Drug discovery initiatives often depend critically on knowledge of ligand–receptor interactions. However, the identity or structure of the target receptor may not be known in every instance. The concept of receptor surrogate, a molecular environment mimic of natural receptor, may prove beneficial under such circumstances. Here, we demonstrate the potential of monoclonal antibodies (mAbs) to act as surrogate receptors for a class of innate immune peptide antibiotics, a strategy that can help comprehend their action mechanism and identify chemical entities crucial for activity. A panel of antibody surrogates was raised against indolicidin, a tryptophan-rich cationic broad spectrum antimicrobial peptide of innate immune origin. Employing an elegant combination of thermodynamics, crystallography, and molecular modeling, interactions of the peptide with a high affinity anti-indolicidin monoclonal antibody were analyzed and were used to identify a motif that contained almost the entire antibiotic activity of native indolicidin. The analysis clarified the interaction of the peptide with previously proposed targets such as bacterial cell membrane and DNA and could further be correlated with antimicrobial compounds whose actions involve varied other mechanisms. These features suggest a multipronged assault pathway for indolicidin. Remarkably, the anti-indolicidin mAb surrogate was able to isolate additional independent bactericidal sequences from a random peptide library, providing compelling evidence as to the physiological relevance of surrogate receptor concept and suggesting applications in receptor-based pharmacophore research.

Launch of any new antibiotic also brings with it the certainty of microbial resistance leaving the field of antimicrobial therapy in a constant need of innovation. But most candidate molecules currently under trial are still based on the same skeleton and consequently target the same components of the pathogens (1). However, templates distinct from conventional drugs both in terms of structure as well as biological targets can be found right within the innate immune system in the form of antimicrobial peptides. They display broad spectrum activity, rapid killing kinetics, and a lower incidence of generating resistance (2). But their use in a clinical setting usually requires design modifications to improve potency, physiological stability, and curb their toxicity. Such rational drug design works best when guided by ligand–“receptor” structure but for many innate immune peptides, the nature of their interaction with potential targets and their precise mechanism of action itself is still under debate.

Absence of a unique receptor presents a stumbling block to the creation of therapeutics based on such peptides. Recourse to conventional “ligand-based” design, which involves multiple cycles of analog synthesis incorporating blind mutations/chemical modifications, has not been very successful (3). Alternatives exist where receptor attributes can be simulated in silico by mapping of the molecular features of known pharmacophores, but their accurate modeling remains complicated, especially while accounting for flexibility in ligand and/or receptor. A possible solution may lie in allowing nature to design a ligand complimentary binding site that may substitute for the natural receptor(s). In fact, such receptors are readily observed in the course of an adaptive immune response as monoclonal antibodies. With a dynamic binding site located on a stable core, they are easily able to provide a snug fit for their ligands, thus fulfilling perfectly the requirements of a receptor “surrogate,” a role that has indeed been proposed earlier (4, 5). Moreover, the well established hybridoma technology or phage-displayed libraries make available a range of variable binding sites from which an appropriate receptor may be chosen. In this context, antibodies as surrogate receptors were explored for an antibiotic peptide indolicidin (ILPWKWPWWPWRR).

Indolicidin is a tryptophan-rich cationic broad spectrum antibiotic (6–8) that can also neutralize LPS (6) and has immunomodulatory effects (9). Its antibacterial activity is realized by which indolicidin-like antibiotic peptides are specifically targeted inside the phagolysosomes of host neutrophils, outside of which it exhibits cytotoxicity (10, 11). The primary mechanism by which indolicidin-like antibiotic peptides are specifically able to target microbes is by utilizing the basic differences between membrane composition of prokaryotic and eukaryotic cells (2). Not surprisingly therefore, initial studies implicated bacterial cell membrane as the primary site of action of indolicidin (6, 12).

An increasing body of evidence however, suggests alternative action mechanisms that involve molecules like DNA (13), topoisomerase-I (14), calmodulin (15), and possibly other uncharacterized proteins. The key to the bactericidal effects of indolicidin appears to lie both in its sequence and its structure. Although it remains unstructured in aqueous solutions, it very
mAb Captures Antibiotic Determinants of Indolicidin

likely adopts a unique conformation in presence of its molecular targets. It was felt that the three-dimensional structure of the peptide in complex with an adequately chosen receptor could help comprehend its mechanism of action better and provide a framework to correlate a wealth of structure-activity data, serving as a rational guide for antibiotic design.

