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More potent linear peptide inhibitors of mammalian ribonucleotide reductase

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Abstract—Mammalian ribonucleotide reductase (mRR) is a chemotherapeutic target. The enzyme is composed of two subunits (mR1 and mR2) and is inhibited by Ac-FTLDADF (denoted P7), corresponding to the C-terminus of mR2, which disrupts mRR quaternary structure by competing with mR2 for binding to mR1. The tripeptide FmocWFF acts similarly. Here we report on the use of small, focused libraries to identify Fmoc derivatives of tetra and hexapeptides having comparable or considerably higher activities than P7 toward inhibition of mRR.

Ribonucleotide reductases (RRs) form a family of allosterically regulated enzymes that catalyze the conversion of ribonucleotides to 2′-deoxyribonucleotides and are essential for de novo DNA biosynthesis.[1] As such, RR is a well-recognized target for cancer chemotherapeutic and antiviral agents.[2–5] Class Ia RRs, which comprise all eucaryotic RRs as well as some from eubacteria, bacteriophages, and viruses, accept the four common nucleoside diphosphates (NDPs) as substrates, with enzymatic activity dependent upon the formation of a complex between two different subunits, R1 and R2. The R2 subunit contains a stable tyrosyl free radical that is necessary for NDP reduction at the active site, which is located within R1.[6] The mammalian form of the enzyme has two active oligomerization states, $mR1_2$ $mR2_2$, and $mR1_6$ $(mR2_2)_3$, depending on allosteric ligand concentration.[7]

Mammalian RR (mRR) is inhibited by the acetylated form of the mR2 C-terminal peptide, N-AcFTLDADF, denoted P7, which competes with mR2 for binding to mR1 and has been considered as the minimal pharmacophore for mRR[8,9] that targets quaternary structure disruption. NMR and modeling studies of peptide binding[9,10] have led to the notion that mR2 C-terminal peptide binding occurs to two contiguous subsites, F^1 and F^7, in mR1. The F^1 subsite, which accomodates the N-terminal portion of the peptide, is broad, shallow, and hydrophobic and, as a consequence, is not strongly sequence specific. By contrast, the F^7 subsite, which accomodates the C-terminal portion, is narrow and deeper, and shows very high specificity for the ultimate C-terminal residue Phe.

We are engaged in an ongoing effort to develop selective peptide and peptidomimetic inhibitors of mRR with higher inhibition potency and shorter peptide length than P7 as potential cancer chemotherapeutics. Recently, building on the observation that the Fmoc group confers substantial affinity for peptide binding to mR1, presumably via binding to subsite F^1, we identified the derivative FmocWFF, from within a targeted tripeptide library, as having the highest binding affinity to mR1 and inhibitory potency toward mRR, with both activities being similar to those observed with P7.[11]

Abbreviations: Ac_5c, 1-aminocyclopentanoic acid; Ac_6c, 1-aminocyclohexanoic acid; Aib, α-aminoisobutyric acid; Cha, β-cyclohexylalanine; CcypG, α-(1-carboxycyclopentyl)glycine; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; HSV-R1, large subunit of herpes simplex ribonucleotide reductase; HSV-R2, small subunit of herpes simplex ribonucleotide reductase; mRR, mammalian ribonucleotide reductase; mR1, large subunit of mammalian ribonucleotide reductase; mR2, small subunit of mammalian ribonucleotide reductase; Phg, phenylglycine; RR, ribonucleotide reductase.

Keywords: Ribonucleotide reductase; Quaternary structure; Peptide inhibitors; Focused libraries.

