obtained cultures were supplemented with 0.5 - 50.0 µM PEG-2S (or pure DMSO in “zero” controls) and grown at 37°C for 24 h with vigorous aeration. At 2, 5, and 24 h, growth was measured as OD₆₀₀ by scanning the plates on a Bio-Rad iMark microplate reader. For samples taken at 5 and 24 h, serial dilutions with the pre-warmed growth medium were prepared using the aliquots of bacterial cultures. The experiments were repeated at least three times.

Evaluation of a PEG-2 against *Clostridium difficile* (*C. difficile* ATCC 700057) was performed by the Drop method on pre-reduced BAK plates according to *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (CLSI 2012).

Assessment of antichlamydial properties of antibiotics. For MIC₅₀ (Minimal Concentration of the antibiotic that inhibits formation of chlamydial inclusions by 50%) determination, HeLa cells were grown in 24-well or 96 well plates overnight prior to chlamydial inoculation. Elementary bodies (10⁵ IFU/ml) were applied to the cells in SPG and after 2 hours of incubation, unattached elementary bodies were removed and infected cells were treated with different concentrations of antibiotics in DMEM with 5% FBS in the presence of cycloheximide (1.0 µg/ml). After 42 h, inclusions were visualized by immunocytochemistry.

To determine the chlamydicidal effect of korormicin and PEG-2, Hela cells were plated on 35mm dishes, infected with *C. trachomatis* and treated with different concentrations of the antibiotic of choice. After 48h, *C. trachomatis* was collected (P1) and used to infect fresh cells.

Subsequent collections of *C. trachomatis* were used to obtain P2, P3, P4 and P5 stocks of *C. trachomatis*. Dilution of *C. trachomatis* used to infect cells after treatment with PEG-2 had to be minimized in order to visualize inclusions. The last passage collection of *C. trachomatis* (P5) after korormicin treatment and all collected passages of *C. trachomatis* treated with PEG-2 were used to establish infectivity of treated *C. trachomatis*. The minimal chlamydicidal concentration (MCC2) for korormicin, PEG-2 and PEG-2S was calculated after 2 passages of infection with treatment and reflect inhibition of infection by more than 90%. In all experiments, the inclusions were visualized by immunocytochemistry.

Immunocytochemistry. Cells were seeded on glass coverslips in 24-well plates ($2-4 \times 10^4$ cells/well) or on glass-bottom 96 well plates (10^4 cells/well) overnight and then inoculated with *C. trachomatis*. After 42 hours, cells were fixed with 90% acetone (or 100% methanol in the case of 96 well plates), probed with anti-Chlamydia specific antibody (Thermo Fisher Scientific) and inclusions were visualized by Alexa Fluor 488–conjugated secondary antibody (Molecular Probes, Invitrogen) with DAPI (300nM) as a counter-staining. Inclusion bodies and cell nuclei were identified using a fluorescent inverted microscope (TE-2000s; Nikon). Cells and inclusions were counted using Adobe Photoshop CS3.Ink software.

Cell Proliferation Assay. Cells were seeded at 5×10^3 cells/well in 96-well plates in growth media and treated with different concentration of antibiotics. After 48 hours, the number of living cells was determined by colorimetric enzyme assay (CellTiter 96 Cell Proliferation Assay, Promega Corporation, Madison, WI) (Cory et al, 1991).

Preparation of sub-bacterial membrane vesicles from *V. cholerae* cells. Membrane vesicles from *V. cholerae* strains were prepared as described previously (Dibrov et al, 2002). The membrane pellet was suspended in buffer A containing 100mM KCl, 50mM NaCl, 5mM

MgSO₄, 20mM HEPES-Tris, pH 8.0 at 20–30 mg protein/mL, snap-frozen in liquid nitrogen and stored at –80°C.

Na⁺-NQR activity assays in sub-bacterial vesicles. The activity of Na⁺-NQR in sub-bacterial *V. cholerae* vesicles was measured at 25°C as oxidation of dNADH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) by following the changes in its fluorescence at 440 nm (excitation light $\lambda = 340 \text{ nm}$) using Shimadzu RF-1501 spectrofluorometer. The assays were conducted in Buffer A supplemented with 15 μM Na⁺-dNADH with constant stirring. The reaction was initiated by the addition of vesicles (aliquots of 50 μg of protein). Fluorescence at 440 nm as a function of [dNADH] in the experimental buffer was linear up to 20 μM of added dNADH (data not shown).

