The Journal of Antibiotics (2012) 65, 153–156

www.nature.com/ja

NOTE

Creatinine inhibits bacterial replication

Thomas McDonald[1], Kristen M Drescher[2], Annika Weber[1] and Steven Tracy[1]

The Journal of Antibiotics (2012) **65,** 153–156; doi:10.1038/ja.2011.131; published online 1 February 2012

Keywords: antibacterial; antibiotic; bacteria; creatinine

Widespread antibiotic use has resulted in increased frequency of clinically important bacteria acquiring single or multiple antibiotic resistance.[1,2] Even antibiotic therapies for relatively trivial afflictions, such as acne,[3] have promoted development of microbial antibiotic resistance. The availability of non-prescription antibiotics in some areas has also resulted in improper and/or irrational self-medication, further exacerbating this problem.[4,5] New antibacterial agents with broad-spectrum impact against both Gram-positive[6] and Gram-negative[7] species, as well as against drug-resistant strains such as methicillin-resistant *Staphylococcus aureus*[8] are needed for wound care and to treat topical and dermatological infections.[9] A chance observation in our laboratory revealed that creatinine (CRN; creatinine hydrochloride, CRN-HCl) halted the growth of bacteria on nutrient agar plates. CRN is the naturally occurring breakdown product of creatine phosphate, a high-energy molecule used to store and then donate, a high-energy phosphate to ADP for the synthesis of ATP in metabolism. Occurring normally in human blood at concentrations ranging approximately between 50–100 μM and in urine at slightly higher levels, CRN is accepted to be a naturally produced inert waste product with no active function,[10] although a recently published study has challenged this dogma.[11] We characterized the ability of CRN-HCl to inhibit the growth of a wide array of bacterial species, including methicillin-resistant *Staphylococcus aureus*.

CRN-HCl (Sigma-Aldrich; St Louis, MO, USA; F.W.=149.6) was evaluated for its ability to inhibit the growth of different bacteria. Bacterial cultures were propagated in LBG (LB broth, Miller; Fisher Scientific; Fair Lawn, NJ, USA) supplemented with 1% w/v glucose) either in liquid or agar (BD Bacto Agar; Becton Dickinson, Sparks, MD, USA) format. Overnight cultures of *Escherichia coli* and *S. aureus* were diluted in fresh LBG to 1–5×10^5 colony-forming units (c.f.u.) per ml, aliquoted to 2 ml tube cultures, to each of which was added CRN-HCl in increasing 5 mM increments from 0–50 mM. After shaking at 250 r.p.m. at 37 °C for 18 h, the OD at 600 nm was recorded. *E. coli* was inhibited from growth in 40 mM (5.98 mg ml^{-1}) CRN-HCl, while *S. aureus* growth was inhibited at a concentration of 15 mM (2.24 mg ml^{-1}) CRN-HCl (Figure 1a). These values were taken as a measurement of the MIC for CRN-HCl under these specific assay conditions. A similar experiment was performed using a 96-well microtiter plate format in which triplicate wells were filled with 100ul total volume of *E. coli* or *S. aureus* (final concentration of 1–5×10^5 c.f.u. ml^{-1}) in LBG containing increasing concentrations of CRN-HCl. Following 18 h stationary incubation at 37 °C, wells were examined visually to ascertain which wells appeared turbid versus clear, then the plate was assayed using an ELISA plate reader (ELX800; Bio-Tek Instruments, Winooski, VT, USA) using a 490 nm wavelength (Figures 1b and c). This format confirmed the results from the tube cultures, demonstrating the inhibition of the growth of *E. coli* and *S. aureus* at 40 and 15 mM CRN-HCl, respectively. Using this microtiter plate format, MIC values for four other bacterial species were established: *Brevibacterium linens* (MIC=10 mM), *Bacillus subtilis* (MIC=20 mM), *Pseudomonas aeruginosa* (20 mM) and *Streptococcus pyogenes* (MIC=20 mM; data not shown).

Drug-resistant bacterial strains and other bacterial species were assayed for sensitivity to inhibition of replication by CRN-HCl using a disc diffusion assay. Assays were performed as described[12] with the following modifications. Twenty-five microliters of 2 M CRN-HCl in water was added to 30 mg of a powdered carrier (Eridex; Cargill Inc., Cedar Rapids, IA, USA) and stirred into a thickened slurry in order to apply the maximum amount of CRN-HCl on the disc. Fifty microliters of the slurry containing 5 mg CRN-HCl were applied to 6 mm diameter sterile dry paper discs (Whatman no. 3 filter paper; Whatman, Piscataway, NJ, USA) that were then inverted onto LBG agar plates containing test bacteria. The LBG agar plates were prepared 15 min before use by spreading the test bacteria (diluted in phosphate-buffered saline to 1–5×10^5 c.f.u. ml^{-1} from overnight cultures) to the plate with a sterile cotton swab. The plates with discs were incubated for 15 h at the temperature appropriate for the particular bacterial species. Clear zones around the discs, indicative of growth inhibition, were measured. As a point of comparison, gentamicin-impregnated discs (GM-10; Becton Dickinson) were tested. For all bacteria but drug-resistant and anaerobic bacteria, for which assays were repeated twice, discs were tested in triplicate on two different days and zones of inhibition were recorded as the average of these six measurements. Variation was ⩽2 mm. Results for the diverse bacterial species assayed using this approach are shown in Table 1. All bacteria tested in this manner, including drug-resistant strains,

