**In Vivo Targeting of Escherichia coli with Vancomycin-Arginine**

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**ABSTRACT** The ability of vancomycin-arginine (V-r) to extend the spectrum of activity of glycopeptides to Gram-negative bacteria was investigated. Its MIC towards *Escherichia coli*, including β-lactamase expressing Ambler classes A, B, and D, was 8 to 16 µg/ml. Addition of 8 times the MIC of V-r to *E. coli* was acutely bactericidal and associated with a low frequency of resistance (<2.32 × 10^{-10}). In vivo, V-r markedly reduced *E. coli* burden by >7 log_{10} CFU/g in a thigh muscle model. These data warrant further development of V-r in combatting *E. coli*, including resistant forms.

**KEYWORDS** *Escherichia coli*, Gram-negative bacteria, antibiotic resistance, arginine, cationic peptides, multidrug resistance, vancomycin conjugate

Novel antibiotics are desperately needed to combat priority 1 or urgent-threat pathogens (1–3). With only four new classes of antibiotics introduced into the market since the early 1960s (4), structural modifications of current antibiotics provide an attractive and possibly speedier approach to fulfill this significant unmet clinical need. Vancomycin is a standard-of-care glycopeptide antibiotic for the treatment of Gram-positive infections (5). Numerous reports have demonstrated augmentation of its antimicrobial activity against resistant strains via different chemical modifications (6–9). Furthermore, its molecular structure has been successfully manipulated to create a broader spectrum of activity in the targeting of Gram-negative bacteria via adjuvant, formulation, and cationic/lipophilic interventions (10, 11) or synergy with existing Gram-negative antibiotics (12, 13). Recently, the covalent conjugation of l-arginine to vancomycin, to produce vancomycin-γ-arginine (V-R), led to promising Gram-negative properties via a cell wall mode of action (14). These findings encouraged us to further characterize the corresponding diastereomer vancomycin-δ-arginine (V-r) in animal models of *E. coli* infection using the δ-isomer of arginine to reduce the risk of conjugate hydrolysis (Fig. 1).

V-r was synthesized in a single chemical step from commercially available vancomycin HCl (StruChem, Wujiang City, China) and δ-arginine amide dihydrochloride (Aladdin Chemical Co., Shanghai, China). The crude compound was purified and isolated as the corresponding HCl salt at 95% purity by high-performance liquid chromatography based on a previously described procedure (14). Identity was confirmed by 1H nuclear magnetic resonance and time of flight mass spectrometry, and HCl content was quantified by ion-exchange chromatography. In various physicochemical screens, V-r behaved similarly to vancomycin, including no observed cellular cytotoxicity at concentrations ranging from 100 to 750 µM on human erythrocytes, HepG2, and primary renal proximal tubule epithelial cells employing fetal bovine serum-deficient media to negate compound quenching (15) (Table 1).
MICs were determined in alignment with CLSI guidelines as previously described for V-R and cationic antimicrobial peptides (14, 16). The MIC range of V-r against 29 different E. coli strains was 8 to 16 μg/ml (MIC<sub>90</sub> 16 μg/ml), including those with multiple resistance mechanisms (Table 2). The MIC of V-r against the efflux pump mutant strain JW0451-2 was 8 μg/ml, suggesting that V-r is unlikely to be a substrate for efflux in this pathogen. Notably, the MIC of V-r was also 8 μg/ml against two out of five of the Acinetobacter baumannii strains tested. In comparison, the MICs of vancomycin were significantly higher, at 64 to 256 μg/ml, against all E. coli and A. baumannii strains tested. Importantly, the antimicrobial potency of V-r towards a number of Gram-positive bacteria remained intact (Table 2). In frequency-of-resistance (FoR) assays at 8 times the MIC of V-r (128 μg/ml), E. coli ATCC 25922 demonstrated an extremely low FoR, at <2.32 × 10⁻¹⁰, which is similar to or lower than those with standard-of-care therapies, such as ciprofloxacin (17, 18). Time-kill assays were performed against uropathogenic E. coli strains, including the sequence type 131 (ST131) NCTC 13341 isolate. V-r, but not vancomycin, demonstrated rapid bactericidal activity to limits of detection (i.e., 100 CFU/ml) within 1 or 4 h of exposure, and this was maintained up to 24 h (Fig. 2).

