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Antibody-Catalyzed Cleavage of the D-Ala-D-Lac Depsipeptide: An Immunological Approach to the Problem of Vancomycin Resistance

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Abstract—Vancomycin resistance is currently a major healthcare problem. The development of a catalytic monoclonal antibody (mAb) that hydrolyzes the D-Ala-D-Lac depsipeptide provides a potentially novel antibiotic strategy. A phosphonate hapten design was used to program antibody catalysis. The characteristics of the hapten were shown to be important for obtaining a viable immune response and several catalytic mAbs that cleave a peptidoglycan model substrate. The best mAb afforded a >500-fold rate enhancement over background. © 2002 Published by Elsevier Science Ltd.

Vancomycin was widely instituted in the 1960s and has arguably been the world's most essential antibiotic over the past three decades. During this time, the vancomycin group of glycopeptides were propelled to the forefront in combating Gram-positive streptococcal, staphylococcal, and enterococcal infections that acquired resistance to the penicillins and cephalosporins, amongst others.[1,2] However, since 1989, healthcare environments have witnessed an escalation in vancomycin-resistant enterococci (VRE) that has resulted in high mortality rates, particularly in immunocompromised individuals.[3,4] The real and serious threat posed by the prevalent and opportunistic VRE arises not only from their own broad antibiotic resistance, but also from the potential for transmission of resistance to more virulent organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA).[5–7] Although yet to be observed clinically, transfer of vancomycin resistance to MRSA and other pathogenic bacteria could have devastating consequences.

For this reason, the explosive emergence of VRE has led to the intensive investigation of the molecular mechanisms of glycopeptide resistance.[8–10] Vancomycin binds noncovalently to the L-Lys-D-Ala-D-Ala portion of pentapeptide cell-wall peptidoglycan (PG) substructures, which inhibits PG cross-linking and thus leads to cell-wall defects and bacterial lysis (Scheme 1). The predominant strategy used by VRE is replacement of pentapeptide D-Ala-D-Ala termini with the D-Ala-D-Lac depsipeptide, resulting in the loss of one hydrogen bond with vancomycin, a 1000-fold decrease in affinity and essentially useless antibiotic activity. Semisynthetic glycopeptides[1,11] and glycopeptide analogues[12,13] that address the resistance problem are in various phases of development and, clearly, other alternative approaches are warranted. Recently, screening of a small-molecule library led to the design of prolinol derivatives that cleaved D-Ala-D-Lac substrates and resensitized bacteria to vancomycin.[14] Herein, we describe the foundation for immunotherapy as a new method in the treatment of VRE based on catalytic antibodies that hydrolyze the D-Ala-D-Lac ester bond in PG precursors.

The phosphonate transition-state (TS) analogue strategy has been paramount for obtaining antibody esterases.[15,16] To this end, hapten **1** (**VCA**) was prepared in homochiral form as a peptidomimetic for the L-Lys-D-Ala-D-Lac fragment starting from known **10** and using literature modifications to obtain **11**–**13** (Fig. 1, Scheme 2).[17–19] Notably, the linker design incorporated a preactivated carboxylic group that afforded regiospecific immunoconjugation. The L-Lys residue was included to improve the elicitation of an immune response, since

