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Affinity-Driven Selection of Tripeptide Inhibitors of Ribonucleotide Reductase

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Abstract—Tripeptide libraries of the type Fmoc(W/F)XF were screened for binding to the large subunit of mouse ribonucleotide reductase (mRR), using a new, affinity chromatography method. A high-affinity tripeptide, FmocWFF, was found that inhibited mRR activity with a K_i equal to that of AcFTLDADF, the heptapeptide corresponding to the C-terminus of the small subunit of mRR.

Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides to 2′-deoxyribonucleotides, the rate determining step in de novo DNA synthesis.[1] It is, thus, an important target for antiviral and cancer chemotherapeutic agents.[2,3] In mammalian RR, the active site and allosteric regulation sites are located in the mR1 subunit, while a tyrosyl radical, required for efficient substrate turnover, is located in the mR2 subunit. mRR activity is completely dependent on the association of mR1 and mR2. The carboxyl terminus of mR2 is critical for such association.[1,4] The *N*-acetylated peptide AcF[1]TLDADF[7] (P7), which corresponds to the C-terminal residues of mR2, inhibits mRR activity by competing with mR2 for association with mR1.[5] Structure–activity,[5–7] transfer NOE,[8,9] and modeling studies[7,10] have indicated that: (a) based on the mR2 C-terminus, P7 provides the minimum and complete length peptide necessary for mRR inhibition; (b) N-terminal acylation is required for high binding affinity; (c) P7 binding affinity to mR1 is strongly dependent on F^1 and F^7 interaction with the F1 and F7 subsites of mR1, respectively; (d) binding to mR1 displays stringent specificity for Phe at F^7, high specificity for Phe at F^1, and little specificity for the *N*-acyl group; (e) increased F^1 subsite interaction can offset lower F^7 subsite interaction; (f) FmocPhe and FmocLeu have surprisingly high binding affinity to mR1; and (g) Fmoc-tripeptides with the general structure FmocF^1XF^3 can be docked into a three-dimensional homology model of the mR2 C-terminal peptide binding site in mR1 with F^1 interacting at the F1 subsite and F^3 interacting at the F7 subsite.

The latter three points suggest that Fmoc-tripeptides of general structure FmocX_1X_2F could bind to mR1 and inhibit RR activity as well or better than P7. The present work uses a new, affinity chromatography-based screening method to test this suggestion.

Synthesis

Fmoc-single peptides and Fmoc- tripeptide sublibraries were synthesized manually using standard Fmoc solid-phase peptide synthesis methods.[11] All single peptides gave molecular ions in the mass spectrum consistent with their molecular masses. In addition, FmocWFF was purified to homogeneity by RP-HPLC[12] and had a ^{1}H NMR spectrum consistent with its structure. Synthesis of typical Fmoc(W/F)XF sublibraries (X position contains approximately equimolar amounts of Lys, Arg, His, Asp and Glu) was performed as follows. FmocPhe-Wang Resin was stirred in *N*-methyl pyrrolidine (NMP) containing 20% piperidine for 30 min, the resin was aliquoted into five vials, each containing one of the above side-chain protected Fmoc-amino acids[13] in 10-fold molar excess over resin, and coupling was initiated by adding 10-fold molar excesses each of HOBt, HBTU, and DIPEA into each vial. Completion of coupling was confirmed via the Kaiser test, the contents of all the vials were pooled, and Fmoc was removed with 20%

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piperidine in NMP. The pool was then divided in two for coupling with FmocPhe-OH and FmocTrp-OH, respectively. Fmoc-peptides were removed from the resin and side-chains deprotected with 95% aqueous TFA.

Screening

mR1[14] (5 mg), dialyzed against O_2-free phosphate buffer (pH 7.6), was coupled with CH Sepharose 4B (1 g) by incubation at 0–4 °C for ~5 h. Reaction completion was verified by leveling off of imidazole release (A_{260}). Residual active groups were blocked by reaction with 2 M glycine in buffer A (50 mM Tris–Cl, pH 7.6, 0.1 mM DTT) for 1 h at 0–4 °C.

The efficacy of the mR1 column in screening peptides was demonstrated with three peptides having known affinities for mR1, with relative K_ds rising in the order P7 (1.0) < AcFALDADF (denoted PA, 4) < AcFTLDAEF (denoted PE, 40)[7] (Fig. 1). A 3-fold molar excess of peptides (measured as Phe equivalents) was applied to the mR1 to ensure competitive binding of peptide to mR1. Demonstrating the validity of the method, the weakest binding component (PE) was most abundant on 0.1 M KCl elution, the intermediate binding component (PA) was most abundant on 0.2 M KCl elution, and the strongest binding component (P7) was eluted in virtually pure form at 0.5 M KCl.

To select X_1 in the target FmocX_1X_2F structure, the mR1 affinity column was next used to screen an approximately equimolar mixture of 10 Fmoc amino acids. As seen in Figure 2, Fmoc derivatives of Leu, Phe, Phg (phenylglycine) and Trp were enriched in the later eluting fraction (KCl = [0.5 M]). The relative K_d values of these four compounds (Table 1), determined using a modified form of the assay developed previously,[15] rise in the order FmocTrp < FmocPhe < FmocPhg < FmocLeu.

