Rapid Optimization of a Peptide Inhibitor of Malaria Parasite Invasion by Comprehensive N-Methyl Scanning

Karen S. Harris, Joanne L. Casey, Andrew M. Coley, John A. Karas, Jennifer K. Sabol, Yen Yee Tan, Olan Dolezal, Raymond S. Norton, Andrew B. Hughes, Denis Scanlon, and Michael Foley

From the Department of Biochemistry, La Trobe University, Victoria 3086, AdAlta Pty. Ltd., 15/2 Park Drive, Bundoolo, Victoria 3083, the BIO21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, Victoria 3010, the Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, CSIRO Molecular and Health Technologies, 343 Royal Parade, Parkville, Victoria 3052, and the Department of Chemistry, La Trobe University, Victoria 3086, Australia

Apical membrane antigen 1 (AMA1) of the malaria parasite Plasmodium falciparum has been implicated in the invasion of host erythrocytes and is an important vaccine candidate. We have previously described a 20-residue peptide, R1, that binds to AMA1 and subsequently blocks parasite invasion. Because this peptide appears to target a site critical for AMA1 function, it represents an important lead compound for anti-malarial drug development. However, the effectiveness of this peptide inhibitor was limited to a subset of parasite isolates, indicating a requirement for broader strain specificity. Furthermore, a barrier to the utility of any peptide as a potential therapeutic is its susceptibility to rapid proteolytic degradation. In this study, we sought to improve the proteolytic stability and AMA1 binding properties of the R1 peptide by systematic methylation of backbone amides (N-methylation). The inclusion of a single N-methyl group in the R1 peptide backbone dramatically increased AMA1 affinity, bioactivity, and proteolytic stability without introducing global structural alterations. In addition, N-methylation of multiple R1 residues further improved these properties. Therefore, we have shown that modifications to a biologically active peptide can dramatically enhance activity. This approach could be applied to many lead peptides or peptide therapeutics to simultaneously optimize a number of parameters.

According to World Health Organization reports, almost half of the world human population is at risk of contracting malaria and approximately one million people are killed by this disease each year, most of which are young children. No reliable vaccine currently exists against Plasmodium falciparum, the parasite that causes the most severe form of malaria, and parasites are becoming increasingly resistant to previously effective pharmaceuticals (1). Thus, novel strategies to combat this disease are urgently required.

Proteins involved in the erythrocytic stage of the parasite life cycle represent attractive targets for anti-malarial therapeutics, because this stage is responsible for the clinical symptoms of malaria. Apical membrane antigen 1 (AMA1) is an integral membrane protein common to all Plasmodium species that is thought to be important for parasite invasion of host red blood cells and hepatocytes (2, 3). The molecular mechanisms of the role of AMA1 in parasite invasion are unknown, but recent evidence suggests that it is involved in stabilizing the binding of the invasive form of the parasite to the erythrocyte (3, 4). In animal models, immunization with recombinant AMA1 induces protection from subsequent parasite challenge (5, 6), and it appears to be an essential protein in the parasite life-cycle as AMA1 genetic knockouts are not viable (3, 7). AMA1 has therefore received much attention as a potential vaccine candidate or drug target. Although lacking the major structural diversity of some other P. falciparum antigens, AMA1 exhibits allelic variation that is a result of point mutations. Because immunization with AMA1 induces inhibitory antibodies that are less effective against heterologous parasite lines (8–10), overcoming this diversity is a key hurdle in the development of AMA1-based anti-malarials. An immune response or drug would therefore ideally target a conserved region that is required for function.

In addition to polyclonal and mAbs that bind to AMA1 and block parasite invasion in vitro (9, 11, 12), peptides that specifically recognize this antigen and demonstrate similar inhibitory activity have been isolated from random peptide libraries displayed on the surface of phage (13, 14). The most potent peptide inhibitor of invasion was the recently identified 20-residue peptide, R1 (13). Peptides that recognize functional sites and act as biological agonists or antagonists can be useful for the design of small molecule mimics, or as therapeutics per se. The R1 peptide is an important lead compound for drug development, because its ability to block parasite growth indicates that it tar-

The abbreviations used are: AMA1, apical membrane antigen 1; MTBD, 7-methyl-1,5,7-triazacyclo-[4.4.0]-dec-5-ene; Fmoc, (9-fluorenyl)methoxycarbonyl; PBS, phosphate-buffered saline; NOE, nuclear Overhauser effect; mAb, monoclonal antibody; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMF, dimethylformamide; DCM, dichloromethane; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SPR, surface plasmon resonance.
Anti-malarial N-Methylated Peptides

gets a site critical for AMA1 function. However, of the four parasite lines tested, the R1 peptide was only effective against the 3D7 and D10 strains, and not HB3 or W2mef, limiting its utility as a therapeutic agent in its current form. A further barrier to the general use of peptides as pharmaceuticals is that they are rapidly degraded by peptidases and proteases in biological fluids, leading to poor bioavailability (15). It was therefore of interest to modify the R1 peptide to improve its pharmaceutical potential and AMA1-binding properties.

