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Identification of E2F-1/Cyclin A Antagonists

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Abstract—A simple method for the synthesis of a rationally designed (*S,S*)-[Pro-Leu]-spirolactam scaffold is described. This was expanded to a small biased library of compounds mimicking the 'ZRXL' motif in order to identify E2F-1/Cyclin A antagonists. The synthesized compounds were evaluated in an E2F-1/Cyclin A binding assay and moderately active analogues were identified. In addition, the critical roles of Phe, Leu, Lys, and Arg residues of the identified motif were determined.

The regulatory p16/Rb/E2F pathway is the most important and commonly altered pathway in cancer.[1–8] Because the ultimate target of an alteration in this pathway is the deregulation of E2F transcription factors, we are examining E2F-1 as a potential target for cancer therapy. Deregulated (overexpressed) E2F-1 produces antiglioma activity in vitro and in vivo.[9,10] Our previous investigation on how cyclin-dependent kinases (cdks) target their substrates led to the identification of a short cyclin/cdk recognition motif, 'ZRXL', that is present in many cell cycle regulatory proteins. Conjugates of the 'ZRXL' peptide and sequences containing a nuclear localization signal (HIV-1 Tat or penetratin) preferentially kill transformed cells relative to immortalized non-transformed cells, suggesting that deregulation of the E2F-1 transcription factor is sufficient to sensitize cells to the Cyclin A/cdk2 inhibitory peptides.[11] Our data indicate that a minor reduction in the Cyclin A/cdk2-mediated shutdown of E2F-1 will push tumor cells into apoptosis. Most importantly, these data suggest that surrogate cdk2 inhibitors may be effective anticancer agents.

Multiple cell-cycle cyclin-binding regulatory proteins, including the cdk inhibitor p27, contain a sequence with homology to the Cyclin A/cdk2 binding sequence of E2F-1 (Table 1). Of note is the relatively high conservation of residues 3–6 in this alignment that can be represented as Z-Arg-X-Leu (ZRXL), where Z is a basic residue or cysteine and X is a basic residue. An E2F-1 derived octapeptide (PVKRRLDL, **1**, ELISA $IC_{50} = 100\,nM$) containing the 'ZRXL' motif efficiently inhibits the complexation of Cyclin A and E/cdk2 to E2F-1 and p21 in vitro.[12] Peptides were synthesized by sequential removal of amino acids from the N-terminal of peptide **2** followed by capping with an acetyl group and these peptides were analyzed for their inhibitory activities in an in vitro ELISA (Table 1). Results of D-amino acid and Ala scan suggest that Arg, Leu, and Phe of the 6-mer peptide are critical residues involved in the E2F-1/Cyclin A inhibition (Table 1, Ala scan results not shown). The minimum sequence required for the E2F-1/Cyclin A interaction is a 6-mer, **3**.

Close inspection of our model of peptide **2** bound in the Cyclin A pocket revealed a potential secondary hydro-

Table 1. IC_{50} values of peptides

Peptide sequence	Sequence origin	ELISA IC_{50} (μM)
PVKRRLDL, **1**	E2F-1 sequence	0.100
PVKARADL	E2F-1 double mutant	>100
SACRNLFG	p27 sequence	0.200
PAKRKLFG, **2**	Consensus sequence	0.100
Ac-PVKRKLFG	Acetylated consensus	0.100
Ac-PVKRKLfG	D-Amino acid scan	0.250
Ac-PVKRKlFG	D-Amino acid scan	55
Ac-PVKRkLFG	D-Amino acid scan	5
Ac-PVKrKLFG	D-Amino acid scan	45
Acetyl-AKRKLFG	Acetylated 7-mer	0.200
Acetyl-KRKLFG, **3**	Acetylated 6-mer	1
Acetyl-RKLFG	Acetylated 5-mer	30
Acetyl-KLFG	Acetylated 4-mer	45

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phobic pocket (Fig. 1).[13] This pocket is occupied by the methyl group of Ala of peptide **2**. In the case of the 6-mer, **3**, the methyl group of the acetyl moiety is situated in this hydrophobic pocket (figure not shown). Further modeling suggested that larger acyl groups, instead of acetyl, would result in greater hydrophobic interactions between the 6-mer **3** and the Cyclin A binding site. The acetyl group of **3** was replaced by several other acyl groups and activities of each analogue measured in an ELISA (Table 2). Replacement of the acetyl group with an isopropanoyl-, pivaloyl-, or cyclopropanoyl-group provided 6-mer analogues that are equipotent to 8-mer **2**.