Based on the results with the Fmoc-amino acids six sublibraries of the type Fmoc(W/F)X_2F-OH were assayed for binding activity to mR1, giving results summarized in Table 1. Fmoc(W/F)X_2F showed highest binding affinity when the residues at position 2 had nonpolar side-chains (X_{2a}), and lowest affinity for residues with charged polar side-chains (X_{2c}). Furthermore, there is little difference in binding affinity with either Phe or Trp in position 1.

The strongest binding sublibrary, FmocWX_{2a}F, supplemented by addition of FmocWGF, which was synthesized separately, was next screened with the mR1 affinity column. A 20-fold excess of total peptides was applied to ensure competitive binding.[16] As the tripeptides show some affinity for the Sepharose column itself, full elution of the most tightly bound components necessitated conditions (30% TFA) resulting in mR1 denaturation. The results (Fig. 3) show the most enriched component in the final fraction to be FmocWFF, which we denote P3. Suitable control experiments with a glycine-blocked CH Sepharose 4B column, not containing mR1, showed some binding of all peptide components, but no selective retention of P3.

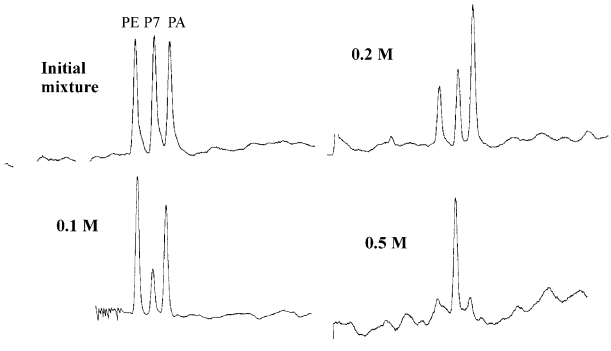

Figure 1. RP-HPLC of three peptide mixture screened with an mR1 affinity column. Peptides, identified by ESI Mass spectra, are eluted from the affinity column with the indicated KCl concentrations in buffer A.

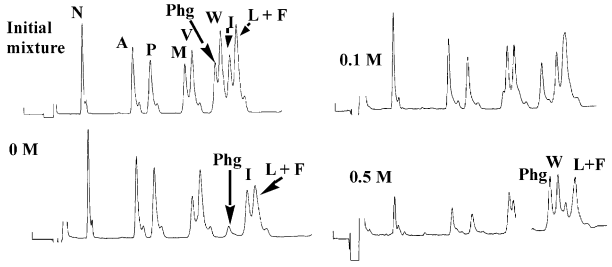

Figure 2. RP-HPLC of 10 Fmoc amino acid mixture screened with an mR1 affinity column. Fmoc amino acids, identified by comparison of elution time with authentic samples, are eluted from the affinity column with the indicated KCl concentrations in buffer A.

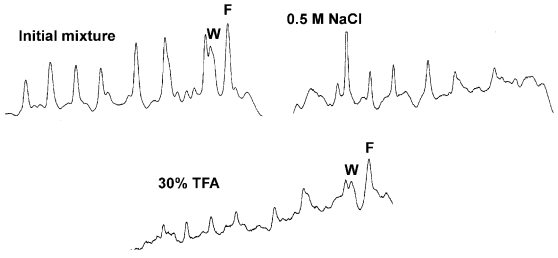

Figure 3. RP-HPLC of the FmocWX_{2a}F sublibrary screened with an mR1 affinity column. Peptides, identified by comparison of elution time with authentic samples, are eluted from the affinity column as indicated.

Table 1. Relative dissociation constants for mR1[a]

mR1 ligand	Rel. K_d	mR1 ligand	Rel. K_d
P7	(1.0)	Fmoc$FX_{2a}F^b$	1.9 ± 0.8
FmocTrp	18 ± 2	Fmoc$WX_{2b}F^c$	4.4 ± 0.6
FmocPhe	22 ± 1	Fmoc$FX_{2b}F^c$	3.5 ± 0.6
FmocPhg	31 ± 9	Fmoc$WX_{2c}F^d$	14 ± 2
FmocLeu	38 ± 2	Fmoc$FX_{2c}F^d$	> 20
Fmoc$WX_{2a}F^b$	1.8 ± 0.3	FmocWFF (P3)	1.0 ± 0.1

[a]All concentrations based on equivalents of Fmoc, except for P7.
[b]X_{2a} contains ~equimolar amounts of A, V, L, I, M, P, F and W.
[c]X_{2b} contains ~equimolar amounts of S, T, N, Q and Y.
[d]X_{2c} contains ~equimolar amounts of K, R, H, D and E.

