Multiple drug-induced stress responses inhibit formation of 
*Escherichia coli* biofilms

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Running Head: Identification of antibiofilm drugs for *E. coli*

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Abstract

In most ecosystems, bacteria primarily exist as structured surface-associated biofilms 
that can be highly tolerant to antibiotics and thus represent an important health issue. 
Here we explored drug repurposing as a strategy to identify new antibiofilm 
compounds, screening over 1000 compounds from the Prestwick Chemical Library of 
approved drugs for specific activities that prevent biofilm formation by *Escherichia 
coli*. Most growth-inhibiting compounds, which include known antibacterial but also 
antiviral and other drugs, had also reduced biofilm formation. However, we also 
identified several drugs that were biofilm-inhibitory at doses where only weak or no 
effect on planktonic growth could be observed. Activities of the most specific 
antibiofilm compounds were further characterized using gene expression analysis, 
proteomics and microscopy. We observed that most of these drugs acted by repressing 
genes responsible for production of curli, major component of *E. coli* biofilm matrix. 
This repression apparently occurred through induction of several different stress 
responses, including DNA and cell-wall damage, and homeostasis of divalent cations, 
demonstrating that biofilm formation can be inhibited through a variety of molecular 
mechanisms. One tested drug, tyloxapol, inhibited biofilm formation independent of 
curli expression or growth, by suppressing bacterial attachment at the surface.
Importance

Prevention of bacterial biofilm formation is one of the major current challenges in microbiology. By systematically screening a large number of approved drugs for their ability to suppress biofilm formation by *Escherichia coli*, here we identified a number of prospective antibiofilm compounds. We further demonstrated different mechanisms of action for individual compounds, from induction of replicative stress to disbalance of cation homeostasis to inhibition of bacterial attachment to the surface. Our work demonstrates the potential of drug repurposing for the prevention of bacterial biofilm formation and suggests that also for other bacteria the activity spectrum of antibiofilm compounds is likely to be broad.

Introduction

Biofilms are the most widespread form of bacterial existence in nature (1-4). Within biofilms, bacteria are typically embedded in matrix that consists of polysaccharides, proteins and extracellular DNA (5, 6). Biofilms are formed at various types of interfaces, including epithelial layers and surfaces of catheters, and many chronic infections are caused by bacteria that are present in biofilms (1). Since cells within biofilms have low sensitivity to many antimicrobial compounds (7), prevention of biofilm formation is important in disease treatment. A number of different alternative approaches are currently explored as the antibiofilm strategies, including surface modification to prevent bacterial adhesion or assembly of the biofilm matrix, specific enzymes to degrade biofilm matrix, and inhibition of bacterial signaling or quorum sensing using small molecules (8-12).

*Escherichia coli* is a common and medically relevant model for biofilm research (13, 14). The major matrix component of *E. coli* biofilms are amyloid protein fibers known as curli (15, 16). Matrix of *E. coli* also includes other components as colanic acid, cellulose, poly-β-1,6-N-acetyl-d-glucosamine, the content of which varies by strain and dependent on growth conditions (17-19). *E. coli* biofilm formation is a highly regulated process that includes initial attachment and biofilm maturation steps, and depends on a number of signaling pathways that regulate curli biosynthesis (14, 19). Expression of structural components of curli, including the major curlin CsgA is under control of the master regulator CsgD that is expressed dependent on the activity of stationary phase sigma factor σ^S^ and the interplay between several diguanylate cyclases and phosphodiesterases that control the level of second messenger cyclic di-GMP (c-di-GMP) (20-22). CsgD regulates the inverse coordination between planktonic motile and biofilm sessile lifestyles is mediated by mutual inhibition between the σ^S^/CsgD curli and flagellar gene expression controls cascades (23, 24). The latter consists of three classes of flagellar genes, where the master regulator FlhDC (class I) induces the expression of class II (middle) genes including the flagellar-specific sigma factor FlIA that, in turn, activates the expression of class III (late) flagellar genes (25).

In this study we screened the Prestwick Chemical Library of over 1000 of FDA-approved drugs with the aim to identify new compounds that are active against...
submerged biofilms of *E. coli*. This library has been recently used to identify drugs that impact human gut bacteria (26) or inhibit growth of several pathogenic bacteria in planktonic culture and also in biofilms (27-29). In contrast, the prime focus of our screen was to identify drugs that specifically inhibit *E. coli* biofilm formation, while having less or no effect on the planktonic growth. Indeed, we report several perspective antibiofilm compounds that were active against both laboratory and pathogenic *E. coli* strains, at doses below the growth-inhibitory concentration. The antibiofilm effect of most of these compounds could be explained by the inhibition of curli expression and, in some cases activation of motility, apparently due to the induction of several different stress responses, or due to inhibition of bacterial attachment.

