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DESIGN OF MHC CLASS II (DR4) LIGANDS USING CONFORMATIONALLY RESTRICTED IMINO ACIDS AT p3 AND p5

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Abstract: High potency synthetic ligands were designed for rheumatoid arthritis linked Class II MHC, DR4 Dw4. The design strategy utilized isosteric replacements at the p1 and p7 positions and conformational restriction with imino acids pipecolic acid (Pec) and proline at the solvent exposed residues p3 and p5. In particular, SC-67655, *(S)*-CBA-Val-Pec-Asp-Pro-Thr-NH-*n*-Pr (IC_{50} = 50 nM) is a potent and stable pentapeptide DR4 ligand.

The presentation of peptide antigens to T-cells by MHC (Major Histocompatibility Complex) molecules is a central process in generating specific immune responses. Drugs that block this cell-based immune response by functioning as synthetic MHC ligands, competing with disease-inducing antigens for the MHC peptide binding cleft, are therapeutics[1] for treating diseases associated with a particular MHC subtype. The MHC Class II haplotype DR4 (DR4 Dw4 or DRB1*0401) is clinically associated with rheumatoid arthritis (RA), an autoimmune disease characterized by chronic inflammation of the synovium.[2] A potential RA treatment could be based on be the blockade of the peptide binding groove of DR4, thus preventing the presentation of disease-inducing peptide antigens to autoreactive T-cells. We have designed small and potent DR4 subtype-selective ligands to compete with disease-inducing antigens enroute to a *selective* immunosuppressive RA therapy.

The DR4 peptide binding cleft or groove is located between two alpha helices, derived from the α and β protein chains. This cleft binds a multiplicity of peptide sequences according to selection rules described in published studies.[3] Numbering from the peptide N-terminus, amino acid residues at p1 (position 1), p4, p6 and p7 are the most important for binding affinity. Our design of DR4 ligands began with a report[3] from the Roche group that the *free* heptapeptide Tyr-Arg-Ala-Met-Ala-Thr-Leu (p1 = Tyr) binds to DR4. We made potent analogues by truncating and introducing cyclic imino acids at p3 and p5 to increase stability to serum and to the intracellular environment of antigen presenting cells (APC's), and to decrease polarity in order to enhance APC membrane penetration.

Results and Discussion

The published free peptide DR4 ligand (above) was modified by "encapping" the N-terminus with an acetyl group and the C-terminus as the amide of ammonia, affording the heptapeptide **Ac**-Tyr-Arg-Ala-Met-Ala-Thr-Leu-**NH_2**. This blocked peptide exhibited an IC_{50} = 180 nM in an ELISA assay[4] that measures the ability of a test compound to compete for binding to DR4 with the potent antigenic peptide derived from influenza virus haemagglutinin, HA306-318 (IC_{50} = 80 nM). Two residue changes were made to bring about an increase in binding affinity and biochemical specificity. First, at the p4 position, the known specificity conferring[5] residue aspartic acid was introduced in place of the p4 Met. Second, the p1 Tyr was replaced with 2-naphthylalanine (Nal) to fill the p1 hydrophobic pocket. These changes yielded the potent *hepta*peptide DR4 ligand SC-64762, Ac-Nal-Arg-Ala-Asp-Ala-Thr-Leu-NH_2, (IC_{50} = 31 nM).

SC-64762 is not stable to human serum, but served as our starting point for the design of smaller, proteolytically stable DR4 ligands. We assumed that the DR4 bound conformation of SC-64762 would resemble that of HA306-318 peptide when complexed to the related allele DR1 as shown by a published x-ray crystal structure.[6] This DR1 bound conformation of HA peptide was used as a model to predict the spacial orientations the side chains, amide carbonyls and NH groups of SC-67462 when bound to DR4. Especially important was the determination of which NH's of the peptide backbone were likely to be *internally* (toward protein) or *externally* (toward solvent) oriented with respect to the DR4 protein.