Plasma pharmacokinetics (PK) of V-r after subcutaneous (s.c.) administration (20 and 121 mg/kg) was determined in naive male CD-1 mice (n = 3/group) using liquid chromatography-tandem mass spectrometry for analysis with a lower limit of quantitation of 5 ng/ml (Table 3). V-r displayed first-order elimination, similar to vancomycin, after s.c. administration (19, 20). Prior to efficacy studies, a single s.c. administration of V-r

![Vancomycin and vancomycin-D-arginine (V-r).](image)

**FIG 1** Vancomycin and vancomycin-D-arginine (V-r).

| TABLE 1 | Physicochemical properties of vancomycin-arginine (V-r) and vancomycin |
|---------|-----------------------------------------------------------------------|
|         | V-r                                                   | Vancomycin                        |
| Mol wt (free base)       | 1,604                                                  | 1,449                              |
| LogD (octanol/buffer)     | < 2.59                                                 | 4.01                               |
| TD solubility in saline (mg/ml) | 373                                      | > 50                                |
| PPB (mouse/human % bound) | 65/76                                                   | 50/50                              |
| Red blood cell lysis (CC<sub>50</sub>, μM) | > 750                                                 | > 750                              |
| HepG2 cell cytotoxicity (CC<sub>50</sub>, μM) | > 750                                                 | > 750                              |
| hRPTEC biomarkers<sup>a</sup> (CC<sub>50</sub>, μM) | > 100                                                 | > 100                              |
| FoR (at 8 × MIC)          | < 2.32 × 10⁻¹⁰                                        | Not determined                     |

<sup>a</sup>TD, thermodynamic; PPB, plasma protein binding; hRPTEC, human renal proximal tubular epithelial cells; CC<sub>50</sub> concentration at which 50% cytotoxicity is observed; FoR, frequency of resistance.

<sup>b</sup>LogD vancomycin reported according to Dave and Morris (29).

<sup>c</sup>Includes cell count, nuclear size, DNA structure, mitochondrial mass, mitochondrial membrane potential, phospholipidosis, and glutathione content.
TABLE 2 Antimicrobial susceptibility profiles of V-r and vancomycin