The methylation of backbone amides (N-methylation) is one strategy of peptide modification that has been employed to confer superior characteristics on a lead peptide. This involves the replacement of residues within a peptide sequence with the corresponding N-methyl amino acid, subtly changing the peptide backbone. The introduction of a methyl group into a peptide has the advantage of substantially improving a number of pharmacokinetically useful parameters, including membrane permeability, conformational flexibility, and proteolytic stability (16). Additionally, it results in a loss of hydrogen bonding potential at the affected site, reducing the role of main-chain hydrogen bonds at a binding interface and potentially altering binding properties (17). There are a number of examples of N-methyl amino acid containing peptides with a range of bioactivities, including antibiotic (18), anti-viral (19), anti-cancer (20), and immunosuppressant activities (21). Importantly, modification of biologically active peptides by the inclusion of N-methyl amino acids into an original sequence has been shown to enhance the potency (22), change the receptor subtype selectivity (23), and stabilize a peptide toward proteolytic degradation in a biological system (24). This approach therefore offers the potential to optimize multiple parameters simultaneously.

In this study, we systematically N-methylate residues within the previously identified AMA1-binding peptide, R1, and describe the synthesis and altered properties of these mono-, di-, and tri-N-methylated R1 derivatives. To introduce an N-methyl group into residues for which an N-methyl analogue is not commercially available, we used on-resin N-methylation (25), facilitating a comprehensive N-methyl scan of the R1 peptide. Analysis of each of these R1 derivatives revealed that inclusion of a single N-methyl group was sufficient to dramatically improve AMA1 affinity, bioactivity, and peptide stability without altering the global peptide structure. Moreover, the introduction of multiple N-methyl groups further increased AMA1 affinity and slightly improved recognition of AMA1 derived from different parasite lines. This represents a substantial step forward in the design of an effective anti-malarial compound able to bind to AMA1 and inhibit malarial parasite growth. Furthermore, this work may contribute to the development of a rational strategy for modulating and optimizing the activity of bioactive peptides.

EXPERIMENTAL PROCEDURES

Fmoc Solid-Phase Peptide Synthesis

**Materials**—Chlorotrityl resin, N,N-diisopropylethylamine, piperidine, hydroxybenzotriazole, HBTU were all obtained from GLBiochem, Shanghai, China. DMF, DCM, diethyl ether, and 2-mercaptoethanol were obtained from Ajax Chemicals (Melbourne, Australia). MTBD, 1,8-diazabicyclo[5.4.0]undec-7-ene, methyl triflate, o-nitrobenzenesulfonyl chloride, chloranil, and TNBS were obtained from Sigma-Aldrich.

The N-methyl amino acids Fmoc-NMe-Val-OH, Fmoc-NMe-Phe-OH, Fmoc-NMe-Ala-OH, Fmoc-NMe-Leu-OH, Fmoc-NMe-Ile-OH, Fmoc-NMe-Met-OH, and Fmoc-Ser-OH were obtained from Peptide Solutions (Melbourne, Australia). Fmoc-NMe-Arg(Mtr)-OH was obtained from Chem Impex (Wood Dale, IL). Fmoc-Lys(Boc)-OH, Fmoc-His(Trg)-OH, Fmoc-Ser(tBu)-OH, and Fmoc-Glu(OctBu)-OH were obtained from GLBiochem and incorporated into the sequence as N-methyl residues by the “on-resin” method of Miller and Scanlan (25).

**Derivatization of the Resin**—Chlorotrityl resin (1.0 g, 1 mmol) was suspended in dry DCM (10 ml). Fmoc-Lys(Boc)-OH (1 mmol) was dissolved in dry DCM (10 ml), and the solution was added to the resin. N,N-diisopropylethylamine (4 mmol, 680 μl) was added, and the solution was stirred for 2 h. After this time methanol (1 ml) was added, and the solution was stirred for another 30 min. The resin was washed sequentially with DCM and ether and then air-dried. The loading of Fmoc-Lys(Boc) onto the resin was estimated by the increase in resin weight and was typically 0.5–0.6 mmol/g.

**Solid-phase Synthesis Protocol**—The scale for synthesis was typically 0.5 g and 0.25 mmol. Resin (0.5 g) was washed with DMF (4 × 10 ml) and then treated with a solution of 25% piperidine/DMF (containing 0.1 μ hydroxybenzotriazole) for 10 min. After this time the resin was washed with DCM (4 × 10 ml), then DMF (4 × 10 ml), and then a small sample (~5 mg) was subjected to a TNBS test (26) for the presence of primary amine. The Fmoc-amino acid (1 mmol and 4-fold excess) was activated by dissolution in DMF (2 ml) followed by the addition of a solution of HBTU in DMF (2 ml, 0.5 μ) to which was added N,N-diisopropylethylamine (340 μl, 2 mmol). This solution was sonicated for 2 min and then added to the resin. This resin-coupling reaction mixture was agitated for 1 h and then drained and washed with DMF (4 × 10 ml). A resin sample was then tested for the presence of residual amine using the TNBS test (26). If the test was positive another aliquot of the Fmoc-amino acid was activated, and the coupling procedure was repeated.

Where an N-methyl was to be incorporated into the peptide sequence following the piperidine treatment, o-nitrobenzenesulfonyl chloride (0.75 mmol, 3 eq) was dissolved in dry DCM (4 ml), and the solution was added to the resin together with 2,4,6-collidine (1.25 mmol, 5 eq). This reaction mixture was stirred for 2 h. The N terminus was then methylated by treatment with a solution of methyl triflate (0.75 mmol, 3 eq) and the base MTBD (2 eq, 77 mg) in DCM (4 ml), and the mixture was stirred for 30 min. After this period the resin was drained, washed with DMF (4 × 10 ml), and then treated repeatedly with a solution of 2-mercaptoethanol/1,8-diazabicyclo[5.4.0]undec-7-ene/DMF (200 μl/200 μl/4 ml). Usually this procedure involved 3 × 30 min treatments until there was no more yellow color evident in the solution (due to the o-nitrobenzenesulfonyl group). The resin was then tested for the presence of a secondary amine due to the N-methyl group on the N terminus. The next amino acid was then activated and coupled in the normal manner (HBTU).