A schematic detailing these internal/external orientations is shown in Figure 1, along with a side chain numbering scheme. A design priority was to eliminate certain peptide secondary amide units that were not likely to be required for protein binding. Secondary amide linkages have been suggested[7] to be significant barriers to cell membrane penetration and consequently, oral absorption; in addition, degradative enzymes such as chymotrypsin, recognize and bind to the secondary amide NH to effect proteolysis.

The published structure of the HA-DR1 complex[6] shows that the p3 side chain (Lys) is in contact with both protein and solvent while the p5 side chain (Asn) is completely solvent exposed. We made the assumption that when SC-67462 binds to DR4 the p3 and p5 side chains would be similarly solvent accessible. Based on the HA-DR1 structure, we also assumed that in the DR4 bound conformation of SC-67462, the NH groups of residues p3 and p5 were likely to be completely solvent exposed, that is, pointing away from the MHC protein in an external orientation. Thus, the hydrogens of the p3 and p5 backbone NH's would be predicted to be unnecessary for binding to DR4.

Conformational restriction was envisioned as a device to retain binding affinity with smaller *backbone-truncated* analogues. Because it was observed[6] that the bound conformation of HA peptide resembled that of a polyproline Type II helix, the introduction of imino acids such as proline seemed a reasonable approach to stabilize the MHC-bound conformation. Another benefit of the use of an imino acid is that it gives rise to a tertiary amide, eliminating a secondary amide NH. Thus, proline and its ring homologues emerged as p3 and p5 replacements to serve dual functions.

The conformations of the p3 Lys and p5 Asn residues in DR1 bound HA were examined. Of particular interest is the dihedral angle[8] defined by H-N-Cα-Cβ (in Figure 1 the asterisked atoms define this dihedral angle). For the p3 Lys of HA peptide, this angle is -45° and for the p5 Asn this angle is -19°. We envisioned a *formal* cyclization, where the p3 and p5 NH's are replaced with carbon and joined to the side chain, forming a cyclic imino acid (Figure 1), with dihedral angles to match the corresponding angles found in the DR1 bound conformation of HA peptide. The corresponding dihedral angle of proline (5-membered ring) has a value of -16°. Thus the particular dihedral angle of proline corresponds well to that of the p5 Asn residue in HA peptide. The dihedral angle of -45° at p3 of HA matches with the angle of -52° found in pipecolic acid (6-membered ring, (Figure 1). Thus, the approach to conformational restriction was to insert pipecolic acid (Pec) at p3 and proline (Pro) at p5.

This design principle was applied to truncated *penta*peptide analogues of SC-64762 to try to achieve "minimal" size DR4 ligands. At the C-terminus, the carboxamido group was eliminated and the isobutyl side chain of the C-terminal leucine was shortened to *n*-propyl. At the N-terminus, *(S)*-3-phenylbutyric acid[9] (*S*-CBA) was introduced in place of the p1 Nal residue, thus formally truncating the peptide backbone between the Nal Cα and the NH, and the naphthyl group was replaced with cyclohexyl. In the event, the insertion of Pec at p3 and Pro at

p5, resulted in the potent *penta*peptide[10] **SC-67655**, shown in Figure 2, (IC_{50} = 50 nM), which is comparable in binding affinity to the heptapeptide lead SC-64762 and the antigenic peptide HA306-318 (IC_{50} = 80 nM).

