R2 C-Terminal Peptide Inhibition of Mammalian and Yeast Ribonucleotide Reductase

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Eucaryotic ribonucleotide reductases (RR) catalyze the reduction of ribonucleoside diphosphates to 2′-deoxyribonucleoside diphosphates. Each has an $R1_2R2_2$ quaternary structure with each subunit playing a critical role in catalysis. Separation of the subunits results in loss of activity. Previous studies have demonstrated that peptides corresponding to the C-terminus of R2 disrupt subunit association by competion with R2 and have potential usefulness as therapeutics. Extensive structure–function studies have been carried out on peptide inhibition of herpes simplex RR in an effort to develop antiviral agents based on the observation that the herpes simplex R2 C-terminus, YAGAVVNDL, is quite different from the corresponding mammalian sequence. In this work we report a detailed structure–function analysis of peptide inhibition of mammalian and, to a more limited extent, *Saccharomyces cerevisiae* RRs. Our results for mammalian RR support the following conclusions with regard to the effect of substitution on inhibitory potency: (a) the N-acetylated R2 C-terminal heptapeptide N-AcPhe^{384}Thr^{385}Leu^{386}Asp^{387}Ala^{388}Asp^{389}Phe^{390} (N-AcF^{7}TLDADF^{1}) is the minimal core peptide length required; deletion of the N-terminus or of middle positions (resulting in penta- and hexapeptides) results in large losses in inhibitory potency; (b) a free carboxylate is required on the C-terminal Phe; (c) Phe is strongly preferred to Leu in positions 1 and 7 and a bulky aliphatic group is preferred in position 5; (d) neither negative charge in positions 2 or 4 nor a polar side chain in position 6 are required for peptide binding, contrary to what evolutionary patterns in the R2 C-terminus of RR would suggest. *S. cerevisiae* RR displays a similar length dependence on the corresponding N-acetylated R2 C-terminal heptapeptide, N-AcFTFNEDF. This peptide has a 4-fold higher inhibitory potency toward *S. cerevisiae* RR than toward mammalian RR. Such selectivity raises the possibility that peptide analogs related to R2 C-termini can be developed as therapeutic agents even against organisms having R2 C-terminal sequences similar to that of mammalian RR.

Introduction

Ribonucleotide reductase (RR) catalyzes reduction of ribonucleotides to 2′-deoxyribonucleotides, an essential step for *de novo* synthesis of DNA. Eucaryotic RRs, which are the focus of this paper, in common with viral and *E. Coli* RRs, use nucleotide diphosphates as substrates and have an $R1_2R2_2$ quaternary structure. Enzyme activity is contingent upon association between the R1 and R2 dimers ($K_a \sim 0.5\text{–}1 \times 10^7$ M^{-1})[1,2] and is inhibited when the C-terminal peptide of R2 competes with intact R2 for association to R1.[1-7] Structure–function studies indicate that the inhibitory potency is critically dependent on the size (7–20 residues for RRs from different organisms) and sequence of the terminal peptide.[8,9] A considerable effort has been devoted to developing antiviral agents by exploiting peptide inhibition of herpes simplex RR,[10,11] based on the observation that the herpes simplex R2 C-terminus, YAGAVVNDL, differs substantially from the corresponding mammalian sequence.

Eucaryotic R2s are highly homologous to one another up to seven residues from the C-terminus, at which point the homology is lost (Table I). We earlier demonstrated[5] that the heptapeptide N-AcFTLDADF, corresponding to the C-terminus of mouse R2, would inhibit mammalian RR. Acetylation is necessary to neutralize the positive charge on the α-amino group, which otherwise interferes with binding. Here we extend this observation by determining how the inhibitory potencies of a series of mammalian R2 C-terminal peptide analogs depend on size and sequence and whether even the slight differences in the C-terminal sequences of *Saccharomyces cerevisiae* vs mammalian R2 can be exploited in developing peptides that show selectivity in the inhibition of the corresponding RR activities. Such selectivity could have important implications for the design of therapeutics targeted toward infectious organisms.

Table I. Homologous R2 C-Terminal Sequences

organism	sequence[a]	organism	sequence[a]
mouse	FTLDADF	*Saccharomyces cerevisiae*	FTFNEDF
clam	FTLDADF	*Schizosaccharomyces pombe*	FTIDEDF
human	FTLDADF	*Plasmodium falciparum*	FCLNTEF
vaccinia	FSLDVDF		

[a] R2 C-terminal sequences extend from Phe^{384} to Phe^{390}, numbered according to the mouse sequence. Sequences may be found in the following references: clam, mouse, vaccinia, and *S. cerevisiae*, ref 23; human, ref 24; *S. pombe*, ref 25; and *P. falciparum*, ref 15.