9362 JOURNAL OF BIOLOGICAL CHEMISTRY
Note that the TNBS test could not be used to detect the secondary amine of proline and the N-methyl amino acids: in this case the chloranil test (27) was used.

Peptide Cleavage from Resin and Purification—Upon completion of the required peptide synthesis cycles the N-terminal amino group was deprotected and the resin was washed with DCM (5 × 10 ml) and lastly ether. The resin (typically 0.8–1.0 g) was air-dried overnight and then treated with a solution of trifluoroacetic acid/dithiothreitol/triisopropylsilylethanol/H2O (90/5/2.2/2.5, 10 ml) for 2 h. The mixture was then filtered, and cold ether (40 ml) was added to the solution, which resulted in the precipitation of the peptide. The peptide slurry was centrifuged (3500 rpm, 3 min) and the crude peptide was isolated by decanting the ethereal supernatant. The peptide was then dissolved in 30% acetonitrile/water, and the solution was lyophilized.

The lyophilized peptides were purified on a Gemini C18 5 μM semi-prep high-performance liquid chromatography column (Phenomenex: dimension, 50 × 21.2 mm; flow, 6 ml/min). Collected fractions were analyzed on an Absorbsphere HD C18 5 μM analytical high-performance liquid chromatography column (Alltech: dimension, 150 × 3.2 mm; flow, 0.7 ml/min). Pure fractions were pooled and lyophilized, and the molecular weight was confirmed by electrospray mass spectrometry.

Phage Display

Production of M13 bacteriophage displaying the R1 peptide was carried out as described elsewhere (28). Briefly, K91 *Escherichia coli* cells harboring the modified phage genome fUSE5, which included DNA encoding the R1 peptide, were incubated in broth culture to produce phage. Phage were harvested by precipitation with polyethylene glycol/NaCl (30% (w/v) polyethylene glycol 8000 (Sigma), 2.6 M NaCl) followed by centrifugation and resuspension of the phage pellet in phosphate-buffered saline (PBS). Phage concentration was determined in colony-forming units per milliliter (cfu/ml) as previously described (28).

Invasion Inhibition Assay

The 3D7 parasite line was cultured essentially as described previously (29, 30), and parasites were synchronized by sorbitol lysis of all but ring stage parasites (31). R1 peptide and methylated R1 analogues were assayed for their ability to inhibit parasite growth as previously described (13). Briefly, peptides were added to each well (4% hematocrit, 0.3% parasitemia) and plates were incubated for 40–42 h at 37 °C in a moist atmosphere of 94% N2, 1% O2, 5% CO2. After washing with ice-cold PBS, parasites were frozen, then thawed, and relative parasitemia levels were determined by assaying for parasite lactate dehydrogenase activity (10). Absorbance was measured at 650 nm (A650 nm), and the percent inhibition of invasion was calculated as: 100 − [(A650 nm peptide sample − A650 nm red blood cells only)/(A650 nm, no peptide control − A650 nm red blood cells only) × 100].

Enzyme-linked Immunosorbent Assay

96-well microtiter plates (Maxisorp, Nunc) were coated with 2 μg/ml recombinant AMA1. The antigen was diluted in coating buffer (15 mM Na2CO3, 34 mM NaHCO3, pH 9.6), and plates were incubated overnight at 4 °C. Unbound protein was removed by washing with PBS, and any unbound surfaces were blocked with 10% skim milk powder in PBS. R1 peptide or N-methylated R1 analogues were applied to the wells in duplicate in the presence of a constant concentration of R1 phage (−2 × 109 cfu/ml). Following incubation for 1 h at room temperature, with shaking, unbound phage was removed by washing with PBS 0.05% Tween 20 (Sigma). Bound phage was detected with horseradish peroxidase-conjugated anti-M13 antibodies (1:5000, Amersham Biosciences). Binding was visualized using tetrathymolbenzidine (Sigma), and the absorbance was read at 450 nm.

Immobilization of AMA1 Proteins to SPR Chip

A Biacore T100 biosensor instrument (32) was used to measure the kinetics of the interaction of R1 peptides and their N-methylated derivatives with AMA1 proteins from different strains of *P. falciparum*. All immobilization and binding experiments were performed at 25 °C using HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.05% surfactant P20, pH 7.4) as the running buffer. AMA1 was immobilized on CM5 sensor chips using standard amine-coupling chemistry. The carboxymethyl dextran surface was activated with a 7-min injection at 10 μl/min of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/0.1 M N-hydroxy succinimide. Coupling of AMA1 to the chip surface was achieved using a manual control approach whereby small volumes (1–15 μl) of AMA1 diluted to 12.5 μg/ml in 10 mM sodium acetate, pH 4.5, were injected over the activated surface at 5 μl/min until immobilization of ~1500–2500 relative units was reached (1 relative units = 1 pg of protein/mm²). The immobilization procedure was completed by a 7-min injection (10 μl/min) of 1 m ethanolamine (pH 8.5) to deactivate residual reactive sites. Final immobilized AMA1 levels achieved were as follows: 3D7 = 1530, HB3 = 2705 relative units and W2mef = 2499 relative units. In each experiment at least one of the flow cells on the chip was used as “mock surface” for referencing purposes. This was prepared by subjecting a surface to the amine coupling procedure with no protein present.