**Results**

**Identification of novel antibacterial and antibiofilm drugs**

In order to identify new antibiofilm compounds, we screened 1280 off-patent drugs approved for human use by the US Food and Drug Administration (FDA) from the Prestwick Chemical Library for their ability to suppress growth as well as formation of submerged biofilms by *E. coli* K-12 strain W3110, a common biofilm model (19, 23). In this screen, *E. coli* cultures were incubated in 96-well microtiter plates at 10 µM final concentrations of individual compounds, and bacterial growth and biofilm formation were quantified (see Materials and Methods).

We observed that under these conditions many compounds had detectable inhibitory effect on *E. coli* growth (Fig. 1, Table 1 and Data set S1). This group of compounds included mostly established antibacterial drugs, but more surprisingly also antiviral and other drugs. These novel antibacterial compounds might be promising as potential drugs, although their activity spectrum and mechanisms of action require future investigation. Most of these growth-inhibitory compounds also proportionally reduced the biomass of surface-attached cells in the crystal violet (CV) assay (Fig. 1), suggesting that their effect on the biofilm formation is the consequence of growth inhibition. Nevertheless, several compounds had a comparatively stronger effect on biofilm formation than on growth. The activities of all potentially specific antibiofilm drugs, which at 10 µM reduced CV stain by >75% but inhibited planktonic growth by <70%, were verified in an additional screen. This left a total of 5 perspective compounds, including 3 established antibacterial drugs clioquinol, pipemidic acid and cefuroxime sodium salt, but also two surfactants tyloxapol and thonzonium bromide (Table 1).

**Perspective compounds suppress biofilm formation by laboratory and uropathogenic *E. coli***

We subsequently subjected these drugs to the more detailed analysis of their antibiofilm activity. Additionally, we included an antiviral drug zidovudine (3'-azido-3'-deoxythymidine; azidothymidine) that showed high activity against both planktonic growth and biofilm formation in our screen (Fig. 1). All of these compounds showed...
dose-dependent inhibition of the biomass of surface-attached cells, and - except tyloxapol - also reduced growth of planktonic *E. coli* cultures (Fig. 2). Nevertheless, the antibiofilm activity of all drugs was consistently higher than growth inhibition, which in most cases was particularly apparent at low concentrations. This turned out to be also true for azidothymidine, which showed antibiofilm activity already in the nanomolar concentration range where it had no measurable effects on or even weakly stimulated planktonic growth.

We further confirmed the potential of these selected drugs to suppress biofilm formation by three uropathogenic (UPEC) strains of *E. coli*, EcoR-50, EcoR-64 and DSMZ 10650. Antibiofilm activity of tested drugs was observed for all three UPEC strains at similar concentrations as for *E. coli* W3110 (Fig. S1). As a minor difference between these isolates, EcoR-50 biofilms were most sensitive to azidothymidine, pipemidic acid, clioquinol and thonzonium bromide, whereas cefuroxime showed highest activity against DSMZ 10650 biofilms. In general, EcoR-64 biofilms were least sensitive to tested drugs, and surprisingly biofilm formation was even stimulated by low concentrations of thonzonium bromide. These strain-specific differences in sensitivity to drugs seemingly correlate with strain’s sensitivity to commonly used antibiotics (Table S1). Here again EcoR-64 showed highest levels of resistance, while EcoR-50 was most sensitive. Nevertheless, even for EcoR-64 biofilm formation could be efficiently suppressed by several compounds, including clioquinol and tyloxapol, indicating that identified drugs should be applicable against biofilms of antibiotic-resistant UPEC strains.

Finally, we tested the effects of these compounds on UPEC biofilm formation on urinary catheters. Here we focused on DSMZ 10650, since in our preliminary experiments this strain showed most pronounced biofilm formation on catheters when growing in donor urine. Several compounds showed inhibitory effects under these conditions, most notably tyloxapol and cefuroxime (Fig. S2). These effects were observed both at 30°C, that is commonly used to study *E. coli* biofilm formation, as well as at the body temperature, 37°C.