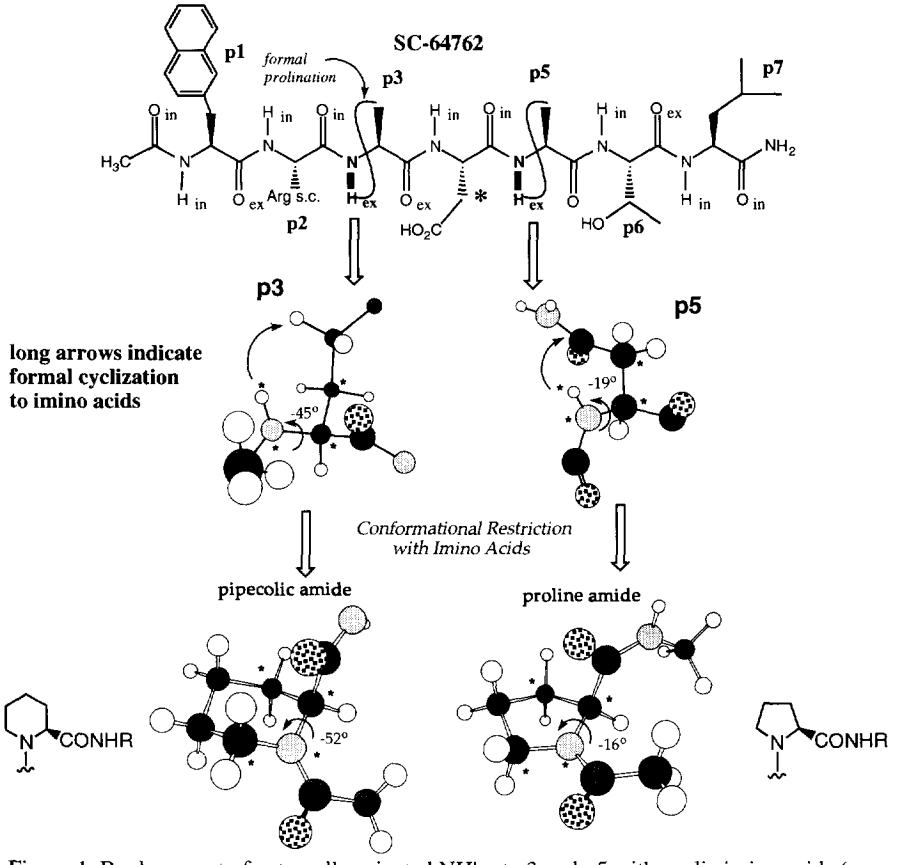

Figure 1. Replacement of externally oriented NH's at p3 and p5 with cyclic imino acids (**ex** = externally oriented toward solvent, **in** = oriented toward protein). The predictions of external or internal orientation were made based structural data given in ref. 6.

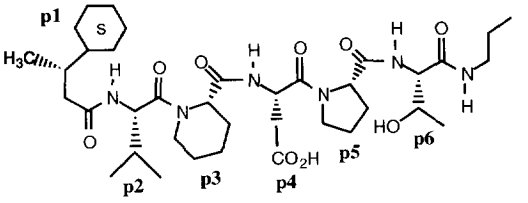

Figure 2. **SC-67655** (with p sites labeled, the p6 residue is natural threonine)

The activity of other new DR4 ligands is shown in Table 1. The pentapeptide analogue SC-67114, with a Lys residue at p2, is also a potent DR4 ligand, although it is a factor of two less potent than SC-67655. When the sequence order of Pec and Pro was reversed in SC-66972, a large loss in potency (160-fold over SC-67114) was

observed. When Pro replaced Pec at p3 in SC-66850, a 25-fold loss in potency resulted. These results suggest that the dihedral angles at p3 and p5 are important for DR4 binding and are consistent with the design hypothesis.

Table 1. **Pentapeptide SC-67655 Analogues as DR4 Ligands**

S-CBA-Aa-**Ia$_1$**-Asp-**Ia$_2$**-Thr-NH-*n*-Pr
p1 p2 p3 p4 p5 p6

SC #	Aa	Ia$_1$	Ia$_2$	IC$_{50}$ (nM)
SC-67655	Val	Pec	Pro	50 ± 24
SC-67114	Lys	Pec	Pro	100 ± 10
SC-66972	Lys	Pro	Pec	16000 ± 1980
SC-66850	Lys	Pro	Pro	2500 ± 260
SC-67176	Lys	NMeAla	Pro	1800 ± 400
SC-68889	Lys	Tic	Pro	178 ± 57
SC-69790	Lys	*cis*-[H]Tic	Pro	303 ± 88
HA306-318 peptide				80 ± 28

