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Multiresistant uropathogenic extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* are susceptible to the carbon monoxide releasing molecule-2 (CORM-2)

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ABSTRACT

Carbon monoxide (CO) releasing molecules (CO-RMs) have been shown to inhibit growth of commensal *Escherichia coli* (*E. coli*). In the present study we examined the effect of CORM-2 on uropathogenic *E. coli* (UPEC) that produces extended-spectrum β-lactamase (ESBL). Viability experiments showed that CORM-2 inhibited the growth of several different ESBL-producing UPEC isolates and that 500 μM CORM-2 had a bactericidal effect within 4 h. The bactericidal effect of CORM-2 was significantly more pronounced than the effect of the antibiotic nitrofurantoin. CORM-2 demonstrated a low level of cytotoxicity in eukaryotic cells (human bladder epithelial cell line 5637) at the concentrations and time-points where the antibacterial effect was obtained. Real-time RT-PCR studies of different virulence genes showed that the expression of capsule group II kpsMT II and serum resistance traT was reduced and that some genes encoding iron acquisition systems were altered by CORM-2. Our results demonstrate that CORM-2 has a fast bactericidal effect against multiresistant ESBL-producing UPEC isolates, and also identify some putative UPEC virulence factors as targets for CORM-2. CO-RMs may be candidate drugs for further studies in the field of finding new therapeutic approaches for treatment of uropathogenic ESBLproducing *E. coli*.

1. Introduction

Carbon monoxide (CO) is often considered as being an environmental toxicant that results from combustion of organic matter. The toxic effects in eukaryotic organisms are manifested when CO binds with high-affinity to hemoglobin that is essential for O_2 delivery to tissues. CO binds to iron-containing heme proteins, such as cytochrome *c* oxidase, and inhibits respiration [1]. CO is also produced endogenously by heme oxygenases (HOs) in mammals [2] and acts as a potent regulatory molecule with anti-inflammatory, anti-apoptotic and vasodilatory effects [3]. Furthermore, an antimicrobial effect of CO has recently been reported. Compounds acting as CO-releasing molecules (CO-RMs) can reduce the viability of different bacterial species such as *Pseudomonas aeruginosa* [4], *Escherichia coli* and *Staphylococcus aureus* [5]. The bactericidal action of CO-RMs in *E. coli* has been linked to alterations in the function of heme–and transition metal-containing proteins which ultimately affect respiration [6]. In addition, generation of reactive oxygen species by CO-RMs has been suggested to contribute to its antibacterial activity [7]. CO-RMs are transition metal carbonyls and the best characterized are the lipid-soluble CORM-2 and the water-soluble CORM-3 [8]. From microarray studies CORM-2 and CORM-3 have been reported to affect several genes families/groups in *E. coli* suggesting that the antibacterial effect does not only involve effects on respiration [9]. However, no data have yet been reported regarding the effects of CO-RMs on uropathogenic *E. coli* (UPEC) isolates.

Urinary tract infection (UTI) is known to be one of the most common human infections. *E. coli* is the most frequent pathogen isolated from acute community-acquired uncomplicated UTIs [10] as well as from hospitalized UTI patients in Europe [11]. UPEC isolates express chromosomally encoded virulence factors such as adhesins (e.g. P fimbriae, type 1 fimbriae), the aerobactin

Abbreviations: CO-RMs, carbon monoxide-releasing molecules; CORM-2, ($[Ru(CO)_3Cl_2]_2$); DMSO, dimethyl sulfoxide; ESBL, extended-spectrum β-lactamase; $ESBL_A$, class A ESBL; IBC, intracellular bacterial communities; LB, Luria Bertani broth; MS, minimal salt medium; TSA, tryptic soy agar; UPEC, uropathogenic *E. coli*.

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system, hemolysin, K capsule, and resistance to serum killing [12]. UPEC is known to invade host cells within the urinary tract [13,14] and intracellular bacterial communities (IBC) and biofilm production facilitates bacterial persistence of UPEC in the urinary tract [15,16].

Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) are an important cause of failure of therapy with cephalosporins [17] and these bacteria often show resistance to several non-β-lactam antibiotic groups as well i.e. multidrug resistance [18]. ESBLs are mainly plasmid-mediated β-lactamases [19] and at present the CTX-M enzymes are the most prevalent [20]. The CTX-M family is classified into five major groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 based on similarities in their amino acid sequences [21] and these comprises at least 146 variants (21 Aug 1013 http://www.lahey.org/studies/other.asp). There are reports showing as high as 21% ESBL-producing *E. coli* in community-acquired UTIs in Turkey [22] and 17.6% in hospitalized European UTI patients [11] of which the majority of the isolates was CTX-M-15. At present CTX-M-15 is the most widespread CTX-M type around the world [20]. The increase of the worldwide CTX-M 15-producing *E. coli* has been suggested to be due to the dissemination of clone ST131 belonging to the virulent phylogroup B2 [23,24]. Thus, the classical dogma regarding an inverse relationship between resistance and virulence does not seem to be valid for ESBL isolates, at least not for the ST131 clonal group. However, a recent study performed on CTX-M-15 producing ST131 UPEC isolates suggests that the virulence potential is lower than that of classical UPEC isolates [25].