Measurements of intracellular pH and sodium in cells. pHrodoTM Green AM and CoroNa Green Sodium Indicator (both Molecular Probes, Invitrogen) were used to measure intracellular pH (pH_i) and intracellular sodium (Na⁺_i) concentration, respectively. HEK293 cells were seeded on 24 well plates and infected with *C. trachomatis* after overnight incubation in DMEM with 5%FBS. After 2 hours of incubation with *C. trachomatis*, cells were treated with different concentrations of PEG-2S and subjected to pH_i and Na⁺_i measurements at different time points according to manufacturer's protocols. In all assessments, calibration curves were performed prior to the experiments. A fluorescent inverted microscope (TE-2000s Nikon) was used to obtain images and mean intensity was quantified using NIS Elements imaging software (Nikon, Mississauga, ON).

Statistical Analysis Data are presented as mean $\pm$ SEM unless otherwise stated. Differences between treatment groups were assessed by one-way analysis of variance using the Student–Newman–Keuls method (Hirono et al 2003). A probability of $P \leq 0.05$ was considered statistically significant.

RESULTS

Chlamydial invasion perturbs ion homeostasis of infected cells. *C. trachomatis* infection of HEK293 cells induced changes in cytoplasmic pH and Na^+ content. As expected, whereas pH did not change in uninfected cells, infected cells became acidic within 2 hrs (Fig. 1A). The significant cytoplasmic acidification caused by the metabolic activity of chlamydial reticulate bodies (RBs) was followed by a significant secondary rise in Na^+_i . Na^+_i did not change in uninfected cells but exhibited a delayed increase in chlamydia-infected cells (Fig. 1B).

Inhibition of chlamydial Na^+ -NQR by korormicin suppresses proliferation of *C. trachomatis* reticulate bodies but is toxic to mammalian cells. Korormicin, an antibiotic targeting Na^+ -NQR that was initially isolated from marine bacterium (Yoshikawa et al 1997), inhibited the growth of *C. trachomatis* at 10-20 μM (Fig. 2A-D). The extrapolated MIC50 for korormicin (after the 1st treatment) was $\sim 18.00 \mu\text{M}$ (Table 1). However, korormicin was also cytotoxic to mammalian cells at nM concentrations (Fig. 2E).

Design of synthetic NQR inhibitor, PEG-2. The toxicity of korormicin encouraged us to redesign the inhibitor to be more suitable as an anti-NQR medicine (Dibrov et al 2016). To eliminate the cytotoxic effect of natural korormicin, its epoxy group was identified as the most probable source of toxicity and removed. Further, the aliphatic “tail” of korormicin (Fig. 3A) was shortened to 7 carbon atoms to lessen the overall hydrophobicity of the molecule and thus lower its effective anti-chlamydial concentration. This approach yielded a furanone compound, named PEG-2 (Fig. 3B) (Dibrov et al 2016). Due to the presence of two chiral centers in the molecule, four stereoisomers of PEG-2 are possible (Fig. 3B). Racemic mixtures of PEG-2 were initially used, which according to the manufacturer’s report, contained $\leq 10\%$ of the active enantiomer, PEG-2S, shown in Fig. 3B.

Direct measurement of inhibition of Na^+ -NQR by PEG-2S. Na^+ -NQR activity can be differentially measured in sub-bacterial membrane vesicles as the NQR-mediated oxidation of an NADH analog, dNADH (Bertsova and Bogachev 2004; Fadeeva et al 2007; 2008; Kostyrko et al 2015). This allows the measurement of NQR activity directly without confounding factors - the enzyme is functioning in its native membrane and feeding the electrons taken from NADH to the respiratory chain. Vesicles isolated from the NQR-positive *Vibrio cholerae* strain demonstrated a PEG-2S-sensitive oxidation of dNADH (Fig. 4A). Removal of functional Na^+ -NQR by the chromosomal *nqrA*-F deletion resulted in the inability of membranes isolated from the mutant *V. cholerae* strain to oxidize dNADH (Fig. 4A). PEG-2S inhibition exhibited an MIC_{50} of 1.76 nM (Fig. 4B).