Table 2. **Pentapeptide DR4 Ligands: SAR at p1, p2, p4 and p6**

X$_1$-Aa$_1$-Ia$_1$-Aa$_2$-Ia$_2$-X$_3$
p1 p2 p3 p4 p5 p6

SC #	SAR	X$_1$	Aa$_1$	Ia$_1$	Aa$_2$	Ia$_2$	X$_3$	IC$_{50}$ (nM)
68667	p1	*R*-CBA	Lys	Pec	Asp	Pro	Thr-NH-*n*-Pr	162 ± 12
68666	p1	CHPA	Lys	Pec	Asp	Pro	Thr-NH-*n*-Pr	178 ± 53
68422	p1	NPA	Lys	Pec	Asp	Pro	Thr-NH-*n*-Pr	76 ± 34
67813	p1	NCG	Val	Pec	Met	Pro	Thr-NH-*n*-Pr	>100000
69020	p1	CPA	Val	Pec	Asp	Pro	Thr-NH-*n*-Pr	130 ± 72
68748	p1	CHA	Lys	Pec	Asp	Pro	Thr-NH-*n*-Pr	>100000
69021	p2	*S*-CBA	CHG	Pec	Asp	Pro	Thr-NH-*n*-Pr	170 ± 8
68977	p2	*S*-CBA	Thr	Pec	Asp	Pro	Thr-NH-*n*-Pr	1300 ± 543
68806	p2	*S*-CBA	PGly	Pec	Asp	Pro	Thr-NH-*n*-Pr	424 ± 33
68246	p2	CPA	His	Pec	Nle	Pro	Thr-NH-*i*-Am	>100000
67814	p2	CPA	His	Pec	Asp	Pro	Thr-NH-*i*-Am	3150 ± 550
68573	p2	*S*-CBA	decL	Pec	Asp	Pro	Thr-NH-*n*-Pr	10000
68807	p4	*S*-CBA	Val	Pec	Prg	Pro	Thr-NH-*n*-Pr	43700 ± 5903
68477	p4	*S*-CBA	Val	Pec	Met	Pro	Thr-NH-*n*-Pr	293 ± 61
68387	p4	CPA	Val	Pec	Nle	Pro	Thr-Leu-NH_2	755 ± 237
68978	p6	*S*-CBA	Val	Pec	Asp	Pro	Thr-NH_2	3500 ± 1446
68952	p6	*S*-CBA	Val	Pec	Asp	Pro	NH_2	23300
68433	p6	*S*-CBA	Val	Pec	β-Ala	—	—	>100000
69504	p6	*S*-CBA	Val	Pec	Asp	Pro	(3-NH_2)Ala-NH-*n*-Pr	>100000
68951	p6	*S*-CBA	Val	Pec	Asp	Pro	threoninol	7000 ± 3704

Abbreviations: "SAR" entry classifies changes by "p" site; Prg = propargylglycinyl; CHG = cyclohexylglycinyl; PGly = phenylglycinyl; decL = ε-N-*n*-decanoyl-Lys; CHPA = 3-cycloheptanylpropionyl; NPA = 3-(2-norbornanyl)propionyl; NCG = N-cyclohexylglycinyl; CPA = 3-cyclohexylpropionyl; CHA= *(R,S)* -3-cyclohexylhexanoyl; *i*-Am = isoamyl.

Potent DR4 ligands were also obtained using the hydrophobic benzo- and cyclohexyl fused pipecolic acid derivatives at the p3 position. Thus, *S*-CBA-Lys-**Tic**-Asp-Pro-Thr-NH-*n*-Pr, employing 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) at p3 shows an IC_{50} = 178 nM. The corresponding analogue with *cis*-perhydroisoquinoline-3-carboxylic acid at p3 exhibits an IC_{50} = 303 nM. The relevant dihedral angle for a peptide derivative of Tic is -40° and the corresponding angle for *cis*-perhydroisoquinoline carboxylic acid is -42°; these values closely match the dihedral angle at the p3 Lys in DR1-bound HA peptide (-45°).