SPR Binding Analysis

All biosensor binding experiments were performed in triplicate in HBS-EP+ running buffer at 25 °C. To generate the binding data, a series of peptide concentrations, typically ranging from 5 to 320 nM for 3D7 and from 0.32 to 40.96 μM for W2mef and HB3, were injected over immobilized AMA1 at a constant flow rate of 60 μl/min for 1 min. Peptide dissociation was then monitored by injecting HBS-EP+ running buffer for 5 min, after which time all of the peptide completely dissociated from the AMA1 surface and thus no further regeneration was required.
**Anti-malarial N-Methylated Peptides**

**SPR Data Analysis**

All Biacore sensorgrams were processed using Scrubber software (Version 2, available from Biologic Software, Australia). Sensorgrams were first zeroed on the y axis and then x-aligned at the beginning of the injection. Bulk refractive index changes were removed by subtracting the reference flow cell responses. The average response of all blank injections was subtracted from all analyte injections and blank sensorgrams to remove systematic artifacts in the experimental and reference flow cells. Scrubber analysis software was used to determine $k_a$ and $k_d$ from the processed data sets by globally fitting to a 1:1 biomolecular binding model that included the mass transport term (33). The $K_d$ was calculated from the quotient $k_d/k_a$. Alternatively, for rapidly dissociating interactions, affinity ($K_{\text{app}}$) estimates were derived using steady-state affinity algorithm available within Scrubber.

**NMR Spectroscopy**

Samples were prepared for NMR analysis by dissolving peptides in 500 µl of 95% H$_2$O/5% D$_2$O containing 10 mM sodium acetate to a final concentration of ~1.7 mM. The pH was adjusted to 4.6–4.7. $^1$H NMR spectra of most peptides showed a single major set of resonances, indicating that any impurities were non-peptidic (the amide and aromatic regions of one-dimensional spectra are shown in supplemental Fig. S1). Additional peaks with intensities <10% of the main peak were present in spectra of N-Me-Leu-8, N-Me-Leu-8/N-Me-Ser-14, and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14. As these peptides were >90% pure, these additional peaks must have arisen from minor conformers. The most likely origin of this conformational heterogeneity is cis-trans isomerism around the peptide bond involving the N-methyl group at Leu-8; this has been observed previously in other N-methylated peptides (34, 35). Two different preparations of N-Me-Leu-8 showed identical ratios of major to minor species.

Two-dimensional homonuclear total correlation spectra with a spin-lock time of 60 ms and double quantum filtered correlation NMR spectra were acquired at 600 MHz on a Bruker DRX-600 spectrometer. Two-dimensional nuclear Overhauser enhancement (NOESY) spectra with a mixing time of 250 ms were also acquired at 600 MHz. For N-Me-Leu-8 and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14, nuclear Overhauser enhancement spectra with a mixing time of 250 ms were also acquired at 800 MHz on a Bruker Avance 800. Water was suppressed using the WATERGATE pulse sequence (36). All spectra were collected at 5 °C unless otherwise stated and were referenced to an impurity peak at 0.15 ppm or to the water resonance.

Translational diffusion measurements were performed for N-Me-Leu-8, N-Me-Leu-8/N-Me-Ser-14, and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14 using a pulsed field gradient longitudinal eddy-current delay pulse sequence (37, 38) as implemented by Yao et al. (39). Spectra were processed using XWINNMR (Version 3.5, Bruker Biospin) and TOPSPIN (Version 1.3, Bruker Biospin) and analyzed using XEASY (Version 1.3.13) (40). Chemical shift assignments for the major conformation of the peptides are tabulated in the supplemental material and have been deposited in the BioMagResBank (41).

**Structural Constraints**

$^3$J$_{\text{HNNH}}$ coupling constants were measured from double quantum filtered correlation spectra at 600 MHz and converted to dihedral restraints as follows: $^3$J$_{\text{HNNH}} > 8$ Hz, $\phi = -120 \pm 40^\circ$; $^3$J$_{\text{HNNH}} < 6$ Hz, $\phi = -60 \pm 30^\circ$. If a positive $\phi$ angle could be excluded on the basis of NOE data (42), $\phi$ angles were restricted to the range $-180$ to $0^\circ$. No $\chi^1$ angle or hydrogen bond restraints were included for N-Me-Leu-8 and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14.

**Structure Calculations**

Intensities of NOE cross-peaks were measured in XEASY and calibrated using the CALIBA macro of the program CYANA (version 1.0.6) (43). NOEs providing no restraint or representing fixed distances were removed. For structure calculations of N-Me-Leu-8 and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14, the constraint list resulting from the CALIBA macro of CYANA was used directly in XPLOR-NIH (44) to calculate a family of 100 structures using the simulated annealing script. The library files supplied with XPLOR-NIH were modified to include a methyl at the backbone amide of the corresponding residue. The 70 lowest energy structures were selected for each peptide. A box of water with a periodic boundary of 18.856 Å was built around the peptide structure, which was then energy-minimized based on NOE and dihedral restraints and the geometry of the bonds, angles, and improper. From this set of structures, final families of 20 lowest energy structures for each peptide were chosen for analysis. In all cases, the final structures had no experimental distance violations >0.2 Å or dihedral angle violations greater than 5°. All structural figures were prepared using the programs MOLMOL (45) and PyMOL.4