[1]Department of Pathology and Microbiology, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, NE, USA and [2]Department of Medical Microbiology and Immunology, Creighton University, Medical Center, Omaha, NE, USA
Correspondence: Dr T McDonald, Department of Pathology and Microbiology, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, NE 68198, USA.
E-mail: tmcdonal@unmc.edu
Received 24 August 2011; revised 31 October 2011; accepted 7 December 2011; published online 1 February 2012

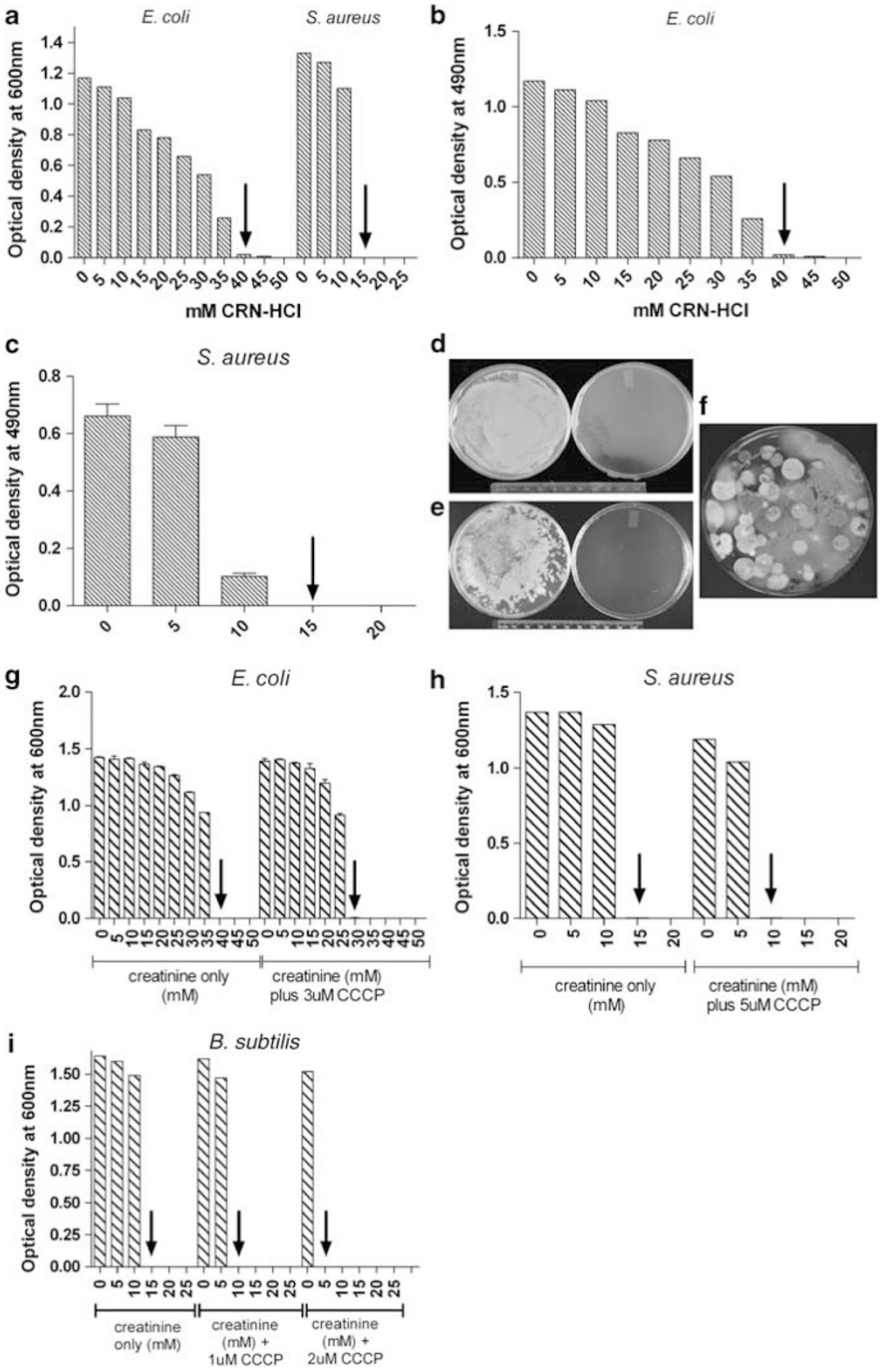

Figure 1 Creatinine inhibits bacterial replication (**a**–**f**) potentially by overwhelming the bacterial cells ability to pump out protons (**d**–**i**). MIC measurements for CRN-HCl against *E. coli* and *S. aureus* determined in shaking tube culture (**a**) or stationary 96-well microtiter dish formats (*E. coli* (**b**); *S. aureus* (**c**)). The MICs for the two bacterial species were the same regardless of the format employed. Arrows indicate MIC as well as the first tube or well in the series that was not visibly turbid. Replication of bacteria derived from samples of (**d**) human feces or (**e**) garden soil was suppressed in the presence of 100 mM CRN-HCl. Left hand plate in each panel contains no CRN-HCl; right hand plates contain 100 mM CRN-HCl. Example of fungal growth following 10 days of incubation at room temperature on an LBG agar plate containing 100 mM CRN-HCl (**c**). The synergy between the inhibitory action of CRN-HCl and the action of the proton efflux inhibitor CCCP in lowering the concentration of CRN-HCl necessary for growth inhibition is demonstrated for *E. coli* (**g**), *S. aureus* (**h**) and *B. subtilis* (**i**). In the presence of CCCP, the MICs are left shifted. Arrows indicate the MIC at which neither detectable turbidity was observed by the eye nor OD when measured at 600 nm.