PEG-2S disrupts chlamydia-induced changes in ionic homeostasis in cells. In accordance with results shown in Figure 1, HEK293 cells infected with *C. trachomatis* displayed a cytoplasmic acidification followed by an increase in Na^+_i levels (Fig. 5). The addition of PEG-2S significantly suppressed the chlamydia-induced acidification in a dose-dependent manner (Fig. 5A). As expected, sodium accumulation was delayed relative to the acidification and it was also sensitive to the low μM PEG-2S concentrations (Fig. 5B).

Anti-chlamydial effects of PEG-2 and PEG-2S. A single treatment with racemic PEG-2 had a significant effect on infection of HeLa cells by *C. trachomatis* (Fig. 6). PEG-2 was more effective than korormicin by at least 2 orders of magnitude (Fig. 6A). As expected, PEG-2S was even more potent as an anti-chlamydial agent than PEG-2 (Table 1). The MIC_{50} for PEG-2 and PEG-2S was 10 μM and 0.7 μM , respectively. After two consecutive treatments, the MIC_{50_2} for PEG-2 was 4 μM versus 0.25 μM for PEG-2S (Table 1). Chlamydial inclusions formed in the presence of PEG-2 were hollow compared to the control non-treated cells (Fig. 6B), and significantly smaller (Fig. 6C).

PEG-2 and PEG2S are non-toxic. PEG-2 up to 20 μM (as well as PEG-2S) had no toxic effects on primary cell cultures (Fig. 6D). Furthermore, PEG-2 and PEG-2S (1-50 μM) did not affect the growth of NQR-negative species, including benign gastrointestinal bacteria, *E. coli*, *E. faecalis* and *L. lactis* (Fig. 7) and pathogenic *C. difficile* (data not shown).

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DISCUSSION

The results presented here are important from two perspectives. First, they demonstrate that the activity of the Na^+ -NQR is critical for the growth and proliferation of *C. trachomatis* and provide an explanation for this. The metabolism of invading *C. trachomatis* increased cytoplasmic $[\text{H}^+]$ in the host cell by 10-fold soon after infection, and subsequently elevated intracellular Na^+ levels as well. Both observed effects have been predicted theoretically in the earlier work (Dibrov et al 2004). The acidification of the cytoplasm in Chlamydiae-infected cells was thought to occur due to the rapid catabolism of glucose provided by the host cell (Dibrov et al, 2004; Iliffe-Lee and McClarty, 1999). Depletion of the host ATP pool caused by the metabolic activity of chlamydial reticulate bodies (RBs) (Iliffe-Lee and McClarty 1999) should have reduce the export of Na^+ by the Na^+/K^+ ATPase. Together with an activation of Na^+ influx *via* Na^+/H^+ exchange at the host cell plasma membrane due to the elevated increased $[\text{H}^+]_i$, this was expected to result in a rise of $[\text{Na}^+]_i$ (Dibrov et al 2004). Measurements of internal Na^+ and pH in uninfected and infected cells confirmed both these predictions (Fig.5A and B). This helps to explain why the membrane energetics of this obligate intracellular parasite relies on the sodium-motive force (SMF). Like other members of the *Chlamydia* genus, *C. trachomatis* encodes Na^+ -dependent symporters for the accumulation of a variety of substrates, including amino acids and dicarboxylates (Dibrov et al 2004). Therefore, it is concluded that the generation and maintenance of the SMF by the Na^+ -NQR becomes a prerequisite for the proliferation of *C. trachomatis*.

The second major advance presented in this study is the design of a novel antibiotic that targets the Na^+ -NQR and in so doing, inhibits the growth and proliferation of *Chlamydia trachomatis*. The best NQR inhibitor identified to date is korormicin, an antibiotic initially isolated from a marine bacterium, *Pseudoalteromonas* sp. (Yoshikawa et al 1997). However, its capacity to inhibit bacterial growth was orders of magnitude less than its toxicity profile. Thus, although the experiments with korormicin