There is an urgent need for new therapy for UTI caused by multiresistant ESBL-producing *UPEC*. The aim of the present study was to evaluate the effect of CORM-2 on uropathogenic ESBL-producing *E. coli* and also to assess the effect of CORM-2 exposure on gene expression of selected UPEC virulence factors.

2. Materials and methods

2.1. Reagents

The source for CO was tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$; CORM-2) (Sigma–Aldrich, St. Louis, MO, USA). Stock solutions of CORM-2 and nitrofurantoin (Sigma–Aldrich) was freshly prepared by dissolution in dimethyl sulfoxide (DMSO, Merck, Schuchardt OHG, Germany), at 1 or 2.5% DMSO for CORM-2 (100 μM and 250/500 μM, respectively) and 0.32% DMSO for nitrofurantoin. The CO-free molecule $Ru(II)Cl_2(DMSO)_4$ (kind gift from R K Poole, Dept. of Molecular Biology and Biotechnology, Sheffield, U. K.) was freshly prepared and used as a negative control for CORM-2.

2.2. Bacterial strains

The commensal *E. coli* K12 strain MG1655 [26] and the UPEC strains J96 and CFT073 originally isolated from patients with acute pyelonephritis [27,28] were used from laboratory stocks. The ESBL-producing *E. coli* isolates 1, 6, 7 and 9 were obtained from the Department of Microbiology at Örebro University hospital, Sweden, and isolated from the urine of anonymized patients with indwelling urinary catheters and symptoms of UTI. Antimicrobial susceptibility testing was performed as recommended by the Swedish Reference Group for Antibiotics (www.srga.org). The isolates were characterized regarding CTX-M type as previously described [29]. The CTX-M types and the antibiotic susceptibility of the different ESBL-producing isolates are shown in Table 1.

Table 1
Characteristics of clinical ESBL isolates from urine of patients with UTI.

Isolate	CTX-M subgroup	CTX-M type	Antibiotic resistance
ESBL1	CTX-M-9	CTX-M-24	CTX, CAZ, CTB, TMP, CIP, MEL, GEN
ESBL6	CTX-M-1	CTX-M-15	CTX, CAZ, CTB, TMP, CIP
ESBL7	CTX-M-1	CTX-M-15	CTX, CAZ, TMP, CIP, MEL
ESBL9	CTX-M-9	CTX-M-14	CTX, CAZ, TMP, CIP, MEL

Abbreviations: cefotaxime (CTX), ceftazidime (CAZ), ceftibuten (CTB), trimethoprim (TMP), ciprofloxacin (CIP), mecillinam (MEL), gentamicin (GEN).

2.3. Bacterial media and growth conditions

Bacteria were maintained on tryptic soy agar (TSA) (Becton Dickinson, Le Pont Claix, France). Overnight cultures of *E. coli* grown with aeration in Difco Luria–Bertani (LB) broth (Lennox, Becton Dickinson, Franklin Lakes, USA) at 37 °C were used to inoculate (at 0.1%) fresh minimal salt (MS) medium [5] or urine. The cultures in MS medium were grown aerobically at 37 °C on shake to early log phase at an optical density of 0.1 (OD_{620}). The bacterial concentration of the initial inoculums used in the present work was in the range of 10^7–10^8 CFU ml^{-1}. Thereafter, the bacteria were treated with CORM-2 or nitrofurantoin or were left untreated (controls) and grown for up to 24 h. Initial experiments confirmed that the vehicle (DMSO) did not affect the bacterial growth at 4 and 24 h. MIC was determined for nitrofurantoin by the broth dilution test. MIC for CORM-2 in *E. coli* has previously been reported to be 500 μM [5]. Urine samples were pooled from 3 healthy female volunteers aged 27–53 years, who had no history of UTI or antibiotic use within two weeks. The urine was filter-sterilized (0.2 μm pore size) and stored at −20 °C for use within two weeks. The cultures in pooled urine were grown aerobically at 37 °C to an optical density of 0.1 and treated with CORM-2 for 24 h.

2.4. Examination of bacterial viability

Bacterial viability was determined by counting the CFU number upon plating serial dilution on TSA plates. Following overnight culture at 37 °C bacterial CFU numbers per milliliter were determined by using mean from two dilutions. Growth was calculated as the numbers of CFU ml^{-1} in treated cultures or controls divided by the number of CFU ml^{-1} formed upon the plating of the initial inoculums and expressed as log CFU ml^{-1}. Positive values in the growth curves indicate growth and negative values indicates reduced growth. A bactericidal effect has been defined as >99.9% (3 log units) decrease in growth as compared to in the initial inoculums [30].