Table 1 CRN-HCl inhibits diverse bacteria but not eukaryotic organisms

		Zone of inhibition (mm)	
	Class	*Creatinine*	*Gentamicin*
Gram-positive bacteria			
Staphylococcus aureus	Lab. strain 29213	26	24
Staphylococcus aureus	UAMS-1	24	ND
Staphylococcus aureus	Methicillin resistant (MRSA)	27	7
Staphylococcus epidermidis	Lab. Strain	27	ND
Enterococcus faecalis	Lab. strain 29212	19	14
Enterococcus faecium	Vancomycin resistant (VRE)	18	8
Micrococcus luteus	Lab. isolate	22	ND
Brevibacterium linens	ATCC 9175	40	ND
Bacillus subtilis	Lab. strain	20	ND
Bacillus cereus	Lab. strain	22	ND
Clostridium difficile	Clinical isolate UNMC 012-SM	23	ND
Clostridium difficile	Clinical isolate UNMC 013-NO	21	ND
Gram-negative bacteria			
Pseudomonas aeruginosa	Lab. strain 27853	27	21
Pseudomonas aeruginosa	HLR	25	8
Pseudomonas fluorescens	Lab. strain	18	ND
Escherichia coli	Lab. strain 35150	21	15
Escherichia coli	Beta lactamase producer (ESBL)	24	19
Acinetobacter baumannii	HLR	16	6
Acetobacter xylinum	Lab. strain	15	ND
Eukaryotes			
Candida albicans	Lab. strain 24433	0	8
Rhodotorula sp.	Lab. isolate	0	ND
Saccharomyces	Lab. isolate	0	ND

Abbreviations: ATCC, American Type Culture Collection; ESBL, extended spectrum beta lactamases; HLR, high-level resistance; Lab. strain, laboratory isolate; MRSA, methicillin-resistant *Staphylococcus aureus*; ND, Not done; *sp.*, species; UAMS, University of Alabama Medical School; UNMC, University of Nebraska Medical Center; VRE, vancomycin-resistant *Enterococcus*. Zones of inhibition were determined using disc diffusion assays as described in text. The use of all organisms and procedures were approved by the Institutional Biosafety Committee (IBC) of the UNMC, Omaha, NE, USA. Bacterial and yeast species were obtained from Department of Pathology and Microbiology at UNMC.

were inhibited by CRN-HCl with a range of zone sizes between 16–40 mm.