confirmed the crucial role of Na^+ -NQR in bacterial proliferation, this inhibitor cannot be advanced clinically as an anti-chlamydial remedy. Conversely, the data with PEG-2 and PEG-2S support this agent as an effective inhibitor of Na^+ -NQR. The experiments with sub-bacterial membrane vesicles allowed the measurement of Na^+ -NQR activity directly when the enzyme was operating in a physiologically relevant background (being placed in its native membrane and supplying electrons taken from NADH to the respiratory chain) but without additional permeability barriers and influences from cytoplasmic metabolism (Bertsova and Bogachev 2004; Fadeeva et al 2007; 2008; Kostyrko et al 2015). In this model, PEG-2S exhibited a low MIC_{50} of 1.76 nM (Fig. 4B). PEG-2S was also effective against *C. trachomatis* at low μM concentrations in infected HeLa cells. It was non-toxic to both mammalian cells (Fig. 6D) and non-pathogenic gastrointestinal bacteria (Fig. 7). Na^+ -NQR is absent in these cell types and this would, therefore, support the contention that PEG-2S is a highly selective inhibitor of Na^+ -NQR. Of note, gram-positive *C. difficile* possesses the ancestral form of NQR, RNF (Reyes-Prieto et al 2014). The Gly140 residue conserved in NqrB subunits of all NQR enzymes is implicated in the binding of korormicin (Hayashi et al 2002) and is absent in a homologous subunit of RNF (Reyes-Prieto et al 2014). Since *C. difficile* was not sensitive to PEG-2S (data not shown), one can conclude that PEG-2S may require Gly140 to bind to Na^+ -NQR.

The data shown for the inhibition of bacterial growth by PEG-2 and PEG-2S have implications beyond the inhibition of the growth of chlamydial pathogens. Na^+ -NQR is present in a variety of pathogenic bacteria including *Chlamydiae pneumoniae*, *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Legionella pneumophila*, *Neisseria meningitides*, *Neisseria gonorrhoeae* and many others (Dibrov et al 2004; Hase et al 2001; Reyes-Prieto et al 2014; Steuber et al 2014). Each may rely on Na^+ -NQR for its energetics to different degrees and, therefore, will have differing sensitivities to anti-NQR drugs. Nevertheless, in the future

PEG-2S may be attractive for clinical use to suppress bacterial growth and proliferation in a number of different diseases by inhibiting Na^+ -NQR activity.

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Table 1. Chlamydicidal properties of natural antibiotic Korormicin and synthetic NQR inhibitor PEG-2 and PEG-2S assayed in HeLa cells.

	Korormicin	PEG-2^a	PEG-2S^e
MIC50₁^b	18 µM (8.28 µg/ml)	10 µM (3.63 µg/ml)	0.7 µM (0.25 µg/ml)
MIC50₂^c	10 µM (4.6 µg/ml)	4 µM (1.45 µg/ml)	0.25 µM (0.09 µg/ml)
MCC2^d	ND	15 µM (5.45 µg/ml)	1.0 µM (0.36 µg/ml)

^a Enantiomeric mixture, PEG-2, contained no more than 10% of the biologically active stereoisomer PEG-2S (according to the manufacturer’s report)

^b**Minimal Concentration of the antibiotic that Inhibits formation of chlamydial incluions by 50%**

^c**Minimal Concentration of the antibiotic that Inhibits formation of chlamydial incluions by 50% after the second treatment**

^d**Minimal Chlamydicidal Concentration calculated after 2 consecutive treatments of infection and reflects inhibition of infection by more than 90%.**

^b Minimal Inhibitory Concentration after the treatment

^c Minimal Inhibitory Concentration after the second treatment

^d Minimal Chlamydicidal Concentration of added antibiotic

^e Pure stereoisomer

ND: Not determined.

Figure legends

Fig. 1. Changes in cytoplasmic pH (**panel A**) and Na^+ content (**panel B**) caused by the *C. trachomatis* infecting HEK293 cells. Cytoplasmic pH and $[\text{Na}^+]_i$ in uninfected and infected cells were monitored using fluorescent dyes as described in Materials and Methods.

Fig. 2. Korormicin is effective against *C. trachomatis* when added at a concentration of ~10 to 20 μM . HeLa cells were infected with *C. trachomatis* (5×10^3 IFU per 5×10^4 cells). Korormicin at indicated concentrations was added with DMEM 2 hours after initial attachment of *C. trachomatis* to target cells. **Panel A**, control (no addition); **panel B**, 10 μM korormicin; **panel C**, 15 μM korormicin. **D.** Quantitation of the effect of korormicin on chlamydial infection. Decline in number of RBs after 5 consecutive treatments with indicated concentrations of korormicin is plotted. Averages of 3 independent experiments are plotted. Standard deviation is shown. See the text for details. **E.** Korormicin is toxic for mammalian cells. Effect of indicated concentrations of korormicin on primary cells was measured as inhibition of proliferation of VSMC as described in Materials and Methods. ($n=8$). Standard deviation is shown.