2.5. Cytotoxicity assay on human bladder epithelial cells

Human bladder carcinoma cell line 5637 (ATCC HTB-9; American Type Culture Collection Manassas, USA) were cultured as a monolayer in Dulbecco's Modified Eagle medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM non-essential amino acids and 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (all from Invitrogen, Paisley, UK) in a humidified incubator at 37 °C with 5% CO_2. The cells were seeded into 96-well plates and grown to confluence in a humidified incubator at 37 °C with 5% CO_2. During the experiments the cells were grown in cell culture medium supplemented with 2% fetal bovine serum, 2 mM L-glutamine and 1 mM non-essential amino acids and exposed for CORM-2 (100, 250 or 500 μM), vehicle or medium only (control) for 4, 8 and 24 h. Cell cytotoxicity was determined, according to the manufacturer's instructions, at the

end of the incubation using a Cytotoxicity Detection Kit plus (Roche, Mannheim, Germany) that detects lactate dehydrogenase (LDH) activity. LDH is released into the cell culture supernatant when the plasma membrane is damaged. The absorbance was measured at 492 nm by using a spectrophotometer (Thermo Labsystems, Multiscan Ascent).

2.6. Isolation of RNA and cDNA synthesis

Overnight cultures of ESBL-producing isolates 6 and 7 (both CTX-M-15) were used to inoculate fresh MS-medium to an OD of 0.1 followed by exposure to 250 μM CORM-2 or 2.5% DMSO vehicle for 15 min at 37 °C. RNA isolation was performed by TrizolMax Bacterial RNA isolation kit (Invitrogen, Bleiswijk, NL) according to manufacturer's instructions, and DNA decontamination treatment performed by Turbo DNase (Ambion, Life technologies, NY, USA) and acid phenol (Ambion) extraction as previously described [31]. The quantity and the purity of the purified RNA samples were determined by measuring the absorbance (A_{260}) and (A_{260}/A_{280}) ratio using a spectrophotometer Nanodrop-1000 (NanodropTechnologies Inc, Wilmington, USA). The cDNA synthesis (0.1 μg of total RNA) was performed by using Omniscript Reverse Transcriptase for first strand cDNA synthesis (Quiagen, Hilden, Germany), RNase inhibitor and random hexamer primers (Quiagen, Hilden, Germany). Genomic DNA contamination was controlled for by cDNA synthesis without reverse transcriptase and considered to be absent if undetected after 35 cycles of amplification.

2.7. Real time RT-PCR

Gene expression studies were performed for genes encoding some established UPEC virulence factors with putative roles in e.g., adhesion, iron acquisition, serum resistance and biofilm formation (Table 2). Real time RT-PCR was performed with SsoFast EvaGreen® Supermix (Bio-Rad laboratories, CA, USA) according to manufacturer's instructions. The passive reference dye ROX (Bio-Rad laboratories), 200–300 nM of primer (Table 2), and 10 ng template cDNA was added to each supermix. Primers were ordered from Eurofins MWG Synthesis GmbH (Ebersberg, Munich) except the CTX-M primers (Scandinavian gene synthesis, Köping, Sweden). The RT-PCR amplification was performed in a 7900 HT Real time RT-PCR system (Applied Biosystems) using the following protocol: enzyme activation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s followed by extension at 60 °C or 65 °C for 30 s. Each PCR was followed by a dissociation curve analysis between 60 and 95 °C. Amplification efficiency analysis was performed on all primers and found to be between 100 ± 10%. All samples were run in duplicates. The Ct values were analyzed by the comparative Ct (ΔΔCt) method and normalized to the endogenous control gapA (encoding glyceraldehyde 3-phosphate dehydrogenase). Fold difference was calculated as $2^{-\Delta\Delta Ct}$.

If no gene expression was detected by real time RT-PCR, the primers were validated by analyzing DNA samples by PCR, using J96 or CFT073 as positive controls. Briefly, 2-3 bacterial colonies grown on TSA-agar plate overnight at 37 °C were suspended in 100 μl of sterile water as previously described [29]. The suspensions were boiled for 15 min, cooled to 4 °C and subsequently centrifuged for 30 s at 12,000× *g*. The supernatant, containing DNA, was transferred to new tubes and stored at 4 °C until PCR analysis.

2.8. Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were assessed by the unpaired two-tailed Students *t*-test. Results were considered statistically significant at $P < 0.05$. n = number of independent biological replicates.