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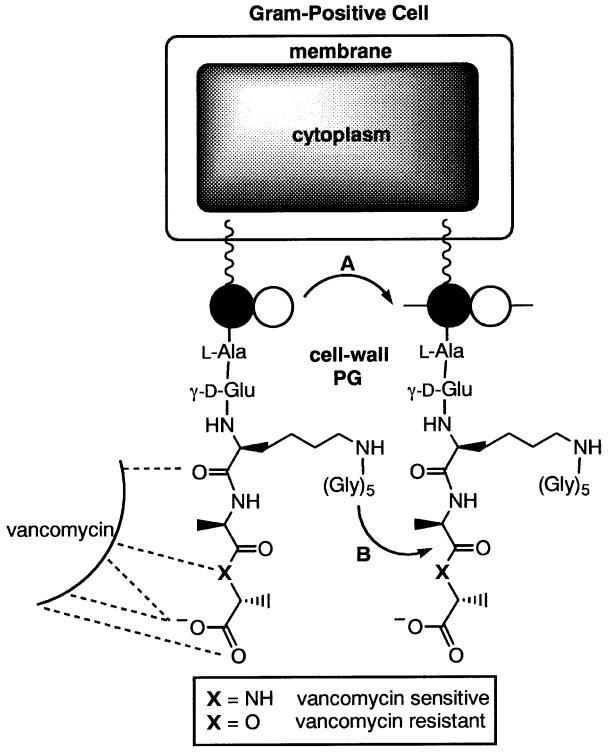
previous studies in our laboratory suggested a smaller hapten would not be sufficiently immunogenic. Immunization of mice with **1** coupled to keyhole limpet hemocyanin (KLH) as a carrier protein yielded a panel of six monoclonal antibodies (mAbs). Indeed, the unusually low number of stable clones obtained was apparently due to a poorly immunogenic hapten which afforded low titer values. Yet, when screened for catalytic activity using **3**, four of the six mAbs afforded a moderate rate enhancement over background. The substrate **3** is structurally congruent to the L-Lys-D-Ala-D-Lac target, but fitted at the distal end of the requisite linker with a chromophoric auxiliary that allows monitoring of cleavage product **6** by HPLC (Fig. 1). The mAb VCA33H3 demonstrated the best esterase activity and was analyzed in further detail.

Well-behaved Michaelis–Menten kinetics were observed, in which it was possible to approach a practical V_{max} at nearly physical saturation with no evidence of product inhibition. Upon mAb saturation with **3**, D-Ala-D-Lac cleavage was >500-fold that of the spontaneous rate (Table 1). The phosphonate **2** competitively inhibited the reaction, which demonstrated that the saturable mAb active site was the same as that elicited by hapten **1**. Surprisingly, the K_i value was much higher than the tight binding ($<10^{-6}$ M) generally observed in



Scheme 1. Illustration of PG biochemistry in Gram-positive bacteria and the vancomycin resistance problem. Glycopeptide precursors are synthesized in the cytoplasm, anchored via a hydrophobic tail, and transported outside the cell membrane for assembly (● = *N*-acetylmuramate, ○ = *N*-acetylglucosamine). In pathway **A**, disaccharide subunits are appended to a growing chain by a transglycosylase. In pathway **B**, a transpeptidase cross-links the N-terminus of a pentaglycine unit, attached to L-Lys in a prior step, with another peptide moiety. Cross-linking between *different* poly(glycopeptides), in which either D-Ala or D-Lac can serve as a leaving group, forms a stable PG matrix. The precise architecture has been hypothesized.[28] Vancomycin sterically blocks transglycosylation and transpeptidation. Loss of one hydrogen bond (**X** = ○) results in drug resistance. A catalytic antibody designed to cleave D-Ala-D-Lac could restore sensitivity to vancomycin.