Results and Discussion

The results of experiments measuring the relative inhibitory potencies (RIs) of mammalian R2 C-terminal peptide analogues are summarized in Table II. RIs of the first seven peptides (1–7) confirm the importance of the C-terminal heptapeptide for binding to the R1 subunit. RIs of the *N*-acetyl octa- and nonapeptides are both 1 (1, 2), showing a lack of effect on binding to R1 of increasing peptide length past the seventh position from the C-ter-

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Table II. Inhibitory Potency of Mammalian R2 C-Terminal Peptide Analogs toward Mammalian Ribonucleotide Reductase Activity

peptide	IC_{50},[a] μM	%RI
1, Ac-NSFTLDADF	9–15	100
2, Ac-SFTLDADF	8–20	100
3, SFTLDADF	80	25
4, Ac-FTLDADF	8–20	100
5, FTLDADF	>400[d]	<3
6, Ac-TLDADF	>400	<3
7, Ac-LDADF	>400	<3
8, Ac-LTLDADF	>400	<3
9, Ac-F(4′-NH_2)TLDADF	100	10
10, Ac-F(4′-N_3)TLDADF	100	10
11, Ac-FSLDADF	40–42	25 ± 1.1
12, Ac-FALDADF	35–40	24 ± 4.1
13, Ac-FTVDADF	30	35
14, AC-FTFDADF	48–58	18 ± 4.2
15, Ac-FTADADF	207	4.9 ± 0.60
16, Ac-FTLNADF	25–29	40 ± 2.4
17, Ac-FTLAADF	286–336	2.9 ± 0.4
18, Ac-FTLDGDF	110–230	9 ± 0.6
19, Ac-FTLDLDF	28	35
20, Ac-FTLDAEF	450–620	1.7 ± 0.15
21, Ac-FTLDANF	32–38	23 ± 1.0
22, Ac-FTLDALF	29–30	34 ± 5.0
23, Ac-FTLDAAF	34–35	28 ± 1.3
24, Ac-FTLDADL	>400	<3
25, Ac-F-LDADF (deletion)	412	2.5
26, Ac-FTLD-DF (deletion)	322	3
27, Ac-FTL-DF (deletion)	364–460	2.5 ± 0.18
28, Ac-FTLDADFAA	500	2.1

[a] IC_{50} is the concentration of peptide producing 50% inhibition of activity obtained in the absence of peptide. Values are derived from Dixon plots of four to six concentration points. Two to four assays were run for each point. Range of IC_{50} values are for two independent determinations conducted on different days. [b] Percent relative inhibitory potency, defined as 100 × (IC_{50} AcFTLDADF)/(IC_{50} peptide analog). Mean values and average deviations based on two or three independent determinations. Other values are single determinations.

minus, whereas the RIs for the *N*-acetyl hexa- and pentapeptides **6** and **7** are at least 40 times smaller, implying that a heptapeptide is important for binding. The low RI of the nonacetylated heptapeptide **5**, repeating our earlier observation,[5] shows that neutralization of positive charge is essential for high inhibitory potency. The RI of nonacetylated octapeptide **3** demonstrates that the effect of acetylation on inhibitory potency becomes less important as the peptide length is extended beyond a heptamer.

Evidence that the Phe residues in positions 1 and 7 are critical for binding is provided by the over 40-fold decreases in RI observed on substitution of Leu for Phe (**8**, **24**). The 10-fold decreases in RI on substitution of either *p*-amino- or *p*-azido-Phe in position 7 (**9**, **10**) indicates the strictness of structural requirements at this position. Each of the remaining positions appears to be more tolerant of substitution although some preferences are clear. Position 6 is rather insensitive to substitution by either Ser (**11**) or Ala (**12**). In position 5, a bulky aliphatic side chain appears to be favored on the basis of the effects seen on Val (**13**), Phe (**14**), or Ala (**15**) substitution. Position 4 appears to have preference for either a polar group or a group of a certain size (or both), since an Asn (**16**) substitution lowers RI only 2-fold, but substitution with Ala (**17**) leads to over a 30-fold decrease in RI. In position 3, a minor drop in RI results from a Leu substitution (**19**), suggesting no specific side-chain requirement in this position, although substitution with Gly (**18**) does lead to a 10-fold decrease in inhibitory potency. The results with position 2 are particularly interesting in that an apparently conservative Glu substitution (**20**) results in over a 50-fold decrease in RI, whereas other conservative (Asn, **21**) or nonconservative (Leu, **22**, Ala, **23**) substitutions result in much smaller (3–4-fold) decreases in RI.