The ability of CRN-HCl to suppress unknown bacterial growth in environmental samples (fecal matter and soil) was also tested (Figures 1d and e). Solids from a human fecal swab were suspended in LBG (~1% w/v) and spread either on control plates containing LBG agar or test plates containing LBG agar poured in 100 mM CRN-HCl. After 24 h incubation at 37 °C, rampant bacterial growth was observed on the control plate but growth was completely suppressed by CRN-HCl (a typical example is shown in Figure 1d; compare control plate on the left to the CRN-HCl-containing plate on the right). Similar experiments using garden soil (1% w/v) as the source of microorganisms gave the same result: complete ablation of bacterial outgrowth in the presence of CRN-HCl (Figure 1e). Sterile swabbing of the surface of the clear CRN-HCl-containing plates (3 days after initial plating) followed by transfer of the swabbed material to control LBG nutrient agar plates, revealed outgrowth of bacterial colonies at varying numbers depending upon the sample used (data not shown). However, none of these outgrowing bacteria replicated when subsequently streaked onto 100 mM CRN-HCl-containing LBG nutrient agar (data not shown). We infer that these represented outgrowth of persistent bacterial spores that had not previously germinated. Occasionally, growth of diverse fungi on CRN-HCl-containing agar days after the plating environmental samples was observed, indicating that eukaryotic microorganisms were not inhibited by CRN-HCl (Figure 1e). We therefore tested the ability of 100 mM CRN-HCl to inhibit the growth of three different yeasts (*Candida albicans*, a *Rhodotorula* species, and a *Saccharomyces* species; Table 1). No growth inhibition of any of the yeast strains was observed on these plates (similar results were obtained in liquid culture containing 100–500 mM CRN-HCl; data not shown).

Preliminary studies revealed that CRN-HCl—but neither the non-salt anhydrous form of CRN (Sigma-Aldrich) nor the creatine monohydrate (Creapure; Degussa AG, Dusseldorf, Germany)—inhibited bacterial growth (data not shown). Using anhydrous CRN, we formed acetate and sulfate salts of CRN and showed that these, too, functioned as antibacterial agents similar to CRN-HCl (data not shown). We hypothesized therefore that only the protonated form[13] of CRN inhibited the growth of diverse bacterial species, perhaps by overwhelming the bacterial cells capacity to pump out protons.[14,15] To test this hypothesis, we used CCCP[14] (carbonyl cyanide 3-chlorophenylhydrazone; Sigma-Aldrich) to disrupt the proton gradient in cultures of *E. coli*, *S. aureus,* and *B. subtilis*. Tube cultures from diluted overnight cultures were established as described above, each containing the concentration of CCCP and CRN-HCl noted in the figure. Following 18 h shaking at 250 r.p.m. at 37 °C, the

OD for each culture was read at 600 nm. Each bacterial species required a 5–10 mM lower CRN-HCl concentration to inhibit replication in the presence of 1–5 μM CCCP as measured by OD of overnight cultures (Figures 1g, h and i).

It is widely accepted that CRN is a pharmacologically inactive waste product of muscle metabolism devoid of biological activity.[10,16] Thus, our discovery that this common nitrogenous waste molecule has antibacterial antibiotic properties is, to the best of our knowledge, unique and potentially of great interest. When tested at physiological levels (50–200 μM) on agar or in broth culture, CRN-HCl did not suppress bacterial growth relative to control cultures (data not shown) but when used between 10–100 mM, suppression of growth and killing of all bacterial species were observed including not only drug-resistant strains, such as methicillin-resistant *Staphylococcus aureus*, but also uncharacterized environmental bacterial strains as well. The mechanism of action is as yet unclear. We know that *Streptococcus* species, which lack the citric acid cycle[17] and *Staphylococcus* species, which use the citric acid pathway, were both inhibited by CRN-HCl (data not shown). CRN-HCl also inhibited the growth of an arginine-deficient mutant of *Staphylococcus epidermidis* compared with the wild-type control strain of *S. epidermidis* (both courtesy of Dr P Fey), indicating the CRN-HCl-induced growth inhibition was not arginine deaminase dependent.[18] As *Clostridium difficile* growth and replication was inhibited under anaerobic conditions, thus, we can infer that a lack of oxygen does not affect the suppressive mechanism of CRN-HCl. Our observations that CRN required protonation for the antibacterial function and that inhibition of proton conductance by CCCP lowered the concentration of CRN-HCl required to suppress bacterial growth are consistent with a working hypothesis that inhibitory CRN-HCl concentrations lead to acidification of the bacterial cytoplasm. Collectively, the results indicate that the mechanism of the suppressive action of CRN-HCl used in this study is likely held in common across diverse bacterial species.

The antibacterial characteristics suggest CRN-HCl or other salts of CRN may have potential for the addition to the armamentarium of agents to inhibit dermatological bacterial infections. We propose that CRN-HCl may find diverse applications in wound care and treatment as a supplement to, or replacement for, currently used antibacterial agents. Various other clinical, veterinary and industrial applications of CRN-HCL can also be envisaged.

ACKNOWLEDGEMENTS

We thank Drs Peter Iwen and Paul Fey (University of Nebraska Medical Center, Omaha NE) for their advice and gracious assistance in assaying restricted bacterial species, and for their useful discussions and for providing various *S. aureus* strains. This study was funded entirely by departmental funds made available to TM and ST.

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