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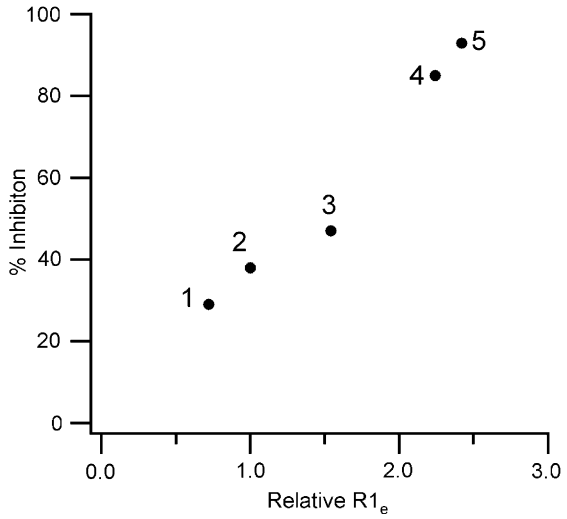

Figure 1. Correlation of mR1 elution and mRR activity inhibition results. The percent inhibition of mRR activity in the presence of 10 μM inhibitor is plotted versus the relative amount (P7 = 1.00) of mR1 eluted from the peptide affinity column by 50 μM inhibitor for five peptides: **1**. FmocWFDF; **2**. P7; **3**. FmocWFVF; **4**. Fmoc(NCH_3)FLDFDF; **5**. Fmoc(NCH_3)FLDChaDF. Eluted mR1 increases as a function of peptide affinity for mR1. The differences in peptide concentration utilized in the two assays reflects stronger competition for binding to mR1 by the affinity column than by $mR2_2$ under the conditions employed in each assay.

Here we report the identification of Fmoc derivatives of tetrapeptides and hexapeptides[12] having comparable or significantly higher inhibitory activities, respectively, than P7. Screening of the peptide libraries was carried out using the affinity column assay,[9] which measures the ability of a test compound to compete with FTL-DADF-Sepharose for binding to mR1, and/or by inhibition of dTTP-dependent GDP reductase catalyzed by $mR1_2$ $mR2_2$.[14] The two methods give results that generally correlate well with one another (Fig. 1).

Fmoc tetrapeptide libraries. Using FmocWFF as a starting point, we first examined tetrapeptide libraries of the types FmocWXFF (**1**), FmocWFXF (**2**) (Table 1), and $FmocWX_2X_3F$ (**1**:**2**, Table 2) by the affinity column assay. Here the amount of mR1 eluted by a single concentration of test peptide, relative to the amount eluted by the lead compound P7, provides a measure of the relative binding strength of the peptide to the P7 binding site of mR1, although measurement at a single peptide concentration does not permit calculation of a relative binding constant. It is an interesting aspect of tetrapeptide and P7 interaction with mR1 that, for the more active tetrapeptides, greater differentiation in relative activity is observed at higher peptide concentrations.[16]

The results make clear that hydrophobic residues are preferred at both positions 2 and 3, with selectivity following the order V,L > F > I,Y in library **1** and V > Y,L,M in library **2**, and that comparable affinities are obtained when two amino acids with aliphatic side chains are placed at these positions as compared with one aliphatic amino acid and one Phe. Also, although charged residues give generally poor binding in both libraries, Asp and Glu are better tolerated in position 3 than position 2.

We next examined library **3**, FmocWFVX, to determine preferences at the C-terminus. Phe is the preferred residue, but Met, Tyr, and Trp also confer high affinity. This is a major difference with N-acetylated heptapeptides, which display very high specificity for a C-terminal Phe. It indicates that tetrapeptide binding to $mR1_2$ is primarily directed toward the F^1 subsite and has little or no interaction with the F^7 subsite. Lastly, we examined library **4**

Table 1. Relative amount of mR1 eluted by single substitution tetrapeptide libraries[a]