With the microbial target(s) of indolicidin not being clearly resolved, we generated a panel of monoclonal antibodies as surrogate receptors for the peptide. The interactions of indolicidin with a high affinity surrogate were analyzed through a multidisciplinary approach involving thermodynamic, crystallographic, and molecular modeling methods. Features of the peptide critical for its antibacterial action were identified through an excellent correlation between computational and biochemical results. It also enabled visualization of a core antibiotic motif that suggests simultaneous targeting of multiple cellular components by indolicidin and presents a template for novel drug design. Finally, ability of the anti-indolicidin mAb to fish out novel bactericidal sequences from a random peptide library not only provides an unequivocal proof of the concept of monoclonal antibodies as surrogate receptors but also opens up avenues for their application in diverse areas of biological research.

EXPERIMENTAL PROCEDURES

Generation and Characterization of Monoclonal Antibodies—Peptides were synthesized by F-moc chemistry on automated synthesizers (Applied Biosystems). Indolicidin conjugated to diphtheria toxoid (Wako Chemical Co.) was used for immunizing 8–10-week-old BALB/c mice. Hybridomas were prepared following standard protocol (16) and screened for anti-indolicidin antibodies by ELISA. Clonal independence was confirmed by cDNA sequencing as mentioned previously (17). mAb V2D2 was purified from ascitic fluid through 40% ammonium sulfate fractionation followed by ion exchange HPLC using a DEAE column (Tosoh, Inc.). Column-bound antibody was eluted with a gradient of 0.3 M NaCl.

Affinity measurements were based on surface plasmon resonance technique, carried out on BIAcore2000 (GE Healthcare). Indolicidin was amine coupled to a CM4 chip (~120 resonance units) and mAb V2D2 was injected as analyte with a 5-min association/10-min dissociation phase. Regeneration was achieved by 0.1 N HCl. Interaction curves were simultaneously fit using bivalent model in BIAevaluation program to determine $K_D = k_a/k_d$ and $\Delta G_{\text{eq}} = -RT \ln K_D/c_w \cdot R$ is the Rydberg's constant ($1.09677 \times 10^3$ 1/m$^2$), $T$ is the temperature in kelvins, and $c_w$ is the standard state concentration (1 mol l$^{-1}$). $\Delta H_{\text{eq}}$ was derived by directly fitting the experimental data to the integrated form of van't Hoff equation $K_D = e^\left(\frac{-\Delta H_{\text{eq}}}{RT}\right)$, $\Delta C/R$ where $\Delta C$ refers to heat capacity change at constant pressure, and the degree symbol refers to parameter values at a reference temperature, taken to be 25 °C (18). Entropy change was then calculated as $\Delta S_{\text{eq}} = \Delta H_{\text{eq}} - T \Delta C_{\text{eq}}$.

Panning a Phage-displayed Peptide Library and Assaying for Antibacterial Activity—A 12-mer peptide library (New England Biolabs) was panned with mAb V2D2 following the manufacturer's recommendations. Briefly, 0.1 mg of mAb V2D2 was coated in a 24-well plate, blocked with 0.5% BSA, and incubated with a phage library containing $\sim 10^{11}$ virions. After

washes with 0.1–0.5% Tris buffered saline-Tween, bound phages were eluted using 0.2 mol glycine-HCl, pH 2.2, amplified and precipitated by 20% PEG 8000/2.5 mol NaCl. They were titrated and used as input for the next round. After four rounds of panning, individual viral plaques were randomly selected and sequenced for the 12-mer peptide.

Radial diffusion assay using double-layered agarose (19) involved incubating $\sim 2 \times 10^7$ mid-log phase bacterial cells with 10 mM peptide solution in nutrient-poor agarose at 37 °C for 3 h. Overlaying with 3% tryptic soy broth agarose revealed circular bacteria-free zones. Minimum inhibitory concentration was determined by broth microdilution (20) in which $\sim 10^4$ bacterial cells in phosphate buffer were incubated with serially diluted peptide solution in a 96-well microtiter plate. Addition of LB medium subsequently was used to ascertain the minimum peptide concentration sufficient to prevent bacterial growth, observable as $A_{600}$.