**Most antibiofilm compounds inhibit curli and activate flagellar expression**

To elucidate possible modes of action of the identified antibiofilm compounds, we first assayed their effects on curli genes that encode major matrix component of *E. coli*. We used green fluorescent protein (GFP) reporter of *csgA* promoter that controls expression of the main curli operon, and determined reporter activity in cells that were recovered from the surface of the incubation well (see Material and Methods). As reported previously (23), *csgA* promoter showed bimodal expression in population of untreated W3110 bacteria, with fractions of *csgA*-positive and -negative cells (Fig. 3A). Since curli genes in *E. coli* are known to be counter-regulated with the flagellar regulon (23, 24), we also monitored activity of flagellin (*fliC*) promoter. Under our conditions, activity of *fliC* promoter was also seemingly bimodal (Fig. 3B). We observed that upon incubation with all antibiofilm drugs except tyloxapol, both the fraction of *csgA*-positive cells and the overall reporter activity decreased dramatically (Fig. 3A,C,D).
Consistently, these compounds also enhanced activity of *fliC* promoter, unmasking its bimodality and strongly increasing fluorescence of positive cells (Fig. 3B,C,D). Thus, all of clioquinol, pipemidic acid, cefuroxime, thonzonium bromide and azidothymidine inhibit expression of curli matrix while also activating motility and therefore cell dispersion, which provides likely mechanism of the antibiofilm activity of these compounds.

Tyloxapol prevents attachment of *E. coli* to plastic surface

In contrast to other drugs, tyloxapol showed no significant effect on curli or flagellar expression (Fig. 3), suggesting that it suppresses *E. coli* W3110 biofilms by a mechanism different from inhibition of matrix biosynthesis. Since tyloxapol is a known surface-active compound (30), we investigated whether it could affect surface attachment of *E. coli*. Indeed, nearly no attachment to the plastic surface of microscopy wells was observed for *E. coli* cells in presence of tyloxapol (Fig. 4), whereas other drugs – including thonzonium bromide that has also being described as a surface-active compound (31) – had little effect on attachment. Nevertheless, microscopy analysis showed that even sub-inhibitory concentrations of pipemidic acid, cefuroxime resulted in substantial elongation of *E. coli* cells. This effect might be explained by the activities of these drugs that are known to respectively inhibit bacterial cell-wall biosynthesis (cefuroxime) or gyrase (pipemidic acid) (Table 1), which both could lead to a (partly) suppression of cell division. Interestingly, certain cell elongation was also observed upon incubation with azidothymidine, consistent with general similarity of effects of azidothymidine and pipemidic acid on *E. coli* (see below).

The observed effect of tyloxapol on attachment is apparently surface-specific, since tyloxapol did not prevent attachment of *E. coli* W3110 to glass surface in a commercial Bioflux chamber that is used to study biofilm formation under flow (Fig. S3). In contrast, all drugs that induced cell elongation at sub-inhibitory concentrations strongly suppressed early stages of *E. coli* biofilm formation in this flow chamber, possibly due to facilitated detachment of elongated daughter cells under flow. Clioquinol and thonzonium bromide had no apparent effects on these early biofilms, presumably because curli expression only becomes important at later stages of submerged biofilm formation by *E. coli* (14, 19).

Antibiofilm compounds induce different stress responses in *E. coli*

In order to better understand the underlying mechanisms of curli inhibition by clioquinol, pipemidic acid, cefuroxime, azidothymidine, we performed analyses of changes in the proteome composition that are induced in surface-attached cells by these drugs at concentrations that inhibit biofilm formation by approximately 50%. Although expected reduction of the levels of structural curli proteins (CsgA, CsgB and CsgC) were indeed observed for all compounds, many changes in protein levels were compound-specific (Fig. 5, Fig. S4, Table 2 and Data set S2). Interestingly, also expression of the regulator CsgD was only inhibited only in some (pipemidic acid and
azidothymidine) but not in other cases. This suggests that curli downregulation might occur through several different mechanisms, which was further confirmed by comparisons between global regulatory changes mediated by individual compounds (Fig. S5). Nevertheless, treatment with azidothymidine and pipemidic acid led to highly similar changes in *E. coli* proteome, indicating that these two drugs have an at least partly shared mechanism of action. This could also be noticed at the level of most highly up- or downregulated proteins, where both drugs appeared to induce the DNA-damage SOS response (Fig. 5A, B and Table 2). This might be consistent with the established activities of these compounds that might interfere with DNA replication (Table 1). Acid stress response and multidrug efflux pump were also activated by both compounds. Pipemidic acid (Fig. 5A, B and Table 2) and to a lesser extent also azidothymidine (Data set S1) further led to reduced levels of transcription factor McbR that was previously implicated in *E. coli* biofilm formation (32).