Fig. 3. Chemical structures of two quinone analogs inhibiting Na^+ -NQR. **Panel A:** The (5S,3'R,9'S,10'R) stereoisomer of natural korormicin (Uehara et al 1999). **Panel B:** Synthetic furanone inhibitor PEG-2 (Dibrov et al 2016). Structure shown is that of an active stereoisomer, PEG-2S. Racemic mixture of stereoisomers used in some experiments is referred to as PEG-2. According to the manufacturer, PEG-2 racemate contained $\leq 10\%$ of desired enantiomer, PEG-2S.

Fig. 4. Inhibition of Na^+ -NQR by PEG-2S measured directly in sub-bacterial *V. cholerae* vesicles as described in Materials and Methods. **Panel A**, PEG-2S-sensitive oxidation of dNADH is absent in Na^+ -NQR-deficient membranes (trace (a)), while Na^+ -NQR-containing membranes oxidize dNADH in the PEG-2S-sensitive manner (trace (b)). **Panel B**, for the calculation of the IC_{50} of PEG-2S inhibition, normalized Na^+ -NQR activity (100% corresponds to the activity in the absence of the inhibitor) was plotted as a function of [PEG-2S]. The IC_{50} value for PEG-2S was 1.76 nM. For the comparison, IC_{50} for HQNO measured in the same experimental model was 130 nM (Fadeeva et al 2008).

Fig. 5. PEG-2S disrupts ionic changes induced by chlamydial infection in HEK293 cell culture. Treatment with 1 and 2.5 μM PEG-2S prevents changes of cytoplasmic pH (**Panel A**) and $[\text{Na}^+]$ (**Panel B**) in HEK293 cells infected by *C. trachomatis*. Plotted are averages of 8 independent experiments. Standard deviation is shown. $*P < 0.003$

Fig. 6. Anti-chlamydial effect of consecutive treatments of HeLa cultures with PEG-2. **Panel A**, comparison of the effectiveness of PEG-2 and natural korormicin. Parallel experiments with two antibiotics were carried out under conditions described for Fig. 2. Number of inclusions was counted after five consecutive treatments with antibiotic. **Panel B**, PEG-2 acts in a dose-dependent manner. Experimental conditions as in Fig. 2, but inclusion numbers were counted after 2nd, 3rd, 4th, and 5th consecutive treatments. **Panel C**, PEG-2 diminishes size of chlamydial inclusions. (*) denotes a statistically significant difference ($P = < 0.001$). Experimental conditions as in Fig. 2, but PEG-2 at 20 μM was used instead of korormicin. **Panel D**, unlike natural korormicin, PEG-2 is non-toxic for mammalian cells. Primary vascular smooth muscle cells were exposed to the indicated concentrations of antibiotic under the same conditions as in Fig. 2. Standard deviation is shown ($n = 8$, $P = 0.149$).

Fig. 7. PEG-2S is highly selective anti-NQR agent. Growth yield of three Na^+ -NQR-negative species belonging to benign intestinal microflora in liquid medium was measured as light absorbance at 600 nm at 2, 5 and 24 hours of aerobic growth as described in Materials and Methods. Plotted are averages of 5 parallel cultures exposed to each concentration of PEG-2S tested. Statistical analysis revealed no difference in growth yields of any tested species at any concentration of added PEG-2S. Pure stereoisomer, PEG-2S, was used in these experiments. **Panel A**, *Escherichia coli*, **Panel B**, *Lactococcus lactis*, **Panel C**, *Enterococcus faecalis*.

Figure 1

229x377mm (300 x 300 DPI)

Figure 2

219x348mm (300 x 300 DPI)

A

korormicin

B

PEG-2S

Figure 3

76x83mm (300 x 300 DPI)

Figure 4

229x377mm (300 x 300 DPI)

Figure 5

229x377mm (300 x 300 DPI)

Figure 6

229x377mm (300 x 300 DPI)

Figure 7

229x377mm (300 x 300 DPI)