| Organism | Strain | Source, resistance mechanism or genotype | Ambler class | V-r | Vancomycin |
|----------|--------|---------------------------------------|--------------|-----|------------|
| E. coli  | ATCC 25922 | CLSI susceptible reference strain | A, D         | 16  | 128        |
| E. coli  | UT879 | Clinical isolate from patient with acute bladder infection | -            | -   | -          |
| E. coli  | NCTC 13441 | Uropathogenic E. coli ST131, blaCTX-M-15, blaOXA-1, blaTEM-1, aac(6')-Ib-cr, mph(A), catB4, tet(A), dfrA7, aadA5, sul1 | -            | -   | -          |
| E. coli  | NCTC 13462 | blaCTX-M-2 | -            | -   | -          |
| E. coli  | NCTC 13846 | Clinical isolate, bacteremia, UK 2013, EUCAST reference isolate, mcr-1 | B, C, D | 16 | 128        |
| E. coli  | AR055 | blaNDM-1, mph(A), blaOXA-1, dfrA17, sul1, tet(A), mrtC, aac(3)-IIa, blaoxa-1, aadA5 | -            | -   | -          |
| E. coli  | AR089 | strB, blacem-2, tet(B), strA, sul2 | C            | 16 | 128        |
| E. coli  | AR0114 | strB, blaTEM-18, blapcr-A, aadB, dfrA5, sul1, strA, sul2, cmrA1 | A            | 16 | 256        |
| E. coli  | AR0137 | blaNDM-1, mph(A), blaoxa-1, dfrA17, mcr-1, TEM-1B, CMY-42 | B            | 16 | 128        |
| E. coli  | AR0150 | blaNDM-1, mph(A), blaTEM-18, blapcr-A, dfrA17, sul1, tet(A), aadA1, aac(3)-IIIa, blaoxa-1, aadA5 | A, B, C | 8 | 128        |
| E. coli  | AR0346 | mcr-1, ESBL | A            | 16 | 256        |
| E. coli  | AR0349 | mcr-1, ESBL | A            | 16 | 128        |
| E. coli  | AR0350 | mcr-1 | -            | 16 | 128        |
| E. coli  | AR0493 | mcr-1, ESBL | A            | 16 | 256        |
| E. coli  | AR0494 | mcr-1 | -            | 8  | 128        |
| E. coli  | B096a | Clinical isolate (UK) 2016, AmpC | C            | 16 | 128        |
| E. coli  | B0608 | Clinical isolate (UK) 2016, blaTEM-1, blaoxa-15 | A            | 16 | 256        |
| E. coli  | ATCC BAA-2340 | blaOXA-1 | A            | 16 | 128        |
| E. coli  | ATCC BAA-2469 | blaOXA-1 | B            | 16 | 128        |
| E. coli  | ExPEC H5 | Clinical isolate (UK) | 8            | 128 | -          |
| E. coli  | H4/5 | Clinical isolate, blaoxa-1, blaoxa-15 | A            | 16 | 256        |
| E. coli  | IR3 | Clinical isolate, blaoxa-1 | B            | 8  | 128        |
| E. coli  | IR45 | Clinical isolate, blaoxa-1 | B            | 16 | 128        |
| E. coli  | IR57 | Clinical isolate, blaoxa-1 | B            | 16 | 128        |
| E. coli  | Swiss 2 (AF45) | Clinical isolate (South Africa) ST101, mcr-1 | 16  | 128  |
| E. coli  | Swiss 13 | Clinical isolate (France) ST69, mcr-1 | 16  | 128  |
| E. coli  | Swiss 15 | Clinical isolate (Switzerland) ST146, mcr-1, blaOXA-15 | A            | 16 | 128        |
| E. coli  | BW25113 | Parent strain of BW25113acrB-kan mutant | 8            | 128 | -          |
| E. coli  | JW0451-2 | BW25113acrB-kan, AcrB-deficient mutant, defective in ArcAB-ToIC multidrug efflux system | 8            | 128 | -          |
| A. baumannii | ATCC 19606 | Isolated from urine, genome-sequenced strain | 32  | 128  |
| A. baumannii | ACC00527 | Clinical respiratory isolate (USA) 2012, blaoxa-24 | D            | 8  | 128        |
| A. baumannii | B8003 | Clinical isolate (UK) 2016 | 32  | 128  |
| A. baumannii | GS2AB1 | Multiresistant clinical isolate (southern Europe) 2017 | 16  | 128  |
| A. baumannii | Naval-81 | Clinical isolate (USA) 2006 | 8  | 128        |
| S. aureus | ATCC 29213 | CLSI susceptible reference strain | 2 | 2 |
| S. aureus | NRS 384 | USA300-0114 MRSA, community associated | 0.5 | 2 |
| E. faecalis | ATCC 29212 | CLSI QC strain | 1 | 2 |
| E. faecalis | B575 | Clinical isolate (northwest UK) | 1 | 2 |
| S. agalactiae | B057 | Clinical isolate (northwest UK) | 0.06 | 0.5 |
| S. agalactiae | B063 | Clinical isolate (northwest UK) | 0.06 | 1 |
| S. pneumoniae | ATCC 49619 | Reference strain | 0.25 | 0.5 |
| S. pneumoniae | 3259-03 | Clinical isolate (northwest UK) | 0.5 | 0.5 |

*ESBL, extended-spectrum β-lactamase.

was shown to be well tolerated in male CD-1 mice (n = 3) at the highest dose tested (800 mg/kg).