3. Results

3.1. Effects of CORM-2 on viability in an ESBL-producing UPEC isolate, a non-ESBL producing UPEC strain and a commensal E. coli strain

CORM-2 showed a concentration-dependent inhibition of growth in an ESBL-producing UPEC isolate (ESBL 7). A low concentration of CORM-2 (100 μM) was without effect, but 250 μM slightly inhibited the bacterial growth at 4 h but at 24 h a resumed growth was seen (Fig. 1a). Exposure to 500 μM CORM-2 caused a sustained bactericidal effect with a reduction of bacterial counts by >3 log units after 4 h (Fig. 1a). Untreated controls showed an increased growth of ~2 log units during the 24 h study period. The antimicrobial effect of CORM-2 (500 μM) was also assessed in the non-ESBL-producing UPEC strain J96. The CORM-2-induced growth inhibition did not differ between ESBL isolate 7 and UPEC strain J96 (Fig. 1b). We next compared the antimicrobial effects of CORM-2 in ESBL isolate 7 with the non-pathogenic commensal *E. coli* strain MG1655. Exposure to 250 and 500 μM CORM-2 revealed no significant differences in CORM-2-induced growth inhibition between ESBL 7 and MG1655 at 2, 4, 8 and 24 h (Fig. 1c). Control experiments with $RuCl_2(DMSO)_4$, where the carbonyl groups have been replaced with DMSO, were performed to confirm that the effect of CORM-2 was caused by CO and not by the ruthenium compound.

Table 2
Summary of target genes and primers used for measuring gene expression. Primer URLs last accession mars 2012 GenBank, National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/.

Gene	Protein function	Putative role	Primer product size bp	Reference for primer sequences
bla_{CTX-M}	CTX-M enzymes	β-lactamases	300	[29]
fimA	Major type 1 subunit fimbriae	Cell adhesion	155	[49]
papC	P fimbriae	Cell adhesion	202	[50]
hlyA	α-hemolysin	Pore-forming toxin	155	[49]
cnf1	Cytotoxic necrotizing factor	GTPase deamidase, toxin	174	[49]
iutA	Aerobactin siderophore receptor	Iron acquisition	144	[51]
iroN	Siderophore receptor	Iron acquisition	142	[51]
iha	Iron-regulated adhesin, catecholate	Iron acquisition	147	[51]
chuA	Heme utilization transport protein	Iron acquisition	183	[49]
flu	Antigen 43	Biofilm formation	198	[49]
yfaL	Putative adhesion	Adhesion, biofilm formation	190	[49]
kpsMT II	Polysialic acid transport protein	Polysaccharide coating	272	[50]
traT	Transfer protein	Serum resistance	287	[50]
gapA	glyceraldehyde 3-phosphate dehydrogenase		142	[51]