**Peptide Stability in Mouse Plasma**

Mouse plasma samples were prepared by adding 1 ml of Milli Q water to the Sigma P-9275 mouse plasma product and making an aliquot of the solution into 5 × 400-µl lots. Peptides (0.4 mg) were dissolved in PBS (400 µl containing 0.1% (v/v) benzyl alcohol as internal standard). Equal volumes of the plasma and peptides solutions were combined (total volume, 800 µl) and incubated at 37 °C. Samples were taken in triplicate (3 × 50 µl) at 0, 10, 20, 40, and 80 min and placed on ice. To each sample, 20 µl of 0.5 M lysine monohydrochloride was added, followed by 65 µl of acetonitrile. The samples were then cooled on ice for 10 min and centrifuged for 10 min at 3000 × g resulting in a biphasic solution. A 50-µl sample was taken from the supernatant, diluted with 100 µl of Milli Q water, and analyzed by reversed-phase high-performance liquid chromatography on a C18 reversed-phase column. The ratio of the disappearing peptide peak area to the benzyl alcohol peak area was calculated for each time point and normalized against the $t = 0$ time point.

---

4 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
(representing 100% peptide remaining) as a percentage peptide remaining.

RESULTS

Synthesis of N-Methylated R1 Derivatives—The R1 peptide consists of 20 residues, and its sequence is shown in Table 1. R1 analogues incorporating a methyl group at the backbone amide of each residue, except Pro-7 and Lys-20, have been synthesized (Table 1 entries 2–19 and Fig. 1). Proline contains a secondary amine and N-methylation at Pro-7 would effectively terminate the peptide sequence. Difficulties were experienced in forming the N-methyl derivative of Lys at the C terminus of the peptide, presumably because of its close proximity to the solid support. Therefore, 18 R1 derivatives were individually synthesized by Fmoc solid-phase peptide chemistry as part of the initial "N-methyl scan." The mode of incorporation of particular N-methyl residues depended on the nature of the amino acid. Relatively few N-Fmoc-N-methyl amino acids are commercially available and ready for the peptide-coupling reaction: these include Fmoc-NMe-Val-OH, Fmoc-NMe-Phe-OH, Fmoc-NMe-Ala-OH, Fmoc-NMe-Leu-OH, Fmoc-NMe-Ile-OH, and Fmoc-NMe-Met-OH. These compounds were readily synthesized by the general method of Aurelio et al. (47) and incorporated into the peptide using standard HBTU-mediated peptide coupling chemistry. When N-methylation of other residues was required the "on-resin" method of Miller and Scanlan (25) was used. Briefly, the Fmoc group was removed from the amino acid to be N-methylated and re-protected with the ortho-nitrobenzenesulfonyl group (o-nosyl). This moiety rendered the amine hydrogen slightly acidic and vulnerable to alkylation by a methylating agent such as methyl triflate (25). Lastly, the nosyl group was removed by treatment with base and mercaptoethanol generating a free secondary amine ready for the next coupling reaction. Using these two techniques the 18 R1 analogues were prepared.

AMA1 Binding Ability of N-Methylated R1 Derivatives—The relative affinity of these N-methylated R1 derivatives for AMA1 was assessed by determining the ability of each peptide to inhibit the interaction of phage displaying the R1 peptide with immobilized AMA1 by enzyme-linked immunosorbent assay (Fig. 2, A–C). The peptides could be classed into four broad categories according to the impact of methylation. Binding was significantly improved by the presence of a methyl group at Leu-8. Methylation of residues Lys-11, Phe-12, Gly-13, and Arg-15 resulted in a striking reduction in AMA1 binding affinity, as judged by the reduced ability of these peptides to prevent phage-displayed R1 peptide from binding to AMA1. Inclusion of an N-methyl group at residues Ala-3, Phe-5, Phe-9, Ser-10, and Met-16 slightly decreased AMA1 binding. The remaining methylations (Val-1, Phe-2, Gly-4, Leu-6, Ser-14, His-17, Iso-18, and Leu-19) exhibited similar binding to native R1 in this assay.

Ability of N-Methylated R1 Derivatives to Inhibit Parasite Invasion—To determine the effect of N-methyl substitution on bioactivity, each R1 analogue was tested for its ability to inhibit parasite invasion in vitro (Fig. 3A). At 50 µg/ml peptide, inhibition correlated well with the binding data, which are summarized in Fig. 3B. For example, R1 with N-methylation of Lys-11, which was unable to bind AMA1, showed little effect on parasite growth, whereas the N-Me-Val-1 analogue both retained its
AMA1-binding capacity and prevented parasite growth in vitro. However, because native R1 peptide completely inhibited parasite growth at this concentration, any improvements in inhibition conferred by methylation could not be assessed. Those peptides exhibiting maximal levels of invasion inhibition were therefore selected for titration in growth inhibition assays (Fig. 3). These titrations revealed that the N-Me-Leu-8 peptide was a more potent invasion inhibitor than native R1, correlating with the enhanced affinity observed for this analogue. Interestingly, N-Me-Val-1 and N-Me-Ser-14 analogues were ∼2-fold more effective inhibitors than the native peptide despite exhibiting similar AMA1 binding to native R1. This suggests that the binding assay may not be sufficiently sensitive to distinguish small changes in affinity.