Using a screening-based strategy, preliminary proof-of-concept studies with V-r employed an abbreviated 9-h thigh muscle infection model in male CD-1 mice rendered neutropenic (21). To that end, an E. coli ATCC 25922 isolate was inoculated at $9.7 \times 10^4$ CFU into both thigh muscles per mouse (n = 5 per experimental group). V-r was administered s.c. every 2 h (110 to 880 mg/kg total dose) starting 1 h postinfection. At 9 h, thigh homogenates were prepared, and CFU were enumerated after culture on CLED (cystine-, lactose-, and electrolyte-deficient) agar. Compared to pretreatment and
vehicle burdens of $5.1 \pm 0.2$ and $7.1 \pm 0.1 \log_{10} \text{CFU/g tissue}$, respectively, $V-r$ exhibited a dose-dependent reduction in bacterial burden of $1.2$ to $3.4 \log_{10}$ compared with vehicle (Kruskal-Wallis one-way analysis of variance using StatsDirect Statistical Analysis Software) (Table 4). $V-r$ doses at $440$ and $880 \text{ mg/kg}$ afforded $1.0$- and $1.3$-log$_{10}$ reductions below stasis, respectively, with an extrapolated static dose of $215 \text{ mg/kg}$. As anticipated, vancomycin failed to significantly impact $E. coli$ burden at a dose equivalent to the highest dose of $V-r$. In a 24-h thigh muscle infection model, $E. coli$ UTI89 was inoculated at $7.8 \times 10^{4} \text{ CFU}$ into one thigh muscle per mouse ($n = 5$ to 8 per group) and treated with $V-r$ (total dose, $200$ to $1,400 \text{ mg}$) using an every-6-h dosing regimen from $1 \text{ h}$ postinfection. All doses of $\geq 200 \text{ mg/kg}$ significantly reduced burden below stasis by up to $2.7 \log_{10} \text{ CFU/g}$. These bactericidal effects of $V-r$ were statistically superior to those of ciprofloxacin, which induced a $1.4 \log_{10}$ reduction from stasis (Fig. 3 and Table 5). Overall, $V-r$ caused an $\sim 4$ to $7.5 \log_{10}$ reduction in bacterial burden, compared with vehicle control, over the entire dose range.

The MIC data confirm previous findings that the coupling of arginine with vancomycin bestows significant antimicrobial activity of the $V-r$ conjugate against $E. coli$ infection while remaining effective against methicillin-resistant $Staphylococcus aureus$ (MRSA) (14). Such $in vitro$ findings were effectively translated into thigh muscle infection models, where a total 24-h dose of $250 \text{ mg/kg}$ $V-r$ reduced $E. coli$ burden to pretreatment (stasis) levels. Since area under the curve over $24 \text{ h}$ in the steady state divided by the MIC (AUC/MIC ratio) is the primary PK/pharmacodynamic predictor of vancomycin (5), this static dose corresponds to a total AUC/MIC of $47.3$. Based on a free ($f$) fraction of $35\%$, as determined in plasma protein binding studies (Table 1), the $f$AUC/MIC of $V-r$ was $16.5$. As an approximation of exposure using allometric scaling (22), this would be equivalent to a human dose of $\sim 20 \text{ mg/kg}$, with a dose of $28 \text{ mg/kg}$

### TABLE 3 PK parameters of $V-r$ in CD-1 mice after s.c. administration

| PK parameter$^a$ | $V-r$ at 20 mg/kg | $V-r$ at 121 mg/kg |
|------------------|------------------|------------------|
| Half-life (h)    | 0.87             | 1.29             |
| $C_{max}$ (mg/liter) | 20.4           | 98.4             |
| Clearance (ml/min/kg) | 7.8            | 5.4              |
| AUC (mg · h/liter)       | 42.7            | 366              |
| $V_d$ (liter/kg)       | 0.59             | 0.60             |

$^aC_{max}$, maximum concentration of drug in plasma; AUC, area under the curve; $V_d$, volume of distribution.
required to elicit an additional $10^{-1}$ kill. Such allometric doses of V-r are in line with the daily and loading doses of vancomycin in humans (5).