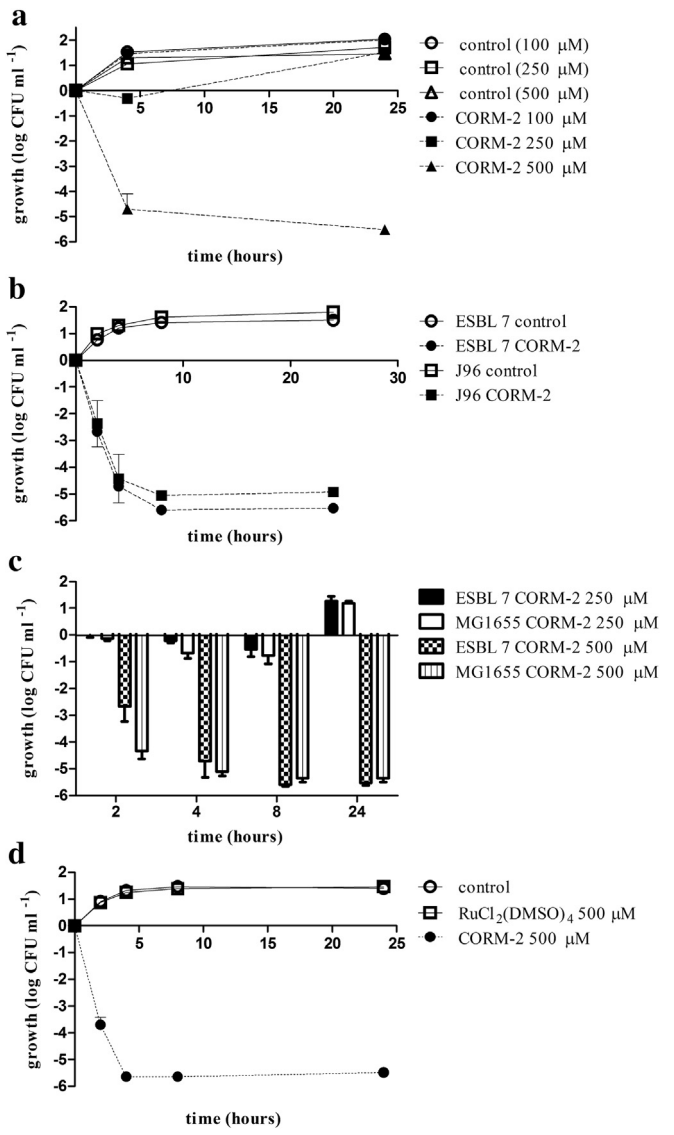

Fig. 1. a) Effect of different concentrations of CORM-2 (100, 250 or 500 μM) on viability studied in ESBL isolate 7. b) A time-course study of CORM-2 (500 μM) exposure on viability studied in ESBL isolate 7 and UPEC strain J96. c) Effect of CORM-2 (250 and 500 μM) exposure on viability studied in ESBL isolate 7 and *E. coli* K-12 strain MG1655. Cultures were grown to early log phase in MS-broth and then exposed to CORM-2 or left untreated (control). d) Effect of CORM-2 or $RuCl_2(DMSO)_4$ on viability studied in ESBL isolate 7. Cultures were grown to early log phase in MS-broth and then exposed to CORM-2 (500 μM), $RuCl_2(DMSO)_4$ (500 μM) or left untreated (control). Growth is shown as log CFU ml^{-1} of treated bacteria or controls and compared to the initial starting inoculum. The data are presented as mean ± SEM from at least three independent experiments in panel a, b, c and from two independent experiments in panel d.

$RuCl_2(DMSO)_4$ (500 μM) had no effect on bacterial viability and the growth response was not different from the untreated controls at 2, 4, 8 and 24 h (Fig. 1d).

3.2. Effect of CORM-2 on viability in additional ESBL-producing UPEC isolates and when grown in urine

The effect of CORM-2 (500 μM) was investigated in three additional ESBL-producing UPEC isolates. After 2 h a reduced growth was seen, and after 8 h a bactericidal effect (>5 log units decrease in growth) of CORM-2 was found in all three isolates (ESBL 1, 6 and 9) (Fig. 2a). The effect of CORM-2 (500 μM) on bacterial

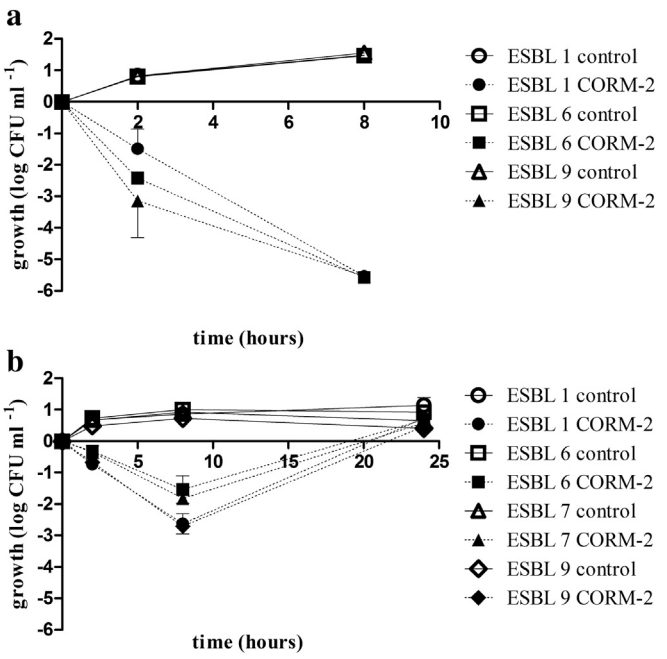

Fig. 2. Effect of CORM-2 on viability studied in additional ESBL-producing isolates and when grown in urine. Cultures were grown to early log phase and then exposed to CORM-2 (500 μM) or left untreated (control). Growth is shown as log CFU ml^{-1} of treated bacteria or controls and compared to the initial starting inoculum. a) ESBL isolates 1, 6 and 9 grown in MS-broth for 2 and 8 h. b) ESBL isolates 1, 6, 7 and 9 grown in urine for 2, 8 and 24 h. The data are presented as mean ± SEM of two independent experiments.

viability when the isolates were grown in urine was also studied in four different ESBL-producing UPEC isolates. Notable, untreated controls showed a lower growth response in urine than in MS-broth. A growth inhibition was detected after exposure to CORM-2 for 2 and 8 h but after 24 h there was resumed growth in all four isolates (Fig. 2b).

3.3. Comparison between CORM-2 and nitrofurantoin on viability in an ESBL-producing UPEC isolate

The effect of CORM-2 was compared to the effect of nitrofurantoin in an ESBL-producing isolate when grown in MS-broth. Nitrofurantoin is an antibiotic used for treatment of uncomplicated UTIs, and is so far effective against many isolates of ESBL-producing *E. coli* (89.3%) [32]. MIC for nitrofurantoin was determined to be 134 μM. The inhibition of bacterial viability exerted by CORM-2 at MIC (500 μM) was significantly ($P < 0.001$) faster in onset than the inhibition induced by nitrofurantoin at its MIC (134 μM) (Fig. 3). A higher concentration of nitrofurantoin (500 μM) was not faster in onset or more effective than 134 μM (Fig. 3). A bactericidal effect of CORM-2, with a reduction of bacterial counts by >3 log units, was in these experiments detected after 3 h of exposure and for nitrofurantoin after 8 h of exposure.