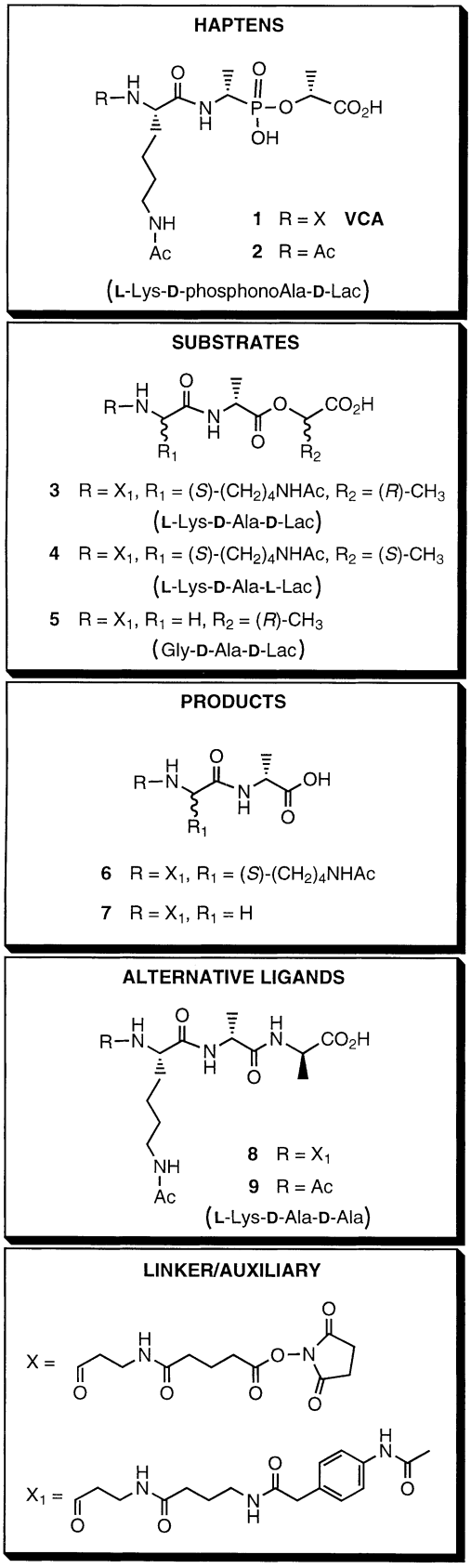
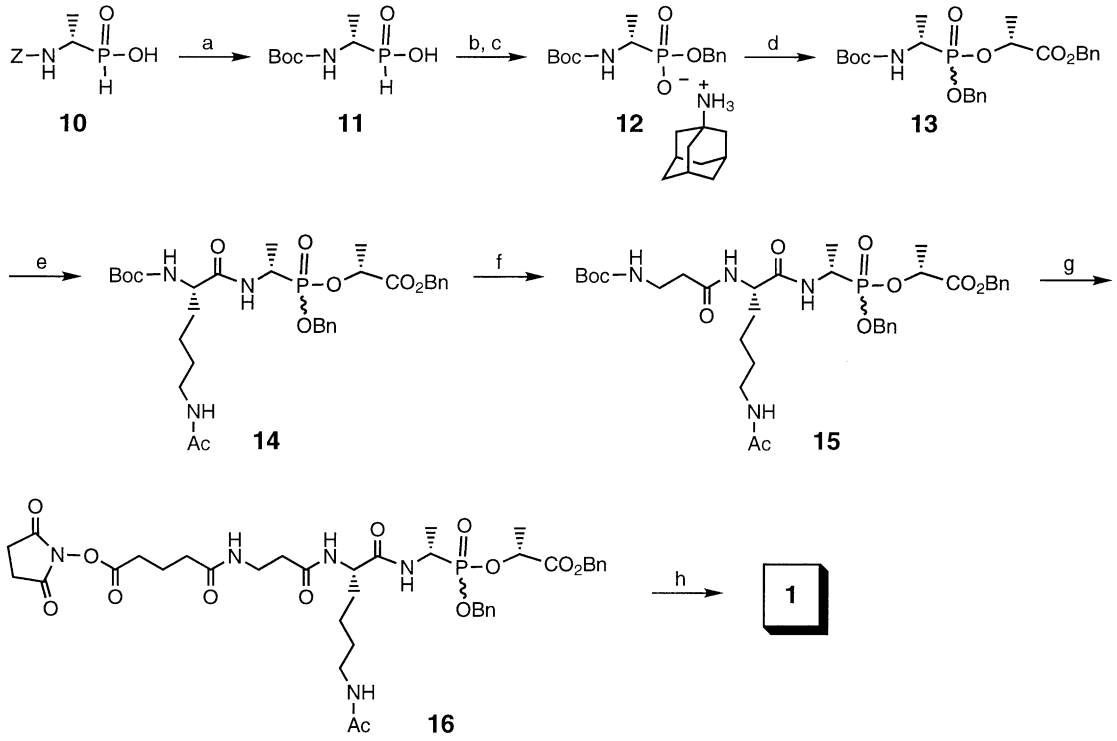


Figure 1. Structures of compounds under discussion.



Scheme 2. Synthesis of the transition-state analogue hapten: (a) (i) 30% HBr; (ii) di-*t*-butyldicarbonate; (b) DCC, benzyl alcohol; (c) (i) $NaIO_4$; (ii) 1-adamantanamine; (d) (i) aq HCl; (ii) benzyl D-lactate, BOP, DIPEA; (e) (i) TFA, CH_2Cl_2; (ii) Boc-Lys(Ac)-OH, HBTU, NMM; (f) (i) TFA, CH_2Cl_2; (ii) Boc-β-Ala-OH, HBTU, NMM; (g) (i) TFA, CH_2Cl_2; (ii) *N*-hydroxysuccinimidyl hemiglutaryl chloride, DIPEA, CH_2Cl_2; (h) H_2, Pd/C, MeOH.