This manipulation of the secondary hydrophobic pocket residue enabled us to remove two amino acids from 8-mer **2** without losing any inhibitory activity.

A spirolactam scaffold was selected from several scaffolds for a focused combinatorial library, because the moiety would impart conformational rigidity while maintaining critical interactions achieved by the 'Arg-Lys-Leu-Phe' segment of the consensus sequence (Scheme 1). The computational docking of this library was intended to identify analogues that should demonstrate significant activity due to their complementarity with the Cyclin A binding site.

Spirolactam derivatives were synthesized on solid phase using a photolabile linker as shown in Scheme 2. The R^1, R^2, R^3, and R^4 groups selected are listed in Table 3. Compounds were purified by reverse-phase HPLC, carried out with Waters HPLC systems on YMC C18 columns using linear gradients of acetonitrile/0.1% aqueous TFA. The elution was monitored at 215, 230, 254, and 280 nm. The purified peptides were analyzed by mass spectrometry (SCIEX API III mass spectrometer). The Fmoc-(*S,S*)-[Pro-Leu]-spirolactam was purchased from Neosystem, France.

An ELISA was developed[14] and the analogues tested in an in vitro assay. A 6-mer peptide, Ac-KRKLFG, **3**, inhibits ($IC_{50} = 1\,\mu M$) the E2F-1/Cyclin A protein–protein interaction in vitro. Lys, Arg, and Leu (**ZRXL**) of the 6-mer peptide are critical residues involved in E2F-1/Cyclin A inhibition. Modulation of the secondary hydrophobic residue of the 6-mer peptide results in better inhibition ($IC_{50} = 0.15\,\mu M$) of the E2F-1/Cyclin A interaction.

The IC_{50} values of all analogues were between 30 and 70 μM. From the spirolactam library, one nonpeptide inhibitor (Fig. 2a) had moderate inhibitory activity ($IC_{50} = 30\,\mu M$) in the E2F-1/Cyclin A in vitro assay. Presumably, the lower activity of the most active spirolactam analogue is due to the insufficient number of H-

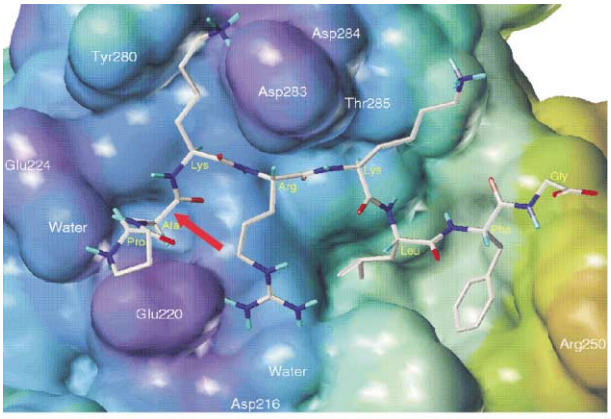
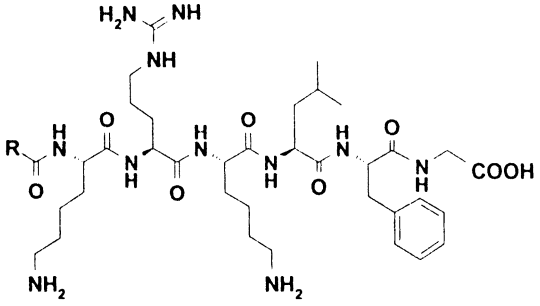
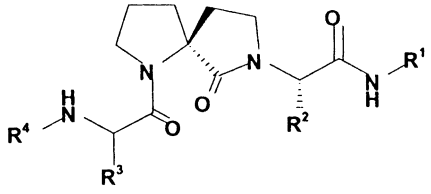


Figure 1. Model of peptide **2** bound to the Cyclin A pocket. Presence of a secondary hydrophobic pocket in the Cyclin A pocket (identified with a red arrow).