In contrast, profiles of expression changes induced by cefuroxime or clioquinol had little overlap with other compounds and only weak correlation between each other. *E. coli* treated with cefuroxime showed elevated expression of several proteins involved in cell wall synthesis and division (Table 2), which is consistent with its function as cell-wall inhibiting drug (Table 1). Cefuroxime also activated expression of fimbriae (FimH, FimG, FimD) and twin-arginine transporter component TatE. Clioquinol induced TatE, along with induced expression of the FeoC uptake system for ferrous iron and ZntA export system for zinc and cadmium (Table 2), which might be potentially consistent with its role as metal ion chelator (33).

**Antibiofilm effect of clioquinol is related to homeostasis of divalent cations**

In order to investigate whether the observed antibiofilm activity of clioquinol is indeed related to the homeostasis of divalent cations, we investigated how this effect is influenced by the addition of divalent cations. Indeed, copper and ferric iron cations restored the ability of *E. coli* W3110 to form biofilms in presence of clioquinol, and also relieved growth of planktonic culture in a dose-dependent manner (Fig. 6A and Fig. S6), largely eliminating the effect of clioquinol at the equimolar concentration (10 μM). In contrast, addition of zinc even further suppressed the growth of planktonic culture, whereas addition of magnesium or calcium cations did not change the growth of planktonic culture or biofilm formation. Consistently, copper and iron relieved the clioquinol-mediated repression of curli genes, whereas zinc further enhanced their repression (Fig. 6B,D and Fig. S7A). An opposite pattern was observed for flagellar gene expression (Fig. 6C,D and Fig. S7B). Notably, none of the tested cations had on itself any effects on gene expression or biofilm formation.

**Discussion**

Most studies dedicated to discovering the new antibiofilm drugs are aimed on specific targeting of biofilm-related pathways (8-10). While this hypothesis-guided approach is potentially promising, it is limited by our poor understanding of the complexity of
regulation that underlies transition to the biofilm state. As an alternative strategy, several recent studies relied on systematic large-scale screening of drugs for their biocidal activity against planktonic cell cultures, with a subsequent retesting of identified biocidal compounds for their antibiofilm activity (28, 29). This approach enables identification of the biocidal compounds that are similarly active against planktonic and biofilm cultures, but it is unlikely to yield antibiofilm compounds that do not suppress planktonic growth.

The aim of this study was in contrast to identify novel drugs that act specifically against biofilms of *E. coli* among the library of the FDA-approved drugs, by designing the screen to focus on drugs that show stronger biofilm suppression than inhibition of growth. This enabled us to identify several antibiofilm compounds, among which three are established antibacterial biocides (clioquinol, pipemidic acid, cefuroxime), one antiviral drug (azidothymidine) and two surfactants tyloxapol and thonzonium bromide (although the latter is also a known antiseptic). Out of identified drugs, only cefuroxime has been previously shown to be active against bacterial biofilms (34, 35). Notably, although the screen was performed for the laboratory strain W3110 of *E. coli* that is commonly used as a model for biofilm formation (19, 23), most of these compounds similarly suppressed biofilms of several tested UPEC strains, and several of them affected UPEC biofilms formed on catheters. This indicates potential clinical applicability of these drugs, although more extensive testing on the clinical isolates of *E. coli* would be necessary to verify it. Furthermore, such repurposing strategy is likely to yield novel antibiofilm drugs for other bacterial species as well.

Besides identifying these perspective compounds, we could at least partly characterize the mechanisms of their antibiofilm activity. For the one of the identified compounds, non-ionic surfactant tyloxapol, this activity is apparently based on direct suppression of the surface attachment, since even at high concentrations tyloxapol had no effect on bacterial growth or on expression of curli genes. Tyloxapol is commonly used as a mucolytic agent for treatment of pulmonary diseases, and it might also possess anti-inflammatory activity (36), but to our knowledge its antibiofilm (or antibacterial) activity has not being reported so far. Although its spectrum of action remains to be tested, it likely conditions the surfaces to prevent attachment of type I fimbriae and/or flagella, the major adhesins of *E. coli* W3110 under our experimental conditions (23).