Double and Triple N-Methylated R1 Derivatives—Because the addition of a single methyl group at Val-1, Leu-8, and Ser-14 enhanced the ability of R1 to block parasite invasion, it was of interest to determine if combining these methylations could further improve R1 activity. Therefore, two analogues incorporating multiple N-methylations were synthesized: a doubly N-methylated peptide with N-methyl groups at Leu-8 and Ser-14, and a triply N-methylated peptide with N-methyl groups at Val-1, Leu-8, and Ser-14. These peptides competed with phage-displayed R1 peptide for binding to immobilized AMA1 to a similar degree to the N-Me-Leu-8 peptide (Fig. 4A). When tested in growth inhibition assays, these peptides reduced parasite growth ∼10-fold more effectively than native R1 (Fig. 4B).
Strain Specificity of N-Methylated R1 Derivatives—To investigate strain specificity of R1 analogues with improved inhibitory characteristics and to precisely measure the affinity of these peptides for the 3D7 form of AMA1, kinetic and steady-state binding to immobilized AMA1 proteins from three P. falciparum strains were examined by SPR (Table 2 and supplemental Figs. S7 and S8). The unmodified R1 peptide bound to 3D7 AMA1 with a $K_D$ of $\sim$80 nm. Progressive methylation of the R1 peptide resulted in an increased affinity for 3D7, although the difference between doubly and triply methylated R1 was not significant. The primary reason for this increased affinity appears to be slower dissociation of the peptide from AMA1 (slower $k_a$ rate constant) suggesting that the presence of N-methyl groups in the R1 peptide backbone increases the overall stability of the peptide-AMA1 complex. Interactions between native and N-methylated R1 peptides and immobilized AMA1 derived from W2mef and HB3 strains of parasite were analyzed by equilibrium analysis. Although binding of all R1 peptides to these isoforms of AMA1 was weaker than to 3D7 AMA1, the inclusion of multiple N-methyl groups in the R1 peptide resulted in increased affinities: $\sim$17 to $\sim$6 μm and $\sim$70 to $\sim$25 μm for W2mef and HB3 AMA1, respectively.

Solution Structure of N-Methylated R1 Derivatives—To determine the impact of N-methylation on the solution structure of R1, one- and two-dimensional $^1$H NMR spectra were recorded on the following R1 analogues: N-Me-Phe-5, N-Me-Leu-8, N-Me-Lys-11, N-Me-Phe-12, N-Me-Gly-13, N-Me-Ser-14, N-Me-Leu-8/N-Me-Ser-14, and N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14. The deviations of the backbone NH and C$^\alpha$H chemical shifts from random coil values are plotted in supplemental Fig. S2. The differences between these backbone chemical shifts for each of the peptides and corresponding values for native R1 are plotted in Fig. 5. A striking feature of these plots is that the backbone chemical shifts caused by N-methylation are predominantly local, mostly within two to three residues either side of the N-methyl substitution, which implies that N-methylation does not cause any long range structural changes in R1. The structures are consistent with the previously described solution structure of R1, which contains two structured regions, both involving turns, the first of which, encompassing residues 5–10, is hydrophobic and the second, involving residues 13–17, is more polar (13). The $i+3$ residues in these turns are Ser-10 and Met-16, respectively, and N-methylation at these positions would be expected to interfere with formation of $i+3$ to $i$ hydrogen bonds and thus destabilize the turns. The observation that N-Me-Ser-10 and N-Me-Met-16 had only slightly decreased binding affinities for AMA1 indicates that the turns present in solution may not persist in the AMA1-bound peptide, at least not in standard turn conformations. Even in solution these turns are unlikely to represent the only conformations sampled by the peptide, because linear peptides lacking any covalent cross-links, as in the case of R1, are known to sample a range of rapidly interconverting conformations in aqueous solution.

**TABLE 2**

| Peptide | $k_m \times 10^7$ | $k_a \times 10^9$ | $k_d \times 10^{-2}$ | $K_D$ | $K_D$ (μM) | $K_D$ (μM) |
|---------|-----------------|-----------------|-----------------|-------|-----------|-----------|
| AMA1 variant | | | | | | |
| Native R1 | 5.50 ± 0.70 | 2.73 ± 0.15 | 21.00 ± 1.73 | 77.37 ± 8.30 | 80.3 ± 5.5 | 16.8 ± 0.1 |
| N-Me-Leu-8 | 2.74 ± 0.54 | 2.84 ± 0.15 | 6.37 ± 0.91 | 22.67 ± 3.10 | 15.0 ± 0.1 | 68.5 ± 0.5 |
| N-Me-Leu-8/N-Me-Ser-14 | 3.50 ± 0.19 | 3.57 ± 0.73 | 3.98 ± 0.48 | 11.28 ± 1.05 | ND | ND |
| N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14 | 3.46 ± 0.23 | 2.25 ± 0.08 | 2.84 ± 0.21 | 12.63 ± 1.30 | ND | 5.7 ± 0.1 |

* Rate parameters ($k_m$, $k_a$, and $k_d$) and corresponding affinity constant ($K_D$) were estimated by globally fitting experimental data (supplemental Fig. S7) to 1:1 binding model that included the mass transport component available within Scrubber.

* Binding data used to estimate $K_D$ values for HB3 and W2mef are shown in supplemental Fig. S8.

* ND, not determined.
It was of interest to explore whether the N-methyl groups nucleated local structure (as opposed to long range structure, which is effectively ruled out by the lack of extensive chemical shift changes). A confounding factor here is that the N-methyl groups give sharp, strong resonances, which are likely to detect inter-proton NOEs over longer distances than the parent backbone amide proton. For this reason we calculated structures for various N-methyl analogues in the presence and absence of NOEs to the N-methyl groups. The various families of calculated structures are shown in the supplemental material (Figs. S3–S6). In essence, the structures of N-methyl analogues calculated without N-methyl NOEs were similar to the structure of R1, but inclusion of N-methyl NOEs and two long range NOEs in the structure calculations for N-Me-Leu-8 caused an apparent stabilization of structure in the vicinity of the N-methyl group, resulting in a more compact global structure. However, because corresponding long range NOEs were not observed in N-Me-Val-1/N-Me-Leu-8/N-Me-Ser-14, its structure was similar to that of R1.