Sheep erythrocytes were used to ascertain mean hemolytic concentration (MHC) (20). Briefly, a 0.02 dilution of 200-µl packed erythrocyte volume in PBS was incubated with serially diluted peptides in round-bottom 96-well microtiter plates at 37 °C for 90 min. Release of hemoglobin in PBS was measured through its absorbance at 405 nm and expressed as percentage of complete erythrocyte lysis achieved by 1% Triton X-100. Therapeutic index was taken as MHC:minimum inhibitory concentration.

Epitope Mapping—Indolicidin residues were substituted with Gly one at a time to generate a series of mutants bound to polyethylene pins, distanced by a molecular spacer (Pin Technology; Cambridge Research Biochemicals). The manufacturer’s recommended protocol involving F-moc chemistry was used for peptide synthesis. For evaluation of antibody binding, pin-bound peptide analogs were blocked by 2% gelatin, incubated with an appropriate concentration of antibody overnight at 4 °C, and assayed following standard ELISA protocol. Incubations were performed by inserting the peptide-bound pins in a 96-well plate containing the appropriate solutions. Absorbance values were plotted as percentage loss or gain of antibody binding of the mutants relative to the native peptide.

Fab Generation and Crystallization—Fab V2D2 was generated by a controlled papain digestion and purified by ion exchange chromatography on a DEAE column (Tosoh, Inc.) Unliganded Fab V2D2 crystallized with 10% PEG 1000 in 20 mM Tris, pH 7.1. X-ray radiation source was a copper-rotating anode generator (Rigaku Corp.) operating at 50 kV and 100 mA. Fab V2D2 crystals were flash-frozen to 120 K soaked in 33% glycerol; diffraction data were recorded on a 345-mm image plate detector and was processed using Automar suite of programs (Marresearch). Structure determination by molecular replacement was performed by Phaser (21), and the solution was refined using the Crystallography and NMR System suite of programs (22). Volume of complementarity determining region (CDR) cavities was estimated by CASTp (23).

In Silico Simulation and Analysis of Peptide-Antibody Interactions—Structure of indolicidin within the antibody binding site was predicted with AutoDock4 (24) using default parameters. Starting conformations for indolicidin were
mAb Captures Antibiotic Determinants of Indolicidin

A 10 Å water box of explicit TIP3P molecules and Cl\textsuperscript{−} counterions beyond the boundary of the Fab-peptide complex was created. The ensemble was minimized till convergence with initial 200 cycles of the steepest descent, and the rest was minimized using the conjugate gradient method. After temperature equilibration to 300 K at constant volume conditions for 0.2 ns and another 0.2 ns of constant pressure conditions, 1 ns MD was performed with a time step of 2 fs and nonbonded cut-off of 10 Å. A unique post-MD structure was arrived at by averaging and minimizing the trajectory conformations at every 10-ps interval.

RESULTS

Generation and Characterization of Antipeptide mAbs—Splenocytes from mice immunized with indolicidin-diphtheria toxoid conjugate were used to raise a panel of anti-indolicidin hybridomas (Fig. 1A). Among the cell lines secreting high antibody titers, V2D2 and 3B4 were established to be clonally independent through RT-PCR-based sequencing of their variable regions (supplemental Table S1). High concentrations of the two monoclonal antibodies were generated by inducing ascites in mice, and purified mAbs were obtained through a combined ammonium sulfate fractionation, anion-exchange chromatography procedure. For subsequent investigations, however, only the antibody purified from the former clone (mAb V2D2) was chosen because it bound indolicidin with nanomolar affinity ($K_D = 56$ nM; Fig. 1B) and could block the antibacterial action of the peptide.

With a high affinity indolicidin receptor in hand, the nature of its interactions were probed further using a surface plasmon resonance-based thermodynamic assay. The robust binding of mAb V2D2 to indolicidin was reflected in the equilibrium free energy ($\Delta G_{eq}$) that remained centered $\sim -10$ kcal/mol at temperatures ranging from 15 to 35°C. Interestingly though, enthalpy ($\Delta H_{eq}$) and entropy ($\Delta S_{eq}$) components of $\Delta G_{eq}$ turned out to have mutually inverse temperature dependence (Fig. 1C), a phenomenon that also has been observed previously (18). An initially favorable entropy nevertheless suggested a primarily hydrophobic mode of interaction involving burial of nonpolar groups. Indeed, mAb V2D2 had strong sequence homology with antisteroid antibodies (PDB codes 1JGL and 1JN6) that enclose their ligands in a pocket of hydrophobic residues.