The mode of action of all other compounds, including clioquinol, pipemidic acid, cefuroxime, azidothymidine and thonzonium bromide, is apparently related to suppression of expression of curli, the major component of *E. coli* biofilm matrix. It might be further enhanced by stimulation of expression of the motility-related genes. Together, this results in a combination of reduced aggregation and enhanced dispersion of bacteria. This finding is overall consistent with the importance of curli for *E. coli* biofilm formation (15, 16) and with the known counter-regulation between curli and motility genes (23, 24). Since curli are important for biofilm formation not only in laboratory but also in pathogenic *E. coli* strains (14, 37), the suppression of curli expression could explain why these drugs are active against UPEC biofilms.
Interestingly, however, our analysis of global changes in protein levels suggested that mechanisms that resulted in curli gene inhibition were different between individual drugs. Two of the drugs, pipemidic acid and azidothymidine, induced similar changes in protein expression, apparently related to the DNA damage response. This is consistent with the established mode of action of pipemidic acid, a known inhibitor of bacterial topoisomerase II (gyrase) (38). Although azidothymidine (zidovudine) is known as an inhibitor of viral reverse transcriptase (39), it can apparently similarly interfere with E. coli DNA replication and/or repair. Notably, the antibacterial activity of azidothymidine has been reported previously, although its mechanism of action remained unknown (40). The exact pathway that links the observed induction of DNA damage stress to the repression of curli remains to be elucidated, but in both cases we observed reduced levels of transcription factor McbR, a positive regulator of E. coli biofilm formation (32). The apparent weak growth-activatory effect of low concentrations of azidothymidine also remains to be understood.

Cefuroxime belongs to cephalosporin group of antibiotics that inhibits cell-wall synthesis (41), and consistently we observed that it caused cell elongation and induced expression of several cell-wall proteins, along with the expression of type I fimbriae. Despite these elevated levels of adhesins, the formation of biofilm was strongly reduced through the repression of curli. Interestingly, cefuroxime may also be applicable as an antibiofilm agent against biofilms of Gram-positive bacterium S. aureus (35).

Finally, clioquinol (5-chloro-7-iodoquinolin-8-ol) affected expression of several proteins involved in iron and zinc transport. Clioquinol is an established chelator of zinc, copper and iron and it is also known to act as an ionophore (33). These properties might explain multiple effects of clioquinol on animal cells (33, 42, 43), as well as its reported antibacterial (44), antifungal (45) and antiprotozoal (46) properties. The same chelator/ionophore activity that perturbs homeostasis of divalent cations is the likely cause of the clioquinol-mediated suppression of curli expression and biofilm formation by E. coli, since the effect of clioquinol could be suppressed by addition of equimolar amounts of copper or ferrous (II) iron. Interestingly, however, the addition of zinc rather potentiated the effect of clioquinol, although zinc itself had no effect on the biofilm formation. Addition of iron was previously shown to stimulate curli expression and biofilm formation by uropathogenic E. coli, by inducing oxidative stress (47), so iron chelation by clioquinol might cause biofilm inhibition. This might also potentially explain the cumulative effects of clioquinol and zinc, since addition of zinc might perturb iron uptake (48, 49). Additionally, zinc is known to affect cellular levels of c-di-GMP, by inhibiting diguanylate cyclase DgcZ (50).

Summarizing, by performing an extensive screen we could identify several drugs that specifically suppress biofilm formation by both commensal and pathogenic E. coli, while having weak or no effect on bacterial growth at a given concentration. While one of the identified antibiofilm drugs specifically prevented adhesion, the effect of other drugs was due to suppressed curli production and therefore of cell aggregation. This apparently occurred due to induction of different stress responses including disrupted homeostasis of divalent cations, DNA damage or perturbations to cell-wall biosynthesis. Same treatments also induced expression of motility genes, thus likely stimulating cell
dispersion. Such stress-induced suppression of biofilm formation and induction of motility was unexpected, since in *E. coli* curli expression is under positive whereas flagellar expression is under negative regulation by general stress response (23, 24), and biofilms typically promote stress resistance (7). It remains to be investigated whether this effect is common and stress induction by these or other drugs might inhibit biofilm formation in other bacteria, too. Finally, suppression of curli expression might have clinical applications besides inhibition of biofilm formation, since curli fibers are known to be generally important for *E. coli* pathogenicity (14, 37).