Additional SAR studies were carried out to discover which modifications of SC-67655 were consonant with good binding to DR4. Table 2 summarizes the results, indicating which positions were modified. The p1 "anchor" position requires[3] hydrophobic structures; a variety of cyclic hydrophobic structures, including cycloheptyl (SC-68666) and norbornanyl (SC-68422) give rise to good binding affinity. However, activity is abolished by the introduction of a β-propyl branch in SC-68748 and a basic nitrogen in SC-67813. The p2 position is best substituted with a small hydrophobic group, such as Val in SC-67655 or cyclohexylglycine in SC-69021; interestingly, the large hydrophobic group, ε-N-*n*-decanoyl-lysine in SC-68573 severely diminishes activity. Incorporation of a steroid-lysine conjugate also gave lower activity (IC_{50} = 5100 nM, data not shown). The p4 position is best substituted with Asp; methionine and norleucine substitutions show diminished activity and propargylglycine abolishes activity. At p6, the 3-aminoalanine residue, introduced in place of threonine, led to a large decrease in potency.

Of particular interest was to define the lower size limit for this series of DR4 ligands, so truncation from the C-terminus of SC-67655 was performed. SC-68978 demonstrates that the *n*-Pr group is required for good binding; when this *n*-Pr is replaced with hydrogen, a 70-fold loss in activity occurs. Termination with threoninol as in SC-68951 results in a 140-fold potency loss. Removal of the p6 threonine residue in SC-68952 results in a total loss of binding affinity; it is not surprizing that further truncation to the tripeptide SC-68433 yields the same result.

To test stability to biological fluids, SC-67655 and SC-64762 were incubated with human serum and lysate from APC's for 1 hr at 37°C. HPLC determination showed that SC-67655 was 93% intact after *both* serum and APC incubation. In contrast, *hepta*peptide SC-64762 was only 3% intact after serum incubation, although it was 90% intact after APC lysate treatment.

Conclusion:

SC-67655, a conformationally restricted pentapeptide, was designed using structural biology data and molecular modeling, and is the smallest DR4 ligand known at its potency level. Its stability to serum and the lysate from antigen presenting cells makes it a candidate for *in vitro* and *in vivo* studies. This compound has sufficient *in vitro* potency to compete with the powerful antigen HA306-318 for binding to the DR4 antigen binding groove, and is being studied for its effects on inhibition of DR4-mediated T-cell proliferation.[11]

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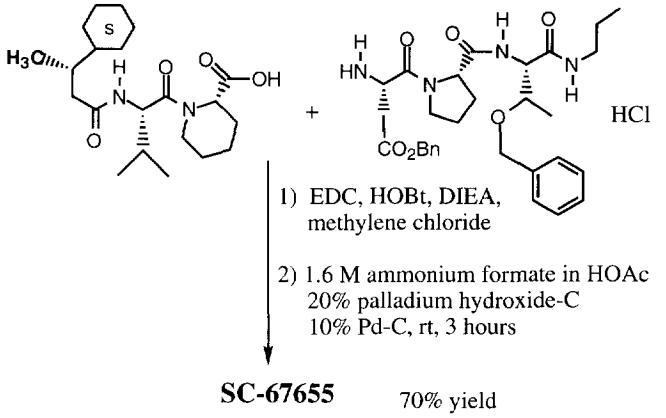
10. The peptides were synthesized using L-amino acids by standard solid phase methods, or by a fragment condensation approach in which *S*-CBA-Aa-**Ia**$_1$-OH was coupled to Asp(OBn)-**Ia**$_2$-Thr(OBn)-NH-*n*-Pr in methylene chloride via EDC/HOBt. The resulting intermediate was *cleanly* deblocked to the final compound using catalytic transfer hydrogenolysis (hydrogen/Pd-C often led to incomplete debenzylation), following the procedure of Felix, A. M.; Heimer, E. P.; Lambros, T. J; Tzougraki, C.; Meinehofer, J. *J. Org. Chem.* **1978**,*43*, 4194; the hydrogenolysis reaction mixture was diluted with methanol, filtered through Celite and the solvent evaporated. Water was added, the solution filtered again through Celite and then lyophilized to give the DR4 ligand. All compounds exhibited spectral properies (^{1}H NMR, mass spectra) consistent with their structures, and were determined to be pure by HPLC as well as TLC analysis (silica F-254 plates, eluting with chloroform/methanol/water/acetic acid 64 : 30 : 4 : 2).



SC-67655 70% yield

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