obtained from Protein Data Bank (PDB$^3$) codes 1G89 and 1G8C. For each docking instance, the five best conformations were analyzed using the program PISA (25) in terms of binding free energy ($\Delta G_{total}$), hydrophobic and hydrophilic components of $\Delta G$, interface area, number of H-bonds, etc. Residue-wise contribution to $\Delta G_{total}$ was calculated using individual residue coordinates as ligand input in PISA. $\Delta G_{total}$ was divided by the total number of peptide residues to determine $\Delta G_{average}$, that was then expressed as percentage of $\Delta G_{total}$/$\Delta G_{average}$ provided a baseline, which separated residues that contributed significantly to $\Delta G_{total}$ from those which played a less essential part. To reveal residues contributing significantly to $\Delta G_{total}$ the following formula was used, $100 \times (\Delta G_{residue} - \Delta G_{average})/ (\Delta G_{total} - \Delta G_{average})$.

Molecular modeling and visualization was performed using PyMOL (26). Molecular dynamics (MD) of the modeled structure was simulated with AMBER9 employing the 2003 force field (27).

$^3$The abbreviation used is: PDB, Protein Data Bank.

FIGURE 1. Generation and characterization of anti-indolicidin antibodies. A, ELISA profile of a panel of anti-indolicidin hybridomas. Clones marked in black were selected for further investigation. B, surface plasmon resonance (SPR) sensogram for the binding of mAb V2D2 (analyte; concentration ranging from 20 to 320 nM) to indolicidin (ligand) immobilized on a CM4 chip. Association ($K_a$), dissociation ($K_d$) rate constants were used to calculate equilibrium constant ($K_D$). C, individual contributions of enthalpy ($\Delta H$) and entropy ($\Delta S$) to equilibrium free energy of binding ($\Delta G$), at temperatures ranging from 15 to 35°C. D, Nonlinear curve fitting to van’t Hoff plot shows a curved slope indicating a change in the specific heat of the system and a negative dependence of enthalpy ($\Delta H$) on temperature in the same range.
The analysis also indicated temperature-dependent changes in heat capacity ($\Delta C_p$) of the system (Fig. 1D) that usually arise from conformational variations of interacting partners (28). As the peptide does not adopt any regular structure in aqueous solutions (29), it must be the antibody that changes conformation such that it binds to indolicidin more readily and accounts for the favorable enthalpy at higher temperatures. This favorable enthalpy could still be originating from hydrophobic interactions as has been demonstrated recently (30). As the temperature increases further however, the increase in enthalpy is partially negated by the increasingly unfavorable entropy term, possibly a reflection of the energy cost of constraining peptide/antibody in a conformation required for complex formation that would have otherwise become more flexible with a rise in temperature.

Overall, the experiment highlighted hydrophobic forces to be responsible for peptide-antibody interaction and a concomitant conformational change in the antibody, probably in its CDRs. To observe the binding event first hand, structural investigations were initiated next.

The Structure of Indolicidin-Fab V2D2 Complex—Crystallization trials set up for indolicidin and the Fab fragment of V2D2 antibody yielded well formed birefringent crystals that diffracted up to a resolution of 2.0 Å (diffraction statistics in supplemental Table S2). Structure solution by molecular replacement, obtained using anti-HCV Fab 19D9D6 (PDB code 1YMH), was refined until $R_{free} = 23.5\%$ (Fig. 2A); however, no density for the peptide could be seen, and despite numerous attempts, only unliganded Fab V2D2 could be crystallized. Nonetheless, solid surface rendering revealed a CDR-enclosed cavity averaging 8 Å in depth and 112 Å$^3$ in volume that appeared to be a potential binding site for indolicidin (Fig. 2B).