The results obtained with peptides **8–24** are only partly in accord with simple expectations based on evolutionary conservation (Table I). As would be expected, positions 1 and 7, which are fully conserved, are quite sensitive to even conservative amino acid substitution, whereas, position 3, which shows the greatest variability, is apparently insensitive to major side-chain modifications. In addition, the limited variabilities observed for positions 4 and 5 in Table I are consistent with the need for a hydrogen binding and a hydrophobic side chain, respectively, at these positions, in accord with the results in Table II. On the other hand, considerable variation is tolerated in position 2, in which an acidic side chain is fully conserved. Additionally, the importance of a hydrogen-bonding side chain in position 6, which could be inferred from Table I, is not supported by the small decrease in RI on substitution with Ala (**12**).

N-Acetylated hexapeptides (**25**, **26**) resulting from deletions of position 6 (Thr) or 3 (Ala) have over 30-fold less inhibitory potency suggesting that, despite their lack of side-chain specificity, these positions serve as necessary spacers between side chains having more stringent structural requirements for tight binding to R1. Similarly, the pentapeptide (**27**) resulting from deletion of positions 3 and 4 (or 2 and 3) also has a low RI value. With respect to the effects of deletion, mammalian R2 C-terminal inhibition of RR differs from the corresponding inhibition of viral RR. In the latter case the hexapeptide YVVNDL, resulting from deletion of the amino acids AGA at positions 6–8 of the R2 C-terminal nonapeptide YAGAVVNDL, has an inhibitory potency essentially equal to that of the parent nonapeptide.[11] The results with peptides **25–27** buttress the conclusion that the acetylated heptapeptide is the minimal length required for effective RR inhibition.

The low RI that results from adding amino acids to the C-terminus (**28**) suggests that a free carboxylate at the highly specific, position 1 may be important for R1 binding. Analogous results have been found for viral RR.[12] This result could pose a problem for efforts to screen random peptide libraries for optimal binding to R1 that utilize C-terminal coupling to either a phage protein or bead.[13,14] The results so far in hand with peptides **1**, **2**, and **28** suggest that such screening is more likely to succeed when the C-terminus is free, although blockage of the N-terminus should be permissible.

It has been possible to synthesize peptides and peptide analogs that selectively inhibit HSV-RR while having little inhibitory potency toward mammalian RR, because the C-terminal sequence of HSV-R2 is very different from that of mammalian R2. The present results demonstrate that even limited changes in the mammalian C-terminal peptide sequence can lead to significant changes in inhibitory potency and raise the question as to whether peptides can be found that will selectively inhibit even those organisms having C-terminal R2 sequences closely related to that of mammalian R2.

In Table III we examine the cross-specificities of mammalian and *S. cerevisiae* C-terminal R2 peptides, which differ from each other at positions 5-3 from the C-terminus (-FNE- vs -LDA-, Table I). As can be seen,

Table III. Cross Inhibitory Potency of *S. cerevisiae* R2 C-Terminal Peptide Analogs on *S. cerevisiae* and Mammalian Ribonucleotide Reductase[a]

peptide	IC_{50},[a] μM yeast RR	mammalian RR
29, AcAGAFTFNEDF	25	
30, AcFTFNEDF	25	100
31, AcTFNEDF	600	600
4, AcFTLDADF	25	8–20

[a] IC_{50}s were determined as in Table II.