**Materials and Methods**

**Strains and culture conditions.** *E. coli* W3110 was used here as the model for biofilm formation (19, 23). Additionally, three uropathogenic *E. coli* (UPEC) strains – EcoR-50, EcoR-64 and DSMZ 10650 – were used for comparison. Bacteria were grown at 30°C, temperature that favors *E. coli* biofilm formation (19, 23), in tryptone broth (TB) medium (10 g tryptone, 5 g NaCl perliter) supplemented with antibiotics where necessary. The same W3110 strain but with genomic eGFP reporter under the rplL promoter (23) was used for microscopy. Promoter activities were measured using the GFP reporter plasmids for *csgA*, *csgD*, *fliA*, *fliC* and *fliD* promoters (23, 51, 52).

**Biofilm growth and quantification.** Biofilms were quantified using standard crystal violet (CV) assay on microtiter plates (53) with modifications. Briefly, overnight cultures of *E. coli* W3110 grown in TB in a rotary shaker at 30°C were diluted 1:100 into fresh TB medium and grown at 220 rpm to the mid-exponential phase (OD<sub>600</sub> = 0.5) at 30°C. Culture was diluted in fresh TB medium to OD<sub>600</sub> = 0.05 and 300 μl were loaded into 96-well plate (Corning Costar, flat bottom, Sigma-Aldrich, Germany). Planktonic culture was measured at OD<sub>600</sub> after 20-24 h of stationary incubation at 30°C, then liquid culture was removed from wells. Wells were washed once with 1x phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>P<sub>4</sub>), then biofilms were fixed by 300 μl of 96% ethanol. After 20 min ethanol was removed, plates were left to dry under the fume hood for 40 min, then stained with 300 μl of 0.1% crystal violet solution for 15 min. Crystal violet was removed, biofilms were washed twice with the same buffer. Remaining CV stain in biofilms was extracted by adding 300 μl of 96% ethanol for 35 min and measured at OD<sub>595</sub>. All the measurements were performed with Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland).

**Biofilm growth on urinary catheters.** The overnight cultures of UPEC strains grown in TB in a rotary shaker at 30°C were diluted 1:100 in fresh TB medium and grown at 220 rpm to the mid-exponential phase (OD<sub>600</sub> = 0.5) at 30°C. Cultures were subsequently diluted in filter-sterilized human urine from a female donor uto OD<sub>600</sub> = 0.05, and 1.5 ml of culture was loaded into 24-well plate (Corning Costar, flat bottom, Sigma-Aldrich, Germany) containing 1 cm pieces of 12Fr silicone Foley catheter (Azid Bonz, Germany) and grown for 48 h at 30°C or 37°C. For biofilm quantification, catheter pieces were taken from the wells, washed with PBS, allowed to dry on a paper towel and stained in 1.5 ml of 0.1% CV solution for 10 minutes. Then the tubing was
rinsed with distilled water using a syringe, and allowed to dry. Remaining CV stain in biofilms was extracted by adding 1.5 ml of 96% ethanol for 35 min, and OD_{595} was then measured in three technical replicated per catheter piece.

**Library screening.** Prestwick Chemical Library (Prestwick Chemical, Illkirch-Graffenstaden, France) contains 1280 compounds at 10 mM in DMSO. These compounds were diluted in DMSO 10-fold using Integra VIAFLO 96/384 robotic liquid-handling system (Switzerland), and 3 μl were added to bacterial culture, resulting in 10 μM final concentration.

**Fluorescence microscopy.** Bacterial cultures were prepared as described above. 200 μl of diluted culture was seeded per well into the 96-well microscopy plates with untreated surfaces (µ-Plate 96 Well Black, ibidi GmbH, Germany). Bacteria were grown in TB medium at 30°C without shaking for 6 hours. Where indicated, tested compounds were added to the medium during growth. After the cultivation, planktonic cells were carefully removed and replaced with 200 μl of PBS. Fluorescent cells were visualized using an inverted Zeiss Axio Observer Laser Scanning Microscope (LSM) 880 equipped with a C-APOCHROMAT 63x/1.2 Water Corr-UV-VIS-IR objective and a 514 nm argon laser.