X	**1**-FmocWXFF[b]	**2**-FmocWFXF[c]	**3**-FmocWFVX[c]	**4**-FmocWXDF[b]
A	1.26 ± 0.02	0.82 ± 0.04	0.93 ± 0.03	0.52 ± 0.01
D	0.27 ± 0.03	0.69 ± 0.05	0.37 ± 0.02	0.12 ± 0.09
E	0.21 ± 0.01	0.71 ± 0.07	0.37 ± 0.01	ND
F	**2.32 ± 0.10**	ND[d]	**1.48 ± 0.10**	**1.05 ± 0.01**
G	1.04 ± 0.06	ND	0.88 ± 0.06	0.31 ± 0.01
H	0.56 ± 0.06	0.27 ± 0.02	0.49 ± 0.06	0.36 ± 0.02
I	**2.10 ± 0.10**	0.53 ± 0.07	0.98 ± 0.03	0.61 ± 0.01
K	0.40 ± 0.02	0.16 ± 0.01	0.14 ± 0.02	0.11 ± 0.03
L	**2.50 ± 0.08**	0.92 ± 0.05	0.93 ± 0.12	0.63 ± 0.01
M	1.60 ± 0.04	0.92 ± 0.06	**1.39 ± 0.08**	0.37 ± 0.03
N	1.83 ± 0.07	0.79 ± 0.04	0.67 ± 0.11	0.36 ± 0.02
P	0.21 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.22 ± 0.02
Q	1.81 ± 0.10	0.15 ± 0.01	ND	0.49 ± 0.20
R	0.27 ± 0.02	0.23 ± 0.01	ND	0.09 ± 0.03
S	0.97 ± 0.02	0.66 ± 0.01	0.79 ± 0.01	0.07 ± 0.01
T	1.70 ± 0.05	0.74 ± 0.07	0.89 ± 0.08	0.15 ± 0.01
V	**2.63 ± 0.10**	**1.48 ± 0.10**	1.00 ± 0.07	0.83 ± 0.01
W	0.71 ± 0.02	0.74 ± 0.08	**1.23 ± 0.04**	**0.95 ± 0.01**
Y	2.08 ± 0.05	0.94 ± 0.06	**1.37 ± 0.02**	0.54 ± 0.01

[a] P7 = 1.00; results indicating tight binding are in bold.
[b] At 100 μM compound.
[c] At 50 μM compound.
[d] Not determined.

Table 2. Relative amount of mR1 eluted for Library 1:2, FmocWX_2X_3F[a]

X_2	X_3					
	E	F	L	N	Y	V[b]
L	0.62 ± 0.05	**1.36 ± 0.04**	**1.20 ± 0.06**	**1.21 ± 0.03**	0.88 ± 0.08	**2.63 ± 0.02**
N	0.83 ± 0.04	**1.19 ± 0.16**	0.93 ± 0.01	0.60 ± 0.08	0.64 ± 0.05	0.89 ± 0.06
Q	0.60 ± 0.09	1.03 ± 0.13	0.73 ± 0.09	0.77 ± 0.05	0.64 ± 0.01	1.11 ± 0.02
T	0.66 ± 0.03	0.71 ± 0.01	0.63 ± 0.09	0.92 ± 0.01	0.64 ± 0.03	0.89 ± 0.06
V	0.47 ± 0.01	**1.34 ± 0.07**	0.90 ± 0.09	**1.38 ± 0.07**	0.94 ± 0.15	**2.46 ± 0.01**
Y	0.47 ± 0.02	1.00 ± 0.25	0.44 ± 0.01	0.50 ± 0.14	0.45 ± 0.01	1.14 ± 0.01

[a] P7 = 1.00; at 50 μM compound except as otherwise indicated; results indicating tight binding are in bold.
[b] P7 = 1.00; at 100 μM compound.

(FmocWXDF, Table 1). Here we had two motivations. First, peptides with—DF in the C-terminal positions mimic the C-terminal residues of P7 and could perhaps bind to both the F^1 and F^7 subsites. Second, inclusion of an Asp residue in the tetrapeptide markedly increases solubility in neutral aqueous solutions, which is desirable for testing in a variety of in vitro assays. FmocWFDF and FmocWWDF are seen to have the highest activities.