Table 2. Effect of R group modification

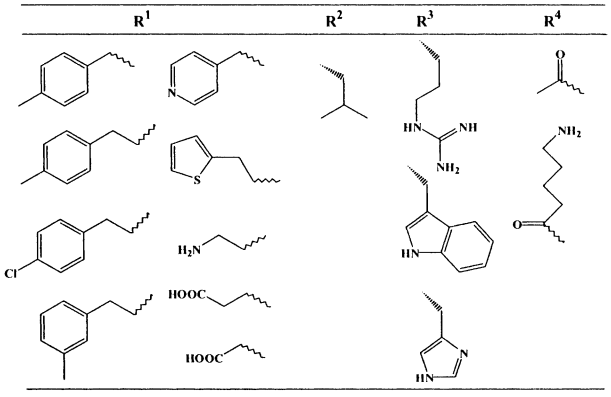


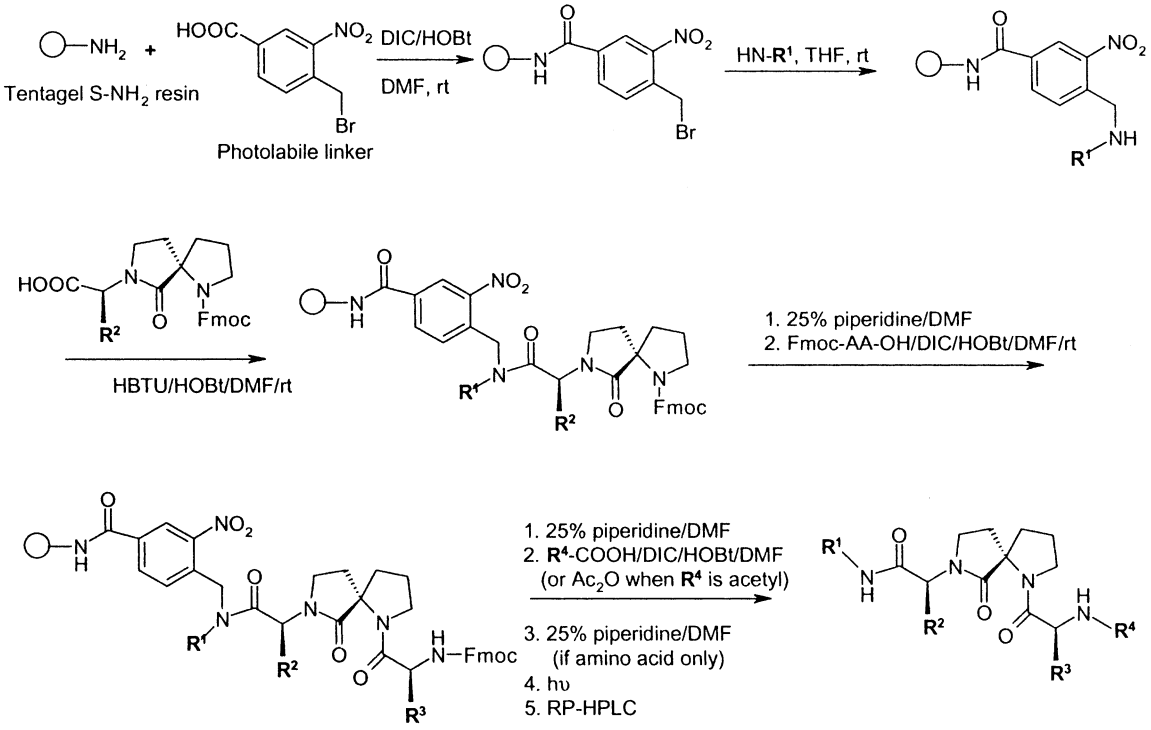
R	ELISA IC_{50} (nM)
Methyl, **3**	1000
Ethyl	450
Isopropyl	150
t-Butyl	150
Cyclopropyl	150
Cyclopentyl	350
Cyclohexyl	450
Phenyl	1500



Scheme 1.