The positive efficacy data support the notion that the cationic feature of arginine within V-r allows for breaching of the stubborn outer membrane of \textit{E. coli} isolates and possibly other Gram-negative bacteria (14). The sequelae of events leading to V-r-mediated \textit{E. coli} eradication likely involve (i) improved cell surface association with negatively charged groups, (ii) effective translocation across the outer membrane leading to enhanced drug uptake, and (iii) disruption of peptidoglycan synthesis within the periplasmic space (6, 14). To our knowledge, the current findings describe the first report of a marked abrogation of \textit{E. coli} burden \textit{in vivo} with a minimally modified vancomycin-cationic transporter conjugate. Previously, it was reported that vancomycin-QC14, a strongly lipophilic/cationic molecule, reduced thigh muscle infection of a carbapenem-resistant \textit{A. baumannii} strain (23). Because V-r was highly effective in time-kill assays against \textit{E. coli} NCTC 13441, a pandemic uropathogenic clone (24), a logical next step would be to evaluate the conjugate in a model of urinary tract infection (UTI). Based on the high renal elimination of vancomycin in humans (25) in a nonmetabolized form (26), it is reasonable to hypothesize that V-r may drive a highly targeted therapeutic intervention to combat \textit{E. coli}-associated UTIs.

These data further underscore a precedent for creating a novel Gram-negative active agent by transforming a commonly used and selective Gram-positive antibiotic by introducing certain cationic features through a simple and scalable synthesis protocol (14). Such an approach, in consort with effective \textit{in silico} predictions (27, 28), might expedite antibiotic development and increase the overall probability of success of

| Group, total dose over 9 h (mg/kg) | Log$_{10}$ (group geometric mean ± SD CFU/g) | Log$_{10}$ change from vehicle (CFU/g) | $P$ value (versus vehicle) |
|-----------------------------------|---------------------------------------------|--------------------------------------|---------------------------|
| Pretreatment                      | 5.1 ± 0.18                                  | −2.01                                | 0.0045                    |
| Vehicle                           | 7.11 ± 0.12                                 | 0                                    | 0                         |
| V-r, 110                          | 5.87 ± 0.60                                 | −1.24                                | 0.0415                    |
| V-r, 440                          | 4.14 ± 0.63                                 | −2.97                                | <0.0001                   |
| V-r, 880                          | 3.76 ± 0.40                                 | −3.35                                | <0.0001                   |
| Vancomycin, 800                   | 6.60 ± 0.66                                 | −0.51                                | Not significant           |

**TABLE 4** Efficacy of V-r in an \textit{E. coli} ATCC 25922 thigh muscle infection model (9 h) in neutropenic CD-1 mice.

**FIG 3** Efficacy of V-r in reducing \textit{E. coli} UT89 burden in a 24-h thigh muscle infection model in neutropenic CD-1 mice.
TABLE 5 Efficacy of V-r in reducing *E. coli* UTI89 burden in 24-h thigh muscle infection model in neutropenic CD-1 mice

| Group, total dose over 24 h (mg/kg) | Log10 (group geometric mean ± SD CFU/g) | Log10 change from vehicle (CFU/g) | P value (versus vehicle) |
|------------------------------------|----------------------------------------|----------------------------------|-------------------------|
| Pretreatment                        | 4.76 ± 0.18                            | −4.95                            | 0.0248                  |
| Vehicle                            | 9.71 ± 0.17                            | 0                                | 0                       |
| V-r, 200                            | 5.60 ± 2.28                            | −4.11                            | 0.0217                  |
| V-r, 400                            | 3.27 ± 1.88                            | −6.43                            | <0.0001                 |
| V-r, 700                            | 2.58 ± 0.25                            | −7.13                            | <0.0001                 |
| V-r, 1,050                          | 2.08 ± 0.89                            | −7.63                            | <0.0001                 |
| V-r, 1,400                          | 2.68 ± 1.38                            | −7.03                            | <0.0001                 |
| Vancomycin, 1,272                   | 8.48 ± 1.31                            | −1.23                            | Not significant         |
| Ciprofloxacin, 20                   | 3.32 ± 0.14                            | −6.39                            | <0.0007                 |

drug candidates. Most important, this would help to arrest the insidious pandemic of difficult-to-treat bacterial infections.

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