Subsequently, a set of probable structures adopted by Fab-bound indolicidin were elucidated using the program AutoDock4. Within the constraints on torsional degrees of freedom, the peptide was kept as flexible as possible, whereas the Fab receptor had to be held rigid. But thermodynamics data had indicated conformational changes in the antibody upon complex formation. This also was supported by high B-factors of the CDRs, especially H3 in the crystal structure of Fab V2D2 (Fig. 2C). A search in PDB for alternate CDR conformations led to Fab MN20B9.34, which had 70% homology with mAb V2D2 CDRs and was co-crystallized with an 11-mer peptide (31). Structural changes in its H3 loop that occurred while binding to the peptide were used to model a corresponding conformation of Fab V2D2 (Fig. 2C). Both the crystal and the modeled conformations, the latter now having a widened CDR cavity, were used as rigid receptors for in silico docking. Furthermore, stability of the docked complexes was ensured by a 1-ns molecular dynamics simulation.

In both cases, the program, without having been biased in any previous way, correctly identified the CDRs as the most likely regions to bind the peptide and further narrowed down the docking site to the cavity formed by the CDR loops. Indolicidin was observed to interact more favorably with the modeled form of Fab V2D2 with an interface area of 661 Å$^2$. In this conformation, the peptide buried itself into the hydrophobic cavity of the antibody by adopting a “$\omega$”-shaped structure with three half-turns each at Pro-3/Trp-4, Pro-7, and Pro-10 (Fig. 3A). It appeared to be tethered in part by Arg-12 that formed a hydrogen bond with Glu-95 (CDR H3) at the floor of the cavity. Significant contributions toward complex formation also came from the placement of all five Trp residues. In particular, a pronounced right-side bulge in the $\omega$-shape inserted both Trp-8 and Trp-9 in to the grooves of the Fab cavity that was mostly composed of aromatic residues like tyrosine, tryptophan, and phenylalanine (Fig. 3, A and B). The bulge in the peptide backbone was evidently caused by Pro-7 and Pro-10 that flanked the two tryptophans and while not interacting directly with Fab V2D2, seemed to facilitate the interaction of the Trp residues. Ile1, Leu2, Pro3, and Arg13, located on either stem of the $\omega$ were found to hang outside the cavity unengaged to any antibody residue (see supplemental Table S3 for a list of peptide-mAb interactions).

In Silico and in Vitro Results Reveal Identical Key Interactions of Indolicidin-Fab V2D2—Analysis of the docking simulations detailed above in terms of free energy changes ($\Delta G_{Total}$) yielded a value of $\sim$10.9 kcal/mol for indolicidin-Fab V2D2 complex formation. This correlated quite well with the thermodynamic analysis presented above lending experimental support to the docked models (Table 1). Additional corroboritation with the thermodynamic data came from the significantly greater $\Delta G_{Hydrophobic}$ contributions ($\sim$90% of $\Delta G_{Total}$) over $\Delta G_{H-bonds}$ that re-emphasized the predominantly nonpolar nature of the mAb-peptide complex. Role of individual residues of indolicidin that interacted with mAb V2D2 was delineated further by analyzing the contribution of each residue toward $\Delta G_{Total}$ (Table 1). A significant role in cementing the complex was played by the Trp residues. Trp-8 in particular accounted for a quarter of $\Delta G_{Total}$ as can be expected from its solvent-shielded conformation. It was closely followed by Trp-4 and Trp-9, and the energy contributions of these three residues together well reflected the structural complementarity between indolicidin and Fab V2D2 (Fig. 3B). Both Trp-4 and Trp-8 were observed to
TABLE 1
Residue-wise analysis of interactions between the complex of Fab V2D2 and the least energy conformation of indolicidin, obtained after a 1-ns MD simulation

| Residue | ΔG_{Total} (kcal/mol) | %ΔG_{Total} – average |
|---------|------------------------|------------------------|
| Ile-1   | -10.1                  | -8.74                  |
| Leu-2   | -10.9                  | -8.62                  |
| Pro-3   | -9.5                   | -8.51                  |
| Trp-4   | -1.4                   | -1.34                  |
| Lys-5   | -1.2                   | -1.17                  |
| Trp-6   | 0.00                   | 0.00                   |
| Pro-7   | -2.49                  | -2.35                  |
| Trp-8   | 1.90                   | 1.76                   |
| Pro-10  | 0.00                   | 0.00                   |
| Trp-11  | -0.68                  | -0.59                  |
| Arg-12  | 0.90                   | -16.48                 |
| Arg-13  | 0.00                   | -8.33                  |

a Includes both number of H-bonds and salt bridges.
b Contribution to ΔG_{Total} in kcal/mol.
%c Percent contribution of each residue to ΔG_{Total} (with ΔG_{average} as baseline).