Table 3. Spirolactam R groups





Scheme 2.

bonds that are present in 6-mer (Fig. 2b). Further modifications, including incorporation of additional H-bond functionality and utilization of the secondary hydrophobic pocket to enhance potency, are ongoing.

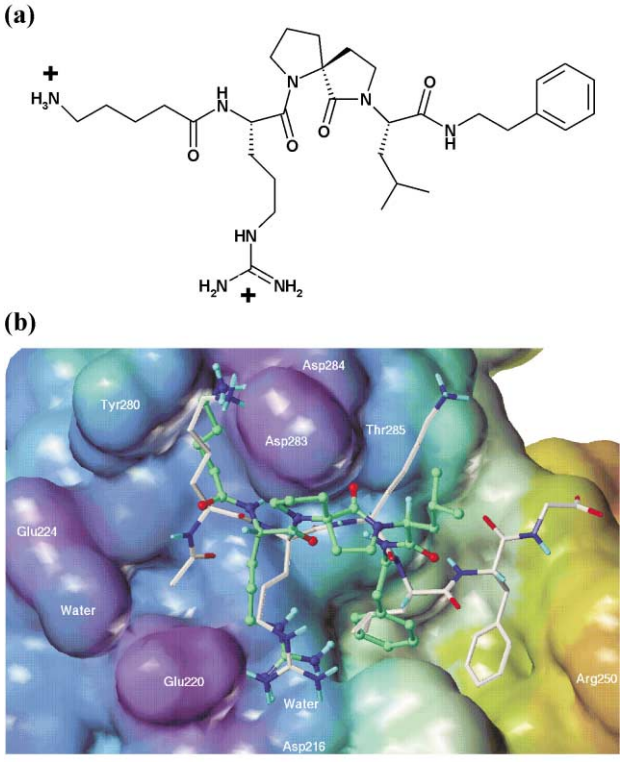


Figure 2. (a) Most active spirolactam analogue; (b) spirolactam analogue superimposed on 6-mer **3** bound to Cyclin A.

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14. Sharma, S. K.; Bair, K. W.; Chen, Y.-N.; Chen, W.; Clune, K.; Jiang, L.; Rogers, L.; Sabio, M.; Ramsey, T. M. 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, LA, March 24–28, 2001. In a typical assay, Nunc Immulon II ELISA plates were coated overnight at 4 °C with 250 μL of 4 mg/mL anti-GST antibody (Pharmacia Biotech) in bicarbonate buffer. Following five washes with wash buffer consisting of 50 mM Tris (pH 7.5), 0.15 M NaCl, and 0.01% Tween-20 (TBST), nonspecific sites were blocked for 2 h at rt with 300 μL of assay buffer consisting of 50 mM HEPES (pH 7.5), 0.15 M NaCl, 0.1% Triton X-100, and 5%

bovine serum albumin (BSA). Plates were then washed five times in TBST, aspirated dry, and treated with 100 μL of GST-E2F-1 in TBS (25 nM). GST-E2F-1 was incubated at RT for at least 1 h with nonspecific binding (NSB) control wells receiving assay buffer with no protein. Plates were washed five times in TBST and multiple dilutions of test compounds diluted in assay buffer were co-incubated with 5 nM Cyclin A/cdk2 diluted in assay buffer. The Cyclin A/cdk2 complex was freshly prepared by mixing a 1:1 ratio of the two proteins in TBS at 4 °C for 30 min prior to addition to the assay plate. Following incubation for 2 h at rt, plates were washed five times in TBST, and 100 μL of a 1:500 dilution of a rabbit anti-cdk2 antibody (Santa Cruz) diluted in assay buffer and were added to all wells. Following incubation for at least 1 h at RT or overnight at 4 °C, the plates were washed five times in TBST and 100 μL of a 1:1000 dilution of an HRP-conjugated anti-rabbit IgG antibody (Pierce) were added to all wells. Following a 30 min incubation at rt, the plates were washed five times in TBST, aspirated dry, and developed by the addition of 100 μL of an HRP substrate ABTS prepared in sodium citrate buffer (pH 4.2). After 10–15 min, plates are read in a microplate reader at 405 nm. Under these conditions, the signal-to-noise ratio between wells with GST-E2F-1 and wells receiving no GST-E2F-1 averaged 10:1.