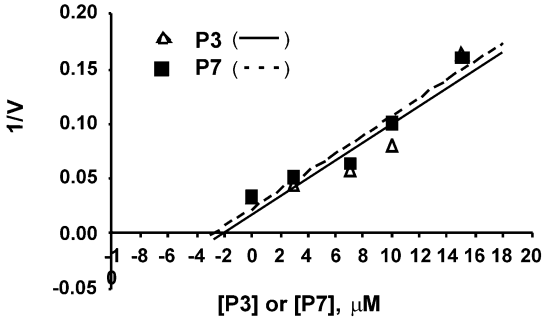

Figure 4. Dixon plots of P3 and P7 inhibition of mRR enzymatic activity.

P3 was synthesized separately and tested for binding affinity to mR1 (Table 1). Other members of the $FmocWX_{2a}F$ sublibrary were also synthesized separately. Preliminary results showed that, except for FmocWGF and FmocWAF, all of these compounds had binding affinities only slightly weaker than that of P3 (within a factor of $\sim$2), accounting for the results found for $FmocWX_{2a}F$ in Table 1. We conclude that, within the $FmocWX_2F$ sublibraries, it is important for affinity that X_2 be large and hydrophobic, but its precise identity is not critical.

That binding to mR1 correlates very well with inhibition of ribonucleotide reductase (mRR) activity[7] is shown by the Dixon plots presented in Figure 4. Direct demonstration of this point is essential, since the large size of mR1 (90 kDa) raises the possibility of a false positive in the binding assay. As can be seen, P3 is as potent an inhibitor of mRR as is P7 (K_i, 3 μM), paralleling exactly the similarity in their binding affinities to mR1 (Table 1).

In summary, here we demonstrate the utility of a new method that screens peptide libraries for binding to mR1. Screening 38 members of the library Fmoc(W/F)X_2F yields P3 as a tightly bound ligand having an affinity for mR1 and a K_i for inhibition of mRR activity essentially identical to that of the much longer peptide, P7, which is based on the C-terminal sequence of mR2. The relatively small size of P3, and its strongly aromatic character makes it a promising lead structure for the development of even higher affinity inhibitors of mRR, both peptide and peptidomimetic, with the eventual goal of developing a specific therapeutic inhibitor of mammalian RR.

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References and Notes

1. Jordan, A.; Reichard, P. *Annu. Rev. Biochem.* **1998**, *67*, 71.
2. Szekeres, T.; Fritzer-Szekeres, M.; Elfore, H. L. *Crit. Rev. Clin. Lab. Sci.* **1997**, *3*, 503.
3. Robins, M. J. *Nucleosides Nucleotides* **1999**, *18*, 779.
4. Stubbe, J.; van der Donk, W. *Chem. Biol.* **1995**, *2*, 793.
5. Yang, F. D.; Spanevello, R. A.; Celiker, I.; Hirschman, R.; Rubin, H.; Cooperman, B. S. *FEBS Lett.* **1990**, *272*, 61.
6. Fisher, A.; Yang, F. D.; Rubin, H.; Cooperman, B. S. *J. Med. Chem.* **1993**, *36*, 3859.
7. Pender, B. A.; Wu, X.; Axelsen, P. H.; Cooperman, B. S. *J. Med. Chem.* **2001**, *44*, 36.
8. Fisher, A. L.; Laub, P. B.; Cooperman, B. S. *Nat. Struct. Biol.* **1995**, *2*, 951.
9. Pellegrini, M.; Liehr, S.; Fisher, A. L.; Laub, P. B.; Cooperman, B. S.; Mierke, D. F. *Biochemistry* **2000**, *39*, 12210.
10. Pender, B. A. PhD thesis, University of Pennsylvania, Philadelphia, PA, 2000.
11. *Catalog and Peptide Synthesis Handbook*; Calbiochem-Novabiochem Corp, 1999; p S3.
12. Peptides were purified and peptide mixtures analyzed using a Vydac C_{18} column and a linear gradient of ACN in 0.1% TFA, with monitoring at either 215 nm (Figs. 1 and 3) or 264 nm (Fig. 2). The elution times shown (and % ACN ranges) were 10–30 min (20–30%), 0–35 min (32–42.5%) and 15–48 min (39–47%) for Figures 1, 2, and 3, respectively.
13. Fmoc-Arg(Pbf)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH.
14. Scott, C. P.; Kashlan, O. B.; Lear, J. D.; Cooperman, B. S. *Biochemistry* **2001**, *40*, 1651.
15. The assay reported previously[7] measured the ability of added ligands to prevent mR1 binding to a P7 affinity column, with protein determined by Bradford assay. As the Fmoc group interferes with assay, protein was determined according to Schaffner and Weissmann.[17]
16. A somewhat different affinity column loading procedure was employed in this case. mR1-Sepharose was equilibrated in a MicroSpin column with the peptide sublibrary in buffer A (5 min at 4 °C), spun gently for 30 s, and the eluate was reloaded onto the mR1 column. Five reload–spin cycles were performed. The column was then eluted as indicated in Figure 3.
17. Schaffner, W.; Weissmann, C. *Anal. Biochem.* **1973**, *56*, 502.