the N-acetylated C-terminal heptapeptide provides the critical length for inhibition of *S. cerevisiae* RR, as it does for inhibition of mammalian RR, since N-acetylated peptides corresponding to *S. cerevisiae* R2 C-terminal deca- and heptapeptides (**29**, **30**) have about the same inhibitory potency toward *S. cerevisiae* RR but the N-acetylated *S. cerevisiae* hexapeptide (**31**) is a much weaker inhibitor. Further, whereas mammalian N-acetylated C-terminal heptapeptide (**4**) has about the same inhibitory potency toward both the *S. cerevisiae* and mammalian enzymes, the *S. cerevisiae* enzyme is about 4-fold more sensitive to inhibition by the *S. cerevisiae* C-terminal peptide (**30**) than is mammalian RR. The RI of peptide **14** (Table II) suggests that it is the Phe in position 5 that is principally responsible for this difference. Our recent observation that the N-acetylated heptapeptide corresponding to the C-terminus of malarial R2 is a rather poor inhibitor of mammalian RR,[15] as might have been anticipated by the low RI of peptide (**20**), may well provide another example that peptides corresponding to the C-terminus of R2s can selectively inhibit even those RRs from different sources having closely related sequences. A more definitive conclusion awaits determination of the effect of the malarial peptide on malarial RR activity. A clear challenge for the future will be to determine whether such selectivity can be enhanced through exploration of a larger range of peptide analogs and derivatives and whether it can be applied to the selective inhibition of infectious organisms. Determination of the peptide conformation when bound to R1 will be an important part of meeting this challenge. Efforts in this direction are underway.

Finally, we note that, as the work presented in this paper was nearing completion we became aware of a European patent[16] presenting results that, to the extent that they overlap, are fully consistent with those presented above. Specifically, these workers, who carried out assays employing crude RR activities from both Hela cells and hamster cellss, demonstrated that (1) extension of the N-terminally acetylated R2 C-terminal peptide beyond seven residues did not increase inhibitory potency toward mammalian R1; (2) the heptapeptide having O-Bzl-Thr in position 6 retained high inhibitory activity, consistent with H-bond donation not being critical from this position; (3) large structural changes are tolerated in position 3, as evidenced by the minor effect on inhibitory potency resulting on replacement of Ala with either Val or Glu; (4) *d*-Ala substitution in position 3 causes a 10-fold decrease in inhibitory potency, consistent with the effect of Gly substitution (18) and indicating that a kink or turn is unfavorable in this position; (5) position 4 was somewhat selective (in decreasing order of inhibitory potency, Asp ~Asn > Phe > Gln > Gly, indicating a size specificity as well as a preference for a polar residue at this position); and (6) the N-acetylated heptapeptide corresponding to the *S. cerevisiae* R2 terminus (**30**) had low inhibitory potency toward mammalian RR.

Experimental Section

Materials. Peptides were synthesized by either tBoc-based or Fmoc-based solid-phase peptide synthesis (SPPS). For tBoc SPPS, Merrifield resin was used (0.65 mequiv of tBoc amino acid/g). Amino acid side chains were protected as follows: Asp, β-O-Bzl; Asp, β-O-cyclohexyl; Glu, γ-O-Bzl; Glu, γ-O-cyclohexyl; Thr, O-Bzl; Ser, O-Bzl; and Cys, trityl (trt). For Fmoc SPPS, *p*-alkoxybenzyl alcohol benzhydrylamine resin (0.36 mequiv of Fmoc amino acid/g) was used. Amino acid side chains were protected as follows: Asp, Glu, Thr, O-Tbu; Asn, Cys, trt. For peptides **9** and **10** Fmoc *p*-amino-Phe(Boc)-OH was used. Both tBoc- and Fmoc-protected amino acids were purchased from Bachem Biosciences. Resins were purchased from Millipore. Peptide synthesis grade trifluoroacetic acid (TFA), diisopropylethylamine (DIPEA), diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBT), acetic anhydride, anisole, dimethyl sulfide (DMS), dimethylformamide (DMF), and piperidine were purchased from Aldrich or Sigma. HPLC-grade CH_3CN, CH_3OH, and CH_2Cl_2 and ACS-grade diethyl ether were purchased from Baker. All nonprotein components in RR assay mixtures and all materials used for synthesis of peptide **10** were reagent grade and purchased from Fisher or Sigma. [5-^{3}H]CDP was purchased from Du Pont NEN.