3.4. Effect of CORM-2 on cell viability in human bladder epithelial cells

Experiments were performed to evaluate the potential cytotoxic effect of CORM-2 on host eukaryotic cells using the human bladder epithelial cell line 5637. The cytotoxicity of CORM-2 (100 μM, 250 μM and 500 μM) or the vehicle (DMSO) after 4 and 8 h of exposure was low (Fig. 4). After 24 h of exposure there was a pronounced cytotoxic effect in response to 250 and 500 μM CORM-2,

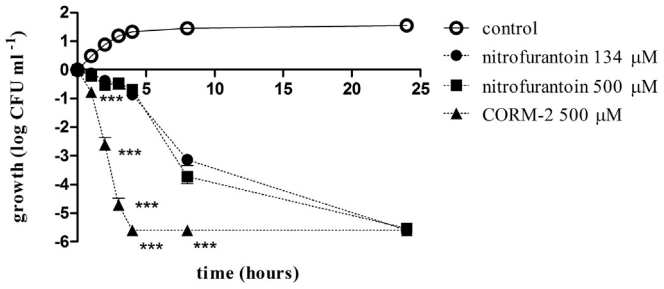

Fig. 3. Effect of CORM-2 or nitrofurantoin on viability studied in ESBL isolate 7 grown in MS-broth. Cultures were grown to early log phase and then exposed to CORM-2 (500 μM), nitrofurantoin (134 μM (MIC) and 500 μM) or left untreated (controls). Growth is shown as log CFU ml^{-1} of treated bacteria or control and compared to the initial starting inoculum. The data are presented as mean ± SEM of three independent experiments. ***$P < 0.001$, CORM-2 versus nitrofurantoin (134 μM).

while the cytotoxicity was lower in response to 100 μM CORM-2. However, the cytotoxicity evoked by CORM-2 (250 and 500 μM) was similar or lower ($P < 0.05$) respectively, to the toxicity evoked by the vehicle. This indicates that the observed cytotoxicity is caused in part by DMSO.

3.5. Effect of CORM-2 on gene expressions in ESBL-producing UPEC isolates

The effect of CORM-2 (250 μM) or vehicle on gene expression of some selected virulence factors was evaluated by real time RT-PCR. A summary of the studied genes and their putative role is found in Table 2. The results showed that the gene expression was significantly reduced for polysaccharide coating *kpsMT II* and serum resistance *traT* when exposed to CORM-2 (Table 3). In addition, CORM-2 exposure significantly increased the expression of the iron-regulated adhesin *iha*. Genes coding for the type 1 fimbriae (*fimA*), P-fimbriae (*papC*), heme utilization transport protein (*chuA*) and biofilm formation (*flu, yfaL*) were not significantly altered by CORM-2 exposure. No expression of *hlyA*, *cnf1* or *iroN* was detected in the ESBL-producing isolates. CORM-2 did not affect the expression of the CTX-M β-lactamase resistance gene (bla_{CTX-M}) (data not shown). Taken together, the results showed that CORM-2 affected the expression of some virulence genes in ESBL-producing *E. coli*.

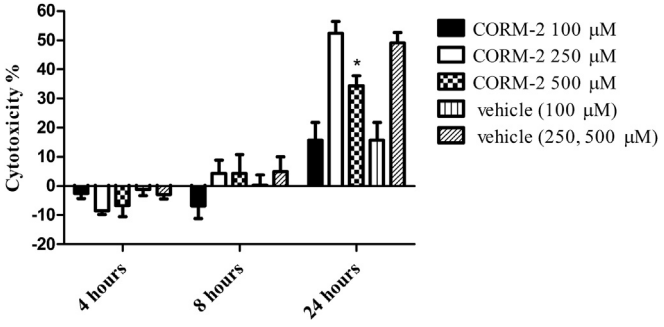

Fig. 4. Cytotoxic effect of CORM-2 or vehicle (DMSO) studied in the bladder epithelial cell line 5637 cells after exposure to different concentrations of CORM-2 (100, 250 or 500 μM) or vehicle. Cytotoxicity was assessed after 4, 8 and 24 h by measuring lactate dehydrogenase (LDH) activity. Cytotoxicity was compared to low controls with untreated cells or high controls with maximum releasable LDH activity and expressed as %. The data are presented as mean ± SEM of three independent experiments. *$P < 0.05$, CORM-2 versus vehicle.