**Proteolytic Stability of N-Methylated R1 Derivatives**—An important property of bioactive peptides is their resistance to proteolytic degradation. To determine whether the N-methyl substitutions that increase affinity and bioactivity can also augment resistance of the peptide to serum proteolysis, peptides incorporating either a single N-methyl amino acid (Val-1 or Leu-8) or multiple N-methyl groups were tested for their stability in serum (Fig. 6). It was evident that the native R1 peptide and the N-Me-Val-1 peptide were rapidly degraded and after 10 min only ~20% of the starting peptide remained. However, addition of a single methyl group at Leu-8, or the inclusion of three methyl groups (Val-1, Leu-8, and Ser-14) resulted in a dramatic improvement in pep-
that the introduction of a methyl group into a peptide backbone largely restricts local conformation, it is possible that decreased flexibility within this region is responsible for the loss of activity observed. Perhaps a flexible core is required for the two regions of well defined structure (residues 5–10 and 13–17) to interact upon binding to AMA1. Indeed, amide exchange data has indicated that this may be the case (13), lending support to this theory. It should also be noted that the introduction of a methyl group removes hydrogen bonding potential at a given position, and this may also contribute to a loss of activity. Nevertheless, it is clear that methylation at a single site within R1 can dramatically alter the affinity of the peptide for AMA1.

The R1 peptide has been reported to be an effective inhibitor of malaria parasite invasion, and the impact of N-methylation on invasion inhibition was examined. In general, the AMA1 binding capacity of N-methylated analogues correlated well with their inhibitory properties (Fig. 3). For example, N-Me-Lys-11 did not inhibit parasite growth, whereas N-Me-Leu-8 showed increased inhibition. Thus, improved AMA1 binding ability translated to superior inhibitory properties. To investigate if the effects of N-methylation were additive, peptides with a methyl group added to the backbone amide at multiple positions were synthesized. Both double and triple methyl substitutions resulted in an increase in AMA1 affinity, as determined by SPR (Table 2), and growth inhibitory ability (Fig. 4B) as compared with the N-Me-Leu-8 peptide indicating that combining single methylations with the desired properties can further improve peptide characteristics.

Although the binding site recognized by native R1 has not yet been described, the peptide is known to compete with the inhibitory mAbs 1F9 and 4G2dc1, as well as with the inhibitory peptide F1 (13). This suggests that these molecules all interact with a similar AMA1 binding site, thus identifying a hot-spot on the antigen that appears to be the target of inhibitory molecules. The crystal structure of domains I and II of \textit{P. falciparum} AMA1 has recently been determined (49). One important feature of this structure was the presence of a conserved hydrophobic pocket surrounded by loops incorporating a high density of polymorphic residues. It has been postulated that this groove may represent an important functional site that is shielded from the immune system by the surrounding polymorphic residues. Interestingly, the crystal structure of AMA1 in complex with the inhibitory mAb 1F9 has indicated that the mAb 1F9 epitope is located at one end of the hydrophobic pocket, whereas mutational analysis suggests that residues involved in mAb 4G2dc1 recognition of AMA1 may be located near the opposite end of this conserved trough (50–53). Because R1 peptide competes with both of these mAbs for binding to AMA1, it is conceivable that the peptide is targeting the conserved pocket between these two sites and is thus able to interfere with the binding of each mAb, despite their apparently distinct binding sites. Such a scenario is in general agreement with the findings of other published binding studies indicating that small molecule binders are better able to access grooves than antibodies, which often exert their effects by occluding, rather than directly interacting with, binding pockets (54, 55). Furthermore, NMR studies have revealed a common feature of the R1 and F1 peptides is the clustering of hydrophobic residues
within turn-like conformations consistent with the ability of these peptides to probe a hydrophobic site (13, 56). Mutational studies are currently underway to investigate this hypothesis and confirm that the hydrophobic trough is the target of inhibitory peptides. This highly conserved trough represents an important target for the design of anti-malarial therapeutics, because it is probable that the fitness of escape mutants will be limited by a requirement to maintain function.

The polymorphic nature of AMA1 is another factor requiring consideration in the investigation of this antigen as a therapeutic target. The native R1 peptide has been shown previously to interact with the 3D7 and D10 strains of AMA1, but no binding to AMA1 derived from the HB3 or W2mef parasite lines was observed. When we examined the strain specificity of R1 analogues, the peptides incorporating multiple N-methyl groups exhibited a slightly increased affinity for the HB3 and W2mef allelic forms of AMA1, as compared with the native peptide (Table 2). This suggests that, with further modification, it may be possible to circumvent AMA1 allelic variation altogether by optimizing access to the conserved region of the putative binding site.

Because R1 adopts an extended structure with only two regions of local structure, it is likely that N-methyl substitution causes some local changes in the mix of interconverting conformations, but no global structural rearrangement. Given that the surface trough on AMA1 likely to be targeted by R1 is non-polar, the effect of N-methylation on affinity may reflect a balance between the enhanced hydrophobicity and the ability of the introduced N-methyl group to fit into the trough without steric clashes. It is feasible then that the introduction of methyl groups at some positions improves the ability of the R1 peptide to interact with the hydrophobic pocket, and that the resulting increase in affinity reduces the reliance upon surrounding polymorphic residues, facilitating improved binding to AMA1 derived from different parasite lines. Data on the site-specific effects of N-methylation will be valuable in assessing any model of how R1 might occupy this trough.