Fmoc peptides corresponding to the C-terminus of mR2. Peptides based on replacing the *N*-acetyl group of P7 with an Fmoc group were next investigated, giving results summarized in Table 3. FmocFTLDADF binds to mR1 somewhat more strongly than does P7. In library **5** we investigated the effects of deleting residues from this sequence, while maintaining the C-terminal Phe. Initial results showed that the Fmoc-heptapeptide derivative bound best, as in the case with the *N*-acetyl derivatives,[8] although it was also clear that removal of Thr at position 2 resulted in only a modest decrease in binding to mR1. By contrast, deletion of Asp at position 6 led to a large decrease in binding.

The result for FmocFLDADF was first followed up by examining library **6** to determine whether higher affinity hexapeptides could be identified through optimization at position 4. Large hydrophobic groups give the best results at this position, with the Fmoc-hexapeptides FmocFLDChaDF and FmocFLDFDF showing affinities for mR1 comparable to FmocFTLDADF. Next, we examined the effect of N-methylation of the N-terminal amino acid residue, based on results of Moss et al.[17] showing that such modification increases peptide affinity for the HSV-R2 C-terminal peptide binding site of HSV-mR1. A preliminary result showed that terminal N-methylation strengthened the binding of two hexapeptides but weakened that of two heptapeptides (Table 3). As a result, a more comprehensive examination of terminal N-methylation for hexapeptides was carried out (library **7**), placing Cha at position 4 based upon the results of library **6**. Here, the highest affinity was found for N-MePhg, although N-MePhe and N-MeCha also had high affinity, with all three compounds displaying binding activities considerably superior to that of FmocFTLDADF.

Table 3. Relative amount of mR1 eluted for modifications of FmocFTLDADF[a]

FmocFTLDADF	1.27 ± 0.03[b]	X	**6**-FmocFLDXDF[b]	**7**-Fmoc(N-Me) XLDChaDF[c]
5A: *Deletion from N-terminus*[b]		Ac_5c	0.67 ± 0.02	ND
FmocADF	0.09 ± 0.01	Ac_6c	0.78 ± 0.07	ND
FmocDADF	0.14 ± 0.02	Aib	0.52 ± 0.07	ND
FmocLDADF	0.22 ± 0.01	Ala	0.86 ± 0.03	1.67 ± 0.02
FmocTLDADF	0.45 ± 0.01	Asn	0.62 ± 0.03	1.66 ± 0.03
5B: *Interior deletions*[b]		Asp	0.07 ± 0.01	ND
FmocFDF, delete 2–5	0.24 ± 0.02	Cha	**1.26 ± 0.01**	**2.11 ± 0.09**
FmocFTF 3–6	0.20 ± 0.02	Gly	0.64 ± 0.04	1.54 ± 0.02
FmocFADF 2–4	0.44 ± 0.03	Ile	ND	1.51 ± 0.01
FmocFTLF 4–6	0.14 ± 0.01	Leu	ND	1.30 ± 0.03
FmocFDADF 2,3	0.24 ± 0.02	Met	ND	1.34 ± 0.07
FmocFTLDF 4,5	0.30 ± 0.02	Phe	**1.12 ± 0.05**	**2.15 ± 0.04**
FmocFLDADF 2	**0.82 ± 0.12**	Phg	0.67 ± 0.06	**2.51 ± 0.08**
FmocFTLDAF 6	0.18 ± 0.01	Ser	ND	1.08 ± 0.02
Fmoc(N-Me)Hexa/hepta-peptides[b]		Tyr	ND	1.48 ± 0.03
Fmoc(NCH_3)ALDADF	**1.26 ± 0.08**	Val	0.98 ± 0.03	ND
Fmoc(NCH_3)FLDADF	**1.37 ± 0.02**			
Fmoc(NCH_3)ATLDADF	0.28 ± 0.02			
Fmoc(NCH_3)FTLDADF	0.68 ± 0.06			

[a] Results indicating tightest binding are in bold.
[b] P7 = 1.00; at 50 μM compound.
[c] P7 = 1.00; at 100 μM compound.