**Microfluidics.** Microfluidic assays were performed by using a Bioflux 200 system (Fluxion Biosciences Inc., USA). Cells were grown in TB medium at 30°C until OD_{600}=0.5. Then, cells were diluted in fresh TB until OD_{600}=0.05 and flushed into the channels for 3 h at 0.5 dyn/cm². Afterwards, cells were removed from the input well and fresh TB medium supplemented with respective compounds at indicated concentrations was flushed overnight at 0.5 dyn/cm². An exception was tyloxapol, where the medium already contained tyloxapol during the first 3 h of incubation. Imaging was performed on a Nikon Eclipse Ti-U fluorescence microscope equipped with a iXon3 897 EMCCD camera using a 40x objective and using GFP (Ex470/40, Em525/50) filter set. 2 positions per channel were imaged per strain. Quantification of whole fluorescence was performed using Fiji software.

**Flow cytometry.** Bacteria were grown as for fluorescence microscopy, except TB medium was supplemented with 50 μg/ml kanamycin to select for reporter plasmids. Planktonic culture was carefully removed, 200 μl PBS was added to the well, and attached cells were removed from the surface by pipetting and scratching using 1 ml pipet tip. Obtained suspension was centrifuged for 5 min at 4500 g, and then pellet was resuspended in PBS and vortexed vigorously to disrupt all remaining cell aggregates. Samples were diluted in 20 times in PBS, and fluorescence was measured on using BD LSRFortessa SORP cell analyzer (BD Biosciences, Germany).

**Peptide analysis using mass spectrometry.** Bacterial cultures were prepared as described above. 1.5 ml of diluted culture was seeded per well into the 12-well culture plates with untreated surfaces (CELLSTAR 12 Well Plates, Greiner Bio-One, Germany). Planktonic culture was carefully removed, 500 μl PBS was added to the well, and attached cells were removed from the surface by pipetting and scratching using 1 ml pipet tip. Obtained suspension was centrifuged for 5 min at 4500 g. Cells were washed with the same amount of PBS and then lysed by incubation with 100 μl of...
2% sodium-lauroyl-sarcosinate (SLS) solution at 95°C for 15 min and subsequent sonication (Vial Tweeter, Hielscher, Germany). Cell lysates were then reduced by adding 5 mM Tris (2-caboxeyl) phosphine and incubating at 95°C for 15 minutes followed by alkylation (10 mM iodoacetamide, 30 min at 25°C). Cell lysates were cleared by centrifugation and the total protein was estimated for each sample with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Germany). Cell lysate containing 50 µg total protein was then digested with 1 µg trypsin (Promega) overnight at 30°C in 2% SLS and 100 mM ammonium bicarbonate for each sample. Next, SLS was removed by precipitation with 1.5% trifluoroacetic acid (TFA) and centrifugation. Peptides were purified using C18 microspin columns according to the manufacturer’s instruction (Harvard Apparatus, USA). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (54) partner repository with the dataset identifier PXD020710.

Purified peptides were dried, resuspended in 0.1% TFA and analyzed using liquid chromatography-mass spectrometry carried out on a Q-Exactive Plus instrument connected to an Ultimate 3000 RSLC nano with a Prowflow upgrade and a nanospray flex ion source (all Thermo Scientific, Germany). Peptide separation was performed on a reverse phase HPLC column (75 µm x 42 cm) packed in-house with C18 resin (2.4 µm, Dr. Maisch GmbH, Germany). The following separating gradient was used: 98% solvent A (0.15% formic acid) and 2% solvent B (99.85% acetonitrile, 0.15% formic acid) to 25% solvent B over 105 minutes and to 35% B for additional 35 minutes at a flow rate of 300 nl/min. The data acquisition mode was set to obtain one high resolution MS scan at a resolution of 70,000 full width at half maximum (at m/z 200) followed by MS/MS scans of the 10 most intense ions. To increase the efficiency of MS/MS attempts, the charged state screening modus was enabled to exclude unassigned and singly charged ions. The dynamic exclusion duration was set to 30 seconds. The ion accumulation time was set to 50 ms for MS and 50 ms at 17,500 resolution for MS/MS. The automatic gain control was set to 3x10^6 for MS survey scans and 1x10^5 for MS/MS scans. Label-free quantification (LFQ) of the data was performed as described previously (55, 56). In short, for LFQ the raw data was loaded into Progenesis (Version 2.0, Nonlinear Dynamics) and exported .mgf files searched by MASCOT (Version 2.5, Matrix Science). Progenesis peptide measurement exports were then further evaluated using SafeQuant for false discovery adjustment and quality control. All experiments were performed in duplicates.