**FIGURE 3.** Binding mode of indolicidin with Fab V2D2. A, stereo view of interactions between the least energy conformation of indolicidin docked in the CDR cavity of Fab V2D2 with alternate conformation of the H3 loop. The structure was average of 100 conformations resulting from a 1ns MD simulation. The indolicidin backbone (schematic) is shaded cyan and interacting residues, Trp-4, Trp-6, Trp-8, Trp-9, Trp-11 (all cyan), Lys-5, Arg-12 (slate blue) are represented as sticks. Pro-7 and Pro-10 (colored yellow) along with Ile-1, Leu-2, Pro-3, and Arg-13 (not shown for clarity) face outward. Fab residues (sticks) are colored gray. B, Connolly surface rendering of the same interaction, shaded according to electrostatic potential (blue to red indicating positive to negative, respectively) reveals complementarity of surface shape and charge between the antibody and indolicidin (surface and sticks), shown inverted with its binding surface facing upwards (right). The box marks the peptide binding site on the surface of the Fab. Trp residues 4, 8, and 9 bury deep in to the grooves of the Fab cavity. C, indolicidin residues interacting with mAb V2D2 as determined by epitope mapping and in silico docking show a near perfect correlation.

**FIGURE 4.** Novel antibiotic motifs revealed by anti-indolicidin mAb V2D2. A, consensus between results of epitope (Epi) mapping and docking (Dock) led to a motif (Indo-411 or IN-411) critical for activity of indolicidin. B, novel sequences panned from random peptide library by mAb V2D2. Numbers in brackets denote the occurrence frequency in a set of 32 phages sequenced. Similar residues are highlighted. C, comparison of antibiotic activities of indolicidin (N-Indo), its core motif IN-411, random library panned peptides, and their N-terminal lysine-derivatized forms against Escherichia coli BL21D3 in a radial diffusion assay. Unrelated peptide (DVFPYPYASGS) was used as negative control.

**mAb Captures Antibiotic Determinants of Indolicidin**

Individual residue contribution to mAb binding was also determined by in vitro mapping of the peptide epitope. Each amino acid in the indolicidin sequence was mutated to glycine, and binding of the 13 resultant analogs to mAb V2D2 was assayed in an ELISA-like format, plotted in Fig. 3C. The overall data pattern of the two experiments concurred very well in terms of the two parameters. The occurrence frequency in a set of 32 phages sequenced. Similar residues are highlighted. C, comparison of antibiotic activities of indolicidin (N-Indo), its core motif IN-411, random library panned peptides, and their N-terminal lysine-derivatized forms against Escherichia coli BL21D3 in a radial diffusion assay. Unrelated peptide (DVFPYPYASGS) was used as negative control.

The contribution of Arg-12, despite forming H-bonds, was still not significant enough probably because of incomplete burial inside the cavity and the small difference in free energy of a protein residue instead of a solvent molecule (32). Other residues either contributed marginally (Leu-2, Lys-5) or did not appear to be involved directly in complex formation (Pro-3, Pro-5, and Pro-7).

Individual residue contribution to mAb binding was also determined by in vitro mapping of the peptide epitope. Each amino acid in the indolicidin sequence was mutated to glycine, and binding of the 13 resultant analogs to mAb V2D2 was assayed in an ELISA-like format, plotted in Fig. 3C. Differences over the extent of individual residue contribution may be attributed to the antibody CDRs adopting a conformation “intermediate” to the two rigid extremes of crystal and the modeled forms.

**mAb V2D2 as Surrogate Receptor of Indolicidin Identifies Novel Antibiotic Motifs**

Biochemical and structural analyses of mAb V2D2 as a receptor for indolicidin led to elucidation of an antibiotic motif that evidently lies at the core of the activity of the peptide (Fig. 4A). All of the experiments carried out in this study indicated that Trp residues played a pivotal role in mediating antibody binding. Therefore, a minimal motif incorporating all of the tryptophans in sequence of indolicidin was synthesized and tested for bactericidal activity. Remarkably, this motif, termed IN-411, retained ~80% of the activity of its parent peptide (Fig. 4C and Table 2). Although positively charged residues may be required to determine target specificity, evidently it is the core Trp residues that appear to be primarily responsible for the antibiotic nature of indolicidin.
Activity of IN-411 was examined further by subjecting a number of different bacterial species to peptide challenge and estimating their minimum inhibitory concentrations. Comparable concentrations of both indolicidin and IN-411 were able to completely inhibit microbial growth across the spectrum of species tested (Table 2), reinforcing the notion of IN-411 as the focal antibiotic motif.