Table 3
Changes in gene expression in ESBL isolates 6 or 7 after exposure to CORM-2 (250 μM) or vehicle for 15 min as revealed by real time RT-PCR. The data are presented as mean fold change ± SEM. $n = 4$.

Gene	Growth condition	Fold change ± SEM	*P* value[a]
fimA	CORM-2	1.6 ± 0.34	0.135
papC [b]	CORM-2	−1.1 ± 0.25	
iutA	CORM-2	−1.3 ± 0.071	
iha	CORM-2	1.7 ± 0.11	0.0011**
chuA	CORM-2	4.8 ± 1.6	0.0595
flu	CORM-2	1.4 ± 0.30	
yfaL	CORM-2	1.6 ± 0.25	0.0666
kpsMT II [c]	CORM-2	−4.0 ± 0.047	0.0001***
traT	CORM-2	−1.8 ± 0.085	0.0018**
	Vehicles[d]	1.0 ± range 0.00065−0.031	

**P values < 0.01, ***P values < 0.001, CORM-2 versus vehicle.
[a] Statistical analysis are only performed for genes showing fold changes >1.5.
[b] No detected expression in ESBL isolate 7.
[c] No detected expression in ESBL isolate 6.
[d] Vehicles (DMSO) performed for every gene.

4. Discussion

The prevalence of ESBL-producing Enterobacteriaceae is increasing [33] and the development of therapeutic drugs designed for use against antibiotic-resistant pathogens is a major health concern. Given their different modes of action compared to currently used antibiotics, CO-RMs have the potential for treatment of antibiotic-resistant bacteria [34]. Multidrug-resistant ESBL-producing *E. coli* may in the future change management of uncomplicated urinary tract infections into difficult-to-treat infections and potential life threatening infections requiring intravenously administered antibiotics and often hospitalization. In this study we examined the antibacterial effect and gene expression profile of CORM-2 on multidrug-resistant ESBL-producing UPEC. CORM-3, a water soluble CO-releaser, has previously been shown to have bactericidal effects against antibiotic-resistant *P. aeruginosa* [4].

Bacterial growth experiments showed a bactericidal effect of 500 μM CORM-2 on ESBL-producing UPEC isolates and the non-ESBL-producing UPEC strain J96. This concentration of CORM-2 did also have bactericidal properties against a non-pathogenic *E. coli* K-12 strain (MG1655), in agreement with an earlier study [5]. The redox-sensing regulators SoxS and OxyR are believed to protect bacteria against CORM-2 [9] and it is possible that pathogenic bacteria have evolved more efficient defense systems against host-derived antimicrobial factors, like CO. To investigate this possibility we performed a more detailed comparison between an ESBL-producing UPEC and the commensal *E. coli* strain MG1655, but no correlation between sensitivity towards CORM-2 and pathogenic potential of the strains was revealed. Ruthenium from CORM-3 has been found to accumulate in bacteria but ruthenium is not believed to have any antibacterial effect *per se* [6] but rather to facilitate CO targeting within bacteria. Indeed, the ruthenium compound $RuCl_2(DMSO)_4$, that lacks carbonyl groups, had no antibacterial effect on ESBL-producing UPEC isolates in our study strongly indicating that the antibacterial effect of CORM-2 is dependent on CO. CORM-2 is highly lipophilic and delivers CO intracellularly close to the cellular targets in bacteria [9,35]. The growth inhibitory effect of CO-RMs involves effects on the bacterial respiratory chain and terminal oxidases [35]. CO release from CORM-2 has been reported to be first order and very rapid with a half-life of 0.5 min [8]. A recent study shows that sodium dithionite, a reducing agent, facilitates a rapid CO release from CORM-2 [36], but so far there is no complete understanding of the mechanism of CO release from CORM-2 and its correlation with biological activity. The data from comparison of CORM-2 with the established

antibiotic nitrofurantoin showed that CORM-2 had a faster onset of action than nitrofurantoin in reducing bacterial growth.

Administration of CORM-2 to ESBL-producing *E. coli* in urine caused a short lasting growth inhibitory effect. It is known that heme-containing proteins and other terminal electron acceptors affect the CO ligands in CORM-2 by scavenging CO [35] and that thiol-containing amino acids reduce the uptake of CO-RMs by *E. coli* [37]. In urine, that displays a more complex composition than the MS medium, more CO may be scavenged or liberated in the extracellular space and less CO may be available for delivery to cellular targets inside the bacteria. This may possibly explain the reduced effect of CORM-2 in urine.