Table 4. IC_{50} values

Compound	IC_{50} (μM)
AcFTLDADF (P7)	13.3 ± 0.7
Fmoc(N-CH_3)PhgLDChaDF	1.9 ± 0.3
Fmoc(N-CH_3)PheLEChaDF	2.4 ± 0.4
Fmoc(N-CH_3)PheLDChaDF	2.4 ± 0.4
FmocWFDF	22 ± 3
FmocWVFF	20 ± 2

Some 70 additional Fmoc-hexapeptides were examined by affinity column or enzyme activity assay in an attempt to further enhance hexapeptide binding activity. These peptides contained the following amino acids at positions 1–6, with those underlined present in at least half of the peptides tested. 1: E, F, N, P, V, Ac_5c, N-MeF, N-MePhg, Phg; 2: E, F, L, N, V, Ac_5c, N-MeL, Phg; 3: D, E, F, H, K, N, Q, R, S, V, Y, Ac_5c, Aib, 4-BrF, CcypG, N-MeD, Phg; 4: F, Cha, N-MeCha; 5: D, CcypG, N-MeD; 6: F, N-MeF. The testing of CcypG at the 5-position was again prompted by the results of Moss et al.,[17] in this case showing that substitution of CcypG for Asp as the penultimate residue led to a marked increase affinity for peptide binding to HSV-R1. However, such substitution led to decreased affinity for mR1, as did virtually all of the other substitutions examined. Substitution of Glu for Asp at position 3 maintained, but did not enhance, affinity.

IC_{50} values. IC_{50} values in a standard dTTP-dependent GDP reductase assay for the most promising compounds described above are presented in Table 4, demonstrating clearly that the best tetrapeptides have potencies similar to that of the heptapeptide lead molecule, P7, whereas the most potent hexapeptide, Fmoc(N-Me)PhgLDChaDF, has an IC_{50} value some seven times lower than that of P7, corresponding to a dissociation constant to mR1 of 310 nM,[16] and is the most potent peptide inhibitor of mRR yet described. These results demonstrate the value of using small, focused libraries to identify inhibitors of target enzymes. Efforts have begun to test the efficacy of some of the molecules in Table 4 as inhibitors of cell proliferation.

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12. Peptides were synthesized with a 96-well manual parallel synthesizer (Robin Scientific) using standard Fmoc solid phase peptide synthesis methodology[13] on a Wang resin. All Fmoc amino acids, 1-hydroxybenzotriazole, HBTU, *N*,*N*′-diisopropylethylamine, *N*-methylpyrrolidone, and trifluoroacetic acid were purchased from Advanced ChemTech. Each coupling step employed 6 equiv of Fmoc mino acid in the presence of 6 equiv of 1-hydroxybenzotriazole, HBTU, and *N*,*N*′-diisopropylethylamine in *N*-methylpyrrolidone, with a reaction time of 3 h (rt). Completion of coupling was confirmed using the Kaiser test. All peptides gave the expected peaks on ESI mass spectral analysis (both $M+H^+$ and $M+Na^+$). Crude peptides, which were generally $\geqslant$85% pure as assessed by analytical reverse-phase HPLC (Synchropak, C18 column, Micra Scientific Inc.), were used directly in the binding assay. Those peptides showing activities greater than that of P7 as crude preparations were purified by preparative RP-HPLC, giving final purities $\geqslant$ 97%, as assessed by analytical HPLC. These purified peptides were used in both the binding and activity assays reported. ^{1}H NMR spectra for P4-FF and Fmoc(NCH_3)FLDFDF were fully consistent with the assigned structures. Peptide concentrations were determined by UV absorption. Sample values are: P7, ε_{256} = 356; FmocWFVF, ε_{264} = 20,600; Fmoc(N-Me)PhgLDChaDF, ε_{264} = 17,500.
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