Because a number of reports in the past have blamed Trp residues for the undesirable cytotoxicity characteristic of indolicidin (20, 33), we also compared the effects of IN-411 and indolicidin on mammalian erythrocytes. Interestingly, the IN-411 motif, despite containing all five Trp residues, exhibited a significant reduction in its mean hemolytic concentration. The overall effect translated in to the motif having a 5-fold higher therapeutic index compared with indolicidin (Table 2), opening up prospects of its use as a template for a new generation of antibiotics.

Novel chemical entities with bioactivity are often obtained by screening a library of synthetic compounds. With a high affinity indolicidin receptor surrogate in hand, we decided to use it as a probe to pan a phage-displayed library of random 12-mer peptides, close in length to the 13-mer indolicidin. After multiple rounds, two sequences named 12P1 and 12P2 were obtained (Fig. 4B). Although they bound to the antibody with low affinity (K_D in micromolar range) and did not seem significantly related to indolicidin at the level of primary structure, they remarkably turned out to have bactericidal properties (Fig. 4C). 12P1 in particular, exhibited broad spectrum antibiotic activity, a hallmark of native indolicidin (supplemental Fig. S1). Also, their bactericidal property could be enhanced by addition of a N-terminal lysine residue, another feature similar to indolicidin (34). Interestingly, analogous to IN-411, a motif contained in the sequence of 12P1, called 12P1–39, displayed the entire activity of the whole 12P1 sequence. Although in this case, antibacterial activity of the peptides panned by mAb V2D2 was less than that of indolicidin, originating from their corresponding low affinity for the antibody, more potent antibiotics/chemical entities can easily be panned by altering the complexity of the library being screened. Yet, the ability of the antibody to identify a common physiological property among distantly related peptide sequences provides a convincing demonstration of mAbs as surrogate receptors.

**DISCUSSION**

Surrogate/pseudo-receptors have become increasingly popular in both fundamental and translational research but inexcitably have remained confined to the virtual world, which makes them suffer from several limitations (35). Our work presents a fresh perspective on this concept in the form of monoclonal antibody V2D2, a “real world” receptor that was successfully used to investigate antibiotic nature of the peptide indolicidin. Through a remarkable concurrence between computational and biochemical results, the antibody revealed a conformation that could be correlated with the antibiotic action of indolicidin. It also helped elucidate a therapeutically relevant antibiotic motif demonstrating the utility of this technique in screening of template pharmacophores. In contrast to previous structure-activity studies, the present work therefore provides a more lucid understanding of the determinants of biological activity.

The peptide binding site in the antibody receptor, a flexible cavity lined with multiple aromatic residues that converged on electronegative atoms, gave an impression of bacterial cell
membrane-like environment. Such features have been observed before in anti-steroid antibodies with which mAb V2D2 shares strong homology. Furthermore, the least energy indolicidin conformer that bound to mAb V2D2 was derived from the wedge-shaped structure that indolicidin adopts in presence of phospholipid like micelles (Fig. 5A) (29). Also, the way Trp residues inserted in to the antibody cavity correlates with experiments that have directly visualized the membrane interaction of indolicidin (36). This suggests that the surrogate bound indolicidin conformation could represent a transitional snapshot of the peptide crossing the bacterial cell membrane.

Two other interesting aspects emerged from the structural overlap of the micelle-bound and antibody-bound indolicidin conformations (Fig. 5A). The side chain orientation of both residues Trp-4 and Trp-6 were on the opposite sides of the backbone, almost appearing as mirror images of each other. This assumes significance because both L- and D-stereoisomers of indolicidin are known to be biologically active (37). On the other hand, Trp-9 and Trp-11 positions were interchanged in the two conformations, suggesting a certain functional equivalence that may provide synergism during receptor binding and may explain its potent membrane activity. It is supported by data on IN-411, K12P1, and K12P2, where a gradual reduction in number of Trp residues can be correlated with a progressive decrease in activity. Such an arrangement may also allow the peptide to adopt an amphipathic structure in multiple ways, enabling it to interact/inhibit a wide range of biomolecules.