Previous work has indicated that methylation can also result in resistance to proteolytic degradation. For example, methylation of a peptide that prevents dimerization of an human immunodeficiency virus, type 1 protease has been shown previously to result in a dramatic improvement in resistance to pepsin (57). We therefore tested a number of methylated R1 analogues for their stability in serum (Fig. 6). It was clear that the presence of a single methyl group at Leu-8, as well as multiple methylation at Leu-8, was able to dramatically improve the amount of intact peptide available after 80 min in serum. This is a significant finding, because poor bioavailability represents a limitation to the use of peptides as therapeutics. Although there are some peptides in clinical use, rapid breakdown often necessitates administration of high doses. For example, T-20, or enfuvirtide, is a 36-residue peptide that is currently in clinical use for human immunodeficiency virus, type 1 infection (58). However, in human trials high doses of T-20 (almost 200 mg/day) were required to achieve anti-viral effects comparable to those observed in vitro, despite much lower doses being effective in cell culture, contributing to the high cost of this drug and clearly indicating a requirement for improved bioavailability (46). Development of strategies to rapidly improve peptide stability will therefore be of value to a wide range of areas within peptide research.

This work has illustrated that the properties of a peptide (R1) that inhibits malaria parasite invasion of erythrocytes can be enhanced by systematic methylation of backbone amides. Importantly, N-methyl substitution of a single residue improved target binding affinity and bioactivity and dramatically enhanced peptide stability in mouse plasma. Furthermore, the incorporation of multiple N-methyl groups further increased target binding affinity and bioactivity. These data demonstrate that comprehensive N-methyl scanning of peptides offers the advantage of enabling bioactivity and proteolytic stability to be enhanced simultaneously. With the renewed interest in peptide therapeutics, this approach could be applied to many lead peptides to rapidly optimize a number of parameters relevant to development of an effective pharmaceutical.

Acknowledgments—We thank Robin Anders and Vince Murphy for provision of recombinant AMA1 and for stimulating discussions.

REFERENCES

1. Ridley, R. G. (2002) Nature 415, 686–693
2. Silvie, O., Franetic, J. F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., van Gemert, G. J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Plushcke, G., and Mazier, D. (2004) J. Biol. Chem. 279, 9490–9496
3. Triglia, T., Healer, J., Caruana, S. R., Hodder, A. N., Anders, R. F., Crabb, B. S., and Cowman, A. F. (2000) Mol. Microbiol. 38, 706–718
4. Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R., and Bannister, L. H. (2004) Infect. Immun. 72, 151–158
5. Anders, R. F., Cowdwerk, P. E., Edwards, S., Margett, M., Matthew, M. L., Pollock, B., and Pye, D. (1998) Vaccine 16, 240–247
6. Collins, W. E., Pye, D., Cowdwerk, P. E., Vandenberk, K. L., Galland, G. G., Sulzer, A. J., Kemp, D. J., Edwards, S. J., Coppel, R. L., Sullivan, J. S., Morris, C. L., and Anders, R. F. (1994) Antimicrob. Agents Chemother. 38, 3179–3183
7. Hehl, A. B., Lekutis, C., Grigg, M. E., Bradley, P. J., Dubremetz, J. F., Oerteg-Barria, E., and Boothroyd, J. C. (2000) Infect. Immun. 68, 7078–7086
8. Healer, J., Murphy, V., Hodder, A. N., Masciantonio, R., Gemmill, A. W., Anders, R. F., Cowman, A. F., and Batchelor, A. (2004) Mol. Microbiol. 52, 159–168
9. Hodder, A. N., Cowdwerk, P. E., and Anders, R. F. (2001) Infect. Immun. 69, 3286–3294
10. Kennedy, M. C., Wang, J., Zhang, Y. L., Miles, A. P., Chitsaz, F., Saul, A., Long, C. A., Miller, L. H., and Stowers, A. W. (2002) Infect. Immun. 70, 6948–6960
11. Dutta, S., Haynes, J. D., Barbosa, A., Ware, L. A., Snively, J. D., Moeh, J. K., Thomas, A. W., and Lanar, D. E. (2005) Infect. Immun. 73, 2116–2122
12. Kocken, C. H., van der Wel, A. M., Dubbeld, M. A., Narumi, D. L., van de Rijke, F. M., van Gemert, G. J., van der Linde, X., Bannister, L. H., Janse, C. W., Waters, A. P., and Thomas, A. W. (1998) J. Biol. Chem. 273, 15119–15124
13. Harris, K. S., Casey, J. L., Coley, A. M., Masciantonio, R., Sabo, J. K., Keizer, D. W., Lee, E. F., McMahan, A., Norton, R. S., Anders, R. F., and Foley, M. (2005) Infect. Immun. 73, 6981–6989
14. Li, F., Dluzewski, A., Coley, A. M., Thomas, A., Tilley, L., Anders, R. F., and Foley, M. (2002) J. Biol. Chem. 277, 50303–50310
15. Egleton, R. D., and Davis, T. P. (1997) Peptides 18, 1431–1439
16. Fairlie, D. P., Abbenante, G., and March, D. R. (1995) Curr. Med. Chem. 2, 654–686
17. Bergseng, E., Xia, J., Kim, C. Y., Khosla, C., and Sulzer, A. J. (2005) J. Biol. Chem. 280, 21791–21796
18. Ebata, M., Takahashi, Y., and Otsuka, H. (1966) J. Biol. Chem. Soc. Jpn. 39, 2535–2538