The toxicity of CORM-2, in concentrations and exposure times that were bactericidal, was evaluated in host bladder epithelial cells. A low cytotoxicity was observed for up to 8 h of exposure, and CORM-2 even seemed to have an initial protective effect. It has been reported that CO and CO-RMs have cytoprotective functions, protecting cardiac, immune, respiratory and gastrointestinal mammalian systems [38,39]. However, at 24 h there was a pronounced cytotoxic effect by the vehicle alone (2.5% DMSO) which indicates that the CORM-2 cytotoxicity is associated in part by DMSO. Supporting this, the low vehicle control (1% DMSO) and 100 μM CORM-2 had almost the same cytotoxicity levels after 24 h of exposure. Other studies have also reported low cytotoxicity of CORM-2 in vascular smooth muscle cells and RAW 264.7 macrophages [8,35]. The low sensitivity of CO-RMs in eukaryotic cells compared to bacteria may be explained by the better accessibility to respiratory enzymes in bacteria [35].

Understanding the targets for CO will be critical for future antimicrobial applications of CO-RMs. Microarray analysis of CORM-2 and CORM-3 treated *E. coli* revealed extensively modified gene expression patterns with both up- and down-regulated genes [6,9]. Besides key aerobic respiratory complexes, genes involved in metal metabolism, homeostasis or transport were also affected. These previous studies were performed using commensal *E. coli* K-12 strain and therefore no data are available on the effect of CORM-2 on UPEC virulence genes. In a whole-genome transcription study [9] of commensal *E. coli*, CORM-2 caused up-regulated expression of inorganic iron transport genes and genes involved in sulfur and methionine metabolism while many down-regulated genes were related to respiratory metabolism. In our study, CORM-2 was found to affect the expression of selected genes encoding known UPEC virulence factors such as putative iron acquisition systems. Expression of iron acquisition systems is fundamental for the ability of the bacteria to colonize and persist in iron-poor niches such as the urinary tract. An increased expression of the iron-regulated adhesion catecholate receptor *iha* was found. Putative Iha function as an outer membrane iron receptor and both *iha* [40] and *chuA* [41] expression is modulated by the ferric uptake regulator (Fur), and *iha* is also regulated by iron availability [40]. Fur participates in regulation of many cellular functions as oxidative stress and respiration [42]. CORM-3 has been shown, by transcriptomics and mathematical modeling, to affect the transcription factor for Fur [6] and it is possible that CORM-2 also mediates its effect on gene expression through Fur. High intracellular concentrations of free iron, associated with degradation of iron–sulfur containing proteins, have been reported after CORM-2 exposure [43], which may explain the changes seen in gene expression of iron-systems in our study.

The gene expression for capsule group II kpsMT II and serum resistance survival gene traT was significantly reduced by CORM-2 exposure. Serum resistance, the ability to avoid the bactericidal activity in serum, has previously been found to be increased among ESBL-producing K. pneumoniae compared to non-ESBL-producers [44]. Likewise, the expression of capsule group II kpsMT II, known to be of importance in UTI pathogenesis [45] was markedly suppressed by CORM-2. Thus, CORM-2 seems to offer an approach to inhibit some specific bacterial virulence factors. The overall fold changes in gene expression were small but the efficient reagents and enzymes used in the qPCR system, as well as the reproducibility and the amplification efficacy of the reactions lead us to believe that we can detect a fold change of 1.5 and above. However, the biological significance of the detected changes in gene expression, including corresponding phenotypic changes and changes in fitness of the isolates needs to be established. The ESBL-producing isolates studied had no detected gene expression for *hlyA, cnf1* and *iroN* therefore no data is available for their expression levels after CORM-2 exposure. These findings are in line with reports on the absence of several classical virulence factors such as HlyA and Cnf1 in ESBL-producing ST131 isolates [24,46]. Genes encoding fimbriae (*fimA, papC*) and biofilm formation (*flu, yfaL*) showed no significant altered expression after CORM-2 exposure in our study. A recent study demonstrates that CORM-2 increases biofilm production in a commensal *E. coli* K-12 strain and that many genes that are transcriptionally modified by CORM-2 are in common with genes modified during the formation of biofilm [9]. Exposure of *P. aeruginosa* to CORM-2 attenuated microcolony formation on human bronchial epithelial cells and reduced the growth of this bacterium within an established biofilm [47]. Beta-lactamase inhibitors are currently of interest as treatment against multidrug-resistant ESBL-producing bacteria [48]. However, the gene encoding the CTX-M β-lactamases ($bla_{\text{CTX-M}}$) remained unaltered by CORM-2 exposure.

This study shows for the first time that CORM-2 is effective against uropathogenic ESBL-producing *E. coli* isolates that currently show a pandemic spread and cause infections with few treatment options [33]. CORM-2 affected the gene expression profile of some virulence factor genes, among them is noticed a reduced expression of the iron acquisition aerobactin siderophore receptor *iutA*, serum resistance survival gene *traT* and capsule group II *kpsMT II*. Thus, CO-RMs may have potential as novel antimicrobial therapeutic agents against multidrug-resistant uropathogenic ESBL-producing *E. coli*.

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