The fact that IN-411 displays an unequal change in minimum inhibitory concentration versus mean hemolytic concentration indicates that membrane interaction and antibacterial activity may not be perfectly correlated, in agreement with previous studies (20, 33). Also, although mAb V2D2 may display membrane-like features in its binding to indolicidin, it does not implicate the lipid bilayer as the sole target of the peptide. Rather, both the results merely highlight membrane interaction as a necessary step to microbial death, which may actually be caused by the interaction of indolicidin with other biomolecules that have an environment similar to the interface of mAb V2D2. In this regard, indolicidin binding to DNA in vitro (13) and producing a morphological state that suggest replication arrest (38) have been reported previously. The molecular basis of these effects can be conjectured from our observation that the mAb-bound conformation of indolicidin superimposes well on DNA-minor groove binders like Hoechst 33258 (Fig. 5B) that also has been shown to have bactericidal action (39). Yet, a number of other indole based antibiotics also are known to bring about microbial death through other mechanisms like inhibition of transcription or down-regulation of protein kinase-mediated signaling (40). Thus, depending on concentration, indolicidin may be targeting multiple cellular components and its surrogate-bound conformation could represent a case of a common structural feature being used for both membrane transport and nonmembrane receptor binding.

It may be pointed out that the case of mAb V2D2 as a surrogate receptor is not a fortuitous event. A growing body of literature exists wherein antibodies raised against biological ligands have been found to possess physiological properties of their natural receptors (41–44). In the present case, the potent activity of IN-411, testifies to the “biological target-like” properties of the mAb surrogate. It is further supported by a previous report where indolicidin residues, presently identified as critical for binding to mAb V2D2, also were found to be critical for its antibiotic nature (33). Simultaneous mutations of Trp-4, -6, -8, -9 and -11 to less hydrophobic (Tyr) or nonaromatic/polar residues (Leu/Ser) were reported to progressively reduce the antibacterial activity of indolicidin by one- and two-log orders, respectively. On the other hand, substitution of all Pro residues with Gly, deletion of Ile-1, Leu-2, or Arg-12, Arg-13 had no significant effect on the activity of the peptide. Together, these results corresponded perfectly to the epitope of indolicidin being recognized by mAb V2D2.

Furthermore, mAb V2D2 was remarkable in its ability to identify novel bactericidal peptides from a random peptide library. Analyzing the results of the latter experiment has been quite instructive. As mentioned, sequence alignment of these peptides with indolicidin did not reveal any contiguous motif to which their bactericidal activity could be ascribed, though similarities at a few positions were observed for prolines and tryptophans (Fig. 4A). Interestingly, at some positions, these sequences had His aligned with the Trp of indolicidin. Although His does not have the membrane partitioning abilities of Trp, and it is difficult to correlate the two in other biochemical properties, but it does form a major component of certain antifungal peptides (45). Thus, apart from establishing mAb V2D2 as a physiologically relevant surrogate, the experiment also demonstrated the ability of the antibody to pick up antibiotic characteristics in distantly related motifs.

In conclusion, we have uncovered a novel bioactive motif within the sequence of indolicidin, which represents a potential template for antibiotic design. Our work reveals possible mode of the interactions of indolicidin with bacterial cell membrane and DNA and indicates a certain conformational “economy,” in terms of the likelihood of same conformation being used to inhibit different microbial components. Second, we have observed a remarkable accord between computational and biochemical data, especially in terms of key molecular interactions in the mAb-peptide complex and associated equilibrium free energies. It suggests that in silico and in vitro approaches to physiological queries need not be mutually exclusive. The combination of methodologies described here can be consolidated in to a ready protocol for potentiating high throughput rational drug design. Most significantly, however, this work demonstrates that the idea of physical surrogate receptors is realistic. At the molecular level, the interactions of mAb V2D2 with indolicidin could be perceived as antibodies fulfilling their evolutionary role of versatile binding. It thereby suggests that receptor surrogacy might be part of their innate character. Suitably chosen surrogate monoclonal antibodies can provide a range of target equivalent binding environments and generate mechanistic data that is biologically credible and can better facilitate pharmacophore identification and optimization.

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mAb Captures Antibiotic Determinants of Indolicidin

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