Table 1. Kinetic data for VCA33H3 that cleaves the D-Ala-D-Lac bond in model PG substrate **3**[a]

k_{cat} (h^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} min^{-1})	k_{cat}/k_{uncat}	K_i (μM)[b]
0.120	2.17	0.921	530	160

[a]Determined in 100 mM MOPS, pH 7.4, 5% DMSO cosolvent at 23 °C. The k_{obs} (**3**) = 2.25×10^{-4} h^{-1}.
[b]The value using compound **2**.

catalytic antibody reactions. On the contrary, the binding of VCA33H3 to the conjugate **VCA**–KLH titered in the nanomolar range similar to other haptens. It is likely that antibody avidity and linker/protein recognition effects[20,21] are operative, which further underscores the borderline immunogenic properties of **VCA**. Even though weak immunogenicity of the TS analogue probably leads to reduced TS stabilization for hydrolysis, additional physicochemical interactions invoked by the hapten could fortuitously contribute to catalysis (e.g., hydrogen bonding, electrostatics, 'bait–switch' effects).[20] In fact, the $K_m/K_i \approx k_{cat}/k_{uncat}$ relationship, according to a standard thermodynamic cycle based on TS theory,[22–24] would suggest a rate enhancement for VCA33H3 of only ~12.5, or ~42-fold less than that observed. Hence, it is possible that some aspect of general/acid–base or nucleophilic chemistry, anchored by the core structure that directs TS stabilization, helps promote catalysis.

Evidence that the locale of the phosphonate group is central to hapten recognition and to the energetics of D-Ala-D-Lac hydrolysis came from the study of diastereomer **4**. This compound showed no detectable activity with VCA33H3, indicating that the configuration of the methyl group is critical to binding and/or catalysis. The lactate methyl group, as programmed by **1**, can help 'lock' the substrate **3** and the developing TS in the correct orientation, affording perhaps 3.2 kcal/mol in binding energy,[25] some of which translates into catalysis. Given the numerous conformational minima that are available to flexible structures such as **1** and **3**, together with the absence of a dominant epitope (e.g., aromatic ring), makes the additivity of weak interactions particularly important. Additional support for this premise was furnished by peptide **5**, devoid of the lysine side chain, where the mAb did not catalyze formation of **7**. Hence, it is clear that all stereoelectronic components programmed by the hapten are essential for substrate activity.

The D-Ala-D-Lac phenotype in VRE is generally induced, although constitutive expression may arise.[3,8–10] Since a given fraction of PG in VRE still relies on D-Ala-D-Ala precursors in the presence or absence of vancomycin, we examined interaction of VCA33H3 with **8** and **9**. Hydrolysis or merely complexation of D-Ala-D-Ala could also serve as immunotherapy. No hydrolytic activity using **8** was observed, apparently from a lack of catalytic power and not ground-state binding, as judged from **9**, which acted as a competitive inhibitor (K_i = 13 mM). In principle, mAb binding could mimic the action of vancomycin and supplement catalytic action. Although the K_i value for **9** is high, transpeptidases show very weak binding ($K_m \approx$ 40–100 mM) to model substrates[26] and so competition in

vivo is possible. New hapten designs should address this issue, since some bacterial species, notably MRSA, can evolve vancomycin resistance simply by increasing the production of D-Ala-D-Ala termini.[5,6]

Bacteria have always demonstrated the ability to readily acquire resistance to small-molecule drugs. Hence, there is a need for alternative approaches to supplement standard antibiotic treatments. Peptide and protein-based structures that operate via catalysis present one such option. In this regard, proteins up to 50 kDa (e.g., scFv, 25 kDa; Fab, 50 kDa) are good candidates to penetrate the cell-wall fabric during normal growth conditions.[27] Studies are underway in our laboratory to determine the bactericidal potential for VCA33H3 and related mAbs, as well as associated strategies for using immunological reagents as antibiotics.

Acknowledgements

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