Peptide Synthesis. Fmoc or tBoc peptides were prepared on an automated Milligen 9600 peptide synthesizer. Couplings of tBoc-protected amino acids (6-fold excess) were achieved with DIC in CH_2Cl_2. For Asn, a combination of HOBT/DIC was used for coupling. tBoc protecting groups were removed with TFA/CH_2Cl_2 (40:60) containing 1% anisole, followed by neutralization with DIEA/CH_2Cl_2 (5:95). After completion of the synthesis and removal of the last tBoc group, peptides were N-acetylated with Ac_2O. Deprotection of amino acid side chains and cleavage of peptides from the resin was accomplished with anhydrous hydrogen fluoride (HF) and anisole (9:1, v:v, 10 mL/g peptide–resin intermediate) at −15 °C for 30 min followed by −5 °C for 30 min. HF was removed *in vacuo* followed by washing of crude peptides with anhydrous ethyl ether and then CH_2Cl_2. Peptides were solubilized in 10% acetic acid. Fmoc amino acids (6-fold excess) were coupled with HOBT/DIC in CH_2Cl_2 and deblocked with 35:35:30 toluene/DMF/piperidine. After removal of the final Fmoc group, the peptides were N-acetylated as described above. Deprotection of amino acid chains and cleavage of the peptide from the resin was accomplished with TFA and water (9.5:0.5, v:v, 10 mL/g peptide resin intermediate) at room temperature for 90 min. Peptides were then precipitated and washed exhaustively with anhydrous diethyl ether. Peptide solutions were lyophilized to yield amorphous powders. For synthesis of peptide **9**, Fmoc-*p*-amino-Phe(Boc)-OH was coupled in position 7. The resulting peptide was acetylated and then cleaved and deprotected using the TFA cleavage procedure. Peptide **10** was synthesized by conversion of the *p*-amino-Phe in position 7 of peptide **9** to *p*-azido-Phe essentially as described previously.[17-19]

Peptide Characterization. Crude peptides were chromatographed by analytical HPLC to assess purity as well as optimize conditions for preparative purification. A sample load of 10–20 mg of crude peptide was chromatographed on preparative HPLC on a Rainin Dynamax C18 preparative column (300 Å, 2 × 35 cm) using a binary solvent system consisting of 0.1% TFA in 10% CH_3CN and 0.1% TFA in 90% CH_3CN, and appropriate gradients. Peptide elution was monitored bath at 215 and 258 nm. Collected fractions were lyophilized and reinjected on analytical HPLC. Every peptide used in this study was >95% pure by HPLC. All peptides had ^{1}H NMR spectra (500 MHz, Bruker AMX) fully consistent with their amino acid compositions. For all peptides, FAB mass spectra (VG ZAB) revealed a single high intensity M + H or M + Na peak consistent with molecular weight. The UV spectra of **9** and **10** were consistent with spectra of similar compounds reported previously.[17,18,20] Complete physicochemical data are available upon request.

Ribonucleotide Reductase Assay. Assays of mammalian RR activity were performed using calf thymus R1, prepared as described by Yang et al.,[5] and cloned mouse R2, prepared from

an *Escherichia coli* strain containing the pET-M2 plasmid encoding mouse R2 as described by Thelander.[21] Assays of *S. cerevisiae* RR activity were performed using partially purified yeast enzyme, prepared similarly to that of Vitols.[22] Briefly, the procedure involves precipitating DNA from a crude yeast supernatant by addition of streptomycin sulfate, ammonium sulfate fractionation, and DEAE-cellulose chromatography. RR activities were assayed at 35 °C by measuring the conversion of [^{3}H]CDP to [^{3}H]dCDP using dithiothreitol (DTT) as an external reductant, essentially as described previously.[4] [^{3}H]dCDP was separated from [^{3}H]CDP on an aminophenylboronate column (Amicon) and radioactivities were determined using a Beckman scintillation counter. The reaction mixture, made up in a final volume of 100 μL, contained 60 mM HEPES, pH 7.6, 2.7 mM $Mg(OAc)_2$, 8.75 mM NaF, 0.05 mM $FeCl_3$, 24 mM DTT, 4 mM ATP, 0.04 mM [5-^{3}H]CDP (22 Ci/mol), and varying amounts of peptide inhibitor. Mammalian RR assays typically contained 1 μg of R1 and 2 μg of R2. The specific activity of R1 was 80 nM dCDP/min/mg and the specific activity of R2 was about 3-fold higher. *S. cerevisiae* assays differed in that 100 μg of crude enzyme replaced mammalian enzyme and assays contained 40 μM phenylmethanesulfonyl fluoride.

Lyophilized peptide was dissolved in 50 mM ammonium bicarbonate just prior to addition to the assay mixture. Peptide concentrations in stock solutions were determined spectrophotometrically at 258 nm, exploiting the absorption of the phenylalanine side chain.

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Supplementary Material Available: Analytical data (NMR and mass spectra, RP-HPLC analysis) used in characterizing the peptides and peptide derivatives reported in this paper are available (8 pages). Ordering information is given on any current masthead page.

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