**Antibiotic sensitivity of UPEC strains.** Sensitivity of *E. coli* strains to antibiotics was tested on LB agar plates supplemented with antibiotics at concentrations indicated in Table S1. 5 µl of the overnight culture of each strain was plated on respective plate and incubated at 30°C for 24 h. Antibiotic sensitivity was determined as the absence of visible colony growth.

**Data evaluation.** All the experiments were done in triplicate. Differences between groups were calculated using Mann-Whitney tests. Flow cytometry data was analyzed in FlowJo v. 10 software (Tree Star, USA). Proteomics data was analyzed in Perseus v.
1.5.2.6. software using Student’s t-test (56). Graphs were drawn in R v. 3.6.0 software (57) using ggplot2 v. 3.2.1. package (58).

**Data availability.** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (59) partner repository with the dataset identifier PXD020710.

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Figure Legends

FIG 1. Screen for antibiofilm compounds within the Prestwick Chemical Library of FDA-approved drugs.

Growth of planktonic *E. coli* cultures and formation of biofilms was measured in presence of 10 µM of individual library compounds. Biofilm formation was quantified using CV staining (see Materials and Methods). Untreated culture was used as a control. Dashed lines indicate cultures where treatment resulted in >75% reduction of biofilm formation while having <70% effect on planktonic growth. Compounds that were chosen for the future work are indicated.

FIG 2. Dose-dependent effects of indicated drugs on planktonic culture growth and biofilm formation. Growth and biofilm formation by *E. coli* W3110 were measured as described in Materials and Methods in presence of varying concentrations of azidothymidine (A), clioquinol (B), cefuroxime sodium salt (C), pipemidic acid (D), tyloxapol (E) and thonzonium bromide (F). All experiments were performed in triplicates. Error bars indicate standard errors.

FIG. 3. Effects of indicated drugs on expression of curli and flagellar genes. (A,B) Representative measurements of activity of csgA (A) and fliC (B) promoter reporters using flow cytometry (see Materials and Methods) in populations of *E. coli* W3110 cell grown in tryptone broth (TB) medium or in TB medium supplemented with 0.1 µM azidothymidine (AZT), 2.5 µM cefuroxime sodium salt (CF), 10 µM pipemidic acid (PA), 1 µM clioquinol (CQ), 2.5 µM tyloxapol (TX) or 5 µM thonzonium bromide (TZ). The Y-axis in (A) and (B) represents cell count, with a total of 50 000 events for each experiment. (C,D) Corresponding fraction of the fluorescent cells (C) and median fluorescence (D). All experiments were performed in triplicates. Error bars indicate standard errors. *P values were calculated using Mann-Whitney test (*P<0.05, **P<0.01, ***P<0.001).

FIG 4. Attachment of *E. coli* cells to the plastic surface under the treatment.

Representative images of cultures grown in microscopy plates (see Materials and Methods) for 6 h in absence of treatment (A), or in presence of azidothymidine (B), cefuroxime sodium salt (C), pipemidic acid (D), clioquinol (E), tyloxapol (F) and thonzonium bromide (G), at concentrations as indicated in Figure 3. Attached cells were washed and imaged in PBS.

FIG 5. Global changes in protein levels upon treatment with indicated drugs.

Protein changes were analyzed using mass spectrometry (see Materials and Methods) for cultures grown in presence of azidothymidine (A), pipemidic acid (B), cefuroxime sodium salt (C), and clioquinol (D) at concentrations as in Figure 3. Data are shown as
volcano plots where X-axis represents fold change of label-free quantification (LFQ)
intensity of each protein, while Y-axis represents -log_{10} of Student’s test p-value. The
most up- and down-regulated proteins are highlighted in red; selected curli proteins are
highlighted in blue.

FIG 6. Effects of divalent cations on growth, biofilm formation and gene
expression in clioquinol-treated *E. coli* cultures.

(A) Changes in cell growth and biofilm formation in presence of clioquinol alone or
combination of clioquinol (CF) with indicated divalent cations (10 µM ZnCl₂, 10 µM
CuSO₄, or 10 µM FeSO₄). All values are normalized to the untreated cultures. (B-D)
Activity of *csgA* (B) and *fliC* (C) promoter reporters, and corresponding changes in
median fluorescence (D) in cultures with or without treatment with 10 µM clioquinol
(CF) and divalent cations, as indicated. The Y-axis in (B) and (C) represents cell count,
with a total of 50,000 events for each experiment. All experiments were performed in
triplicates. Representative measurements are shown in (B) and (C). Error bars in (A)
and (D) indicate standard errors.
