Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 1, pp. 37-40, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(96)00576-8

STRUCTURE-ACTIVITY STUDIES OF CTL INHIBITORY PEPTIDES DERIVED FROM HLA CLASS I MOLECULES

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Abstract. A series of dimeric peptides derived from a conserved HLA Class I hexapeptide sequence have been synthesized and tested for their ability to inhibit T cell-mediated lysis and to disrupt membranes. Structure-activity studies of the C-N/N-C dimer show that activity is especially sensitive to substitution of isoleucine residues. The results further define and delimit the basis for activity by HLA-derived peptides.

Peptides derived from conserved regions of Human Leukocyte Antigen (HLA) Class I have been shown recently to exert allele-nonspecific immunosuppressive effects.[1] For example, a decapeptide corresponding to residues 75-84 of the α_1 domain of HLA-B2702 suppressed rejection of skin allografts in a mouse model, and an inverted/direct repeat dimer of this peptide blocked lysis of target cells by cytotoxic T lymphocytes (CTL). While the basis for these activities has not been firmly established, it has been found that HLA-derived peptides which inhibit T cell-mediated cell lysis bind to 70 kDa heat shock proteins (HSP70 and HSC70), whereas those that are inactive do not. In this communication we report identification of smaller HLA-derived dimeric constructs that retain the ability to block T cell-mediated lysis, and explore structure-activity relationships in one of these compounds.

The dodecapeptides RIALRY-RIALRY, RIALRY-YRLAIR, YRLAIR-RIALRY, and YRLAIR-YRLAIR represent couplings of direct/inverted repeats of B2702 residues 79-84. These dimers and derivatives of the inverted/direct repeat C-N/N-C dimer YRLAIR-RIALRY in which corresponding residues in each half of the dimer were replaced by serine or other residues were prepared by solid-phase peptide synthesis and purifed by reversed-phase HPLC.[2,3] The constitutions of the peptides were confirmed by FABMS or ESMS (Table I). The inverted/direct repeat dodecapeptide **4** (84-79/79-84) was selected for structure-activity analysis via serine substitution in light of the potent CTL inhibitory properties exhibited by the analogous inverted/direct repeat eicosapeptide **16** (84-75/75-84). Serine substitutions were chosen over the more usual "alanine scan"[4] in an effort to maximize the water solubility of the peptides.

These peptides were tested for their ability to inhibit in vitro lysis of the Epstein-Barr virus transformed B cell line JY by the HLA-A2 specific, $CD8^+$ cytotoxic T cell line AJY.[5] For comparison, we carried out parallel studies with **16** and a threonine-for-isoleucine substitution of this eicosapeptide which is inactive (**17**, 84-75/75-84-80T_2).[1] Plots of percent specific lysis versus peptide concentration (Figure 1) revealed several effects. The N-C/N-C, N-C/C-N, C-N/C-N, and C-N/N-C dodecapeptide dimers (peptides **1-4**, respectively) inhibited T cell-mediated cell lysis, but with diminished potency relative to **16** (Figure 1A+B). Inhibitory potency decreased in the order **16** > **1** ≈ **2** > **3** > **4** > **17**. The acylated/amidated hexapeptide corresponding to B2702 residues 79-84 (Ac-RIALRY-NH_2) did not inhibit specific lysis at concentrations up to 240 μM (data not shown). Serine substitutions within the context of peptide **4** had position-dependent effects. Substitution for tyrosine at position 84 (**5**), leucine at position 82 (**7**), or isoleucine at position 80 (**9**) decreased or eliminated the ability to inhibit

Table I. Immunosuppressive Peptides Derived From HLA Class I.

Number	Name	Sequence	MW (calcd.)	*m/z* (MH+) (obsd.)
1	79-84/79-84	RIALRY-RIALRY	1564.0	1563.8
2	79-84/84-79	RIALRY-YRLAIR	1564.0	1563.7
3	84-79/84-79	YRLAIR-YRLAIR	1564.0	1563.7
4	84-79/79-84	YRLAIR-RIALRY	1564.0	1564.0
5	84-79/79-84-84S_2	SRLAIR-RIALRS	1411.9	1411.9
6	84-79/79-84-83S_2	YSLAIR-RIALSY	1425.8	1426.0
7	84-79/79-84-82S_2	YRSAIR-RIASRY	1511.9	1512.0
8	84-79/79-84-81S_2	YRLSIR-RISLRY	1595.9	1596.0
9	84-79/79-84-80S_2	YRLASR-RSALRY	1511.9	1511.8
10	84-79/79-84-79S_2	YRLAIS-SIALRY	1425.8	1425.7
11	84-79/79-84-80T_2	YRLATR-RTALRY	1539.9	1540.0
12	84-79/79-84-80T*$_2$	YRLAT(**OMe**)R-RT(**OMe**)ALRY	1567.9	1568.0
13	84-79/79-84-80V_2	YRLAVR-RVALRY	1536.0	1535.8
14	84-79/79-84-80L_2	YRLALR-RLALRY	1564.0	1564.0
15	84-79/79-84-84F_2	FRLAIR-RIALRF	1532.0	1532.0
16	84-75/75-84	YRLAIRLNER-RENLRIALRY	2589.1	2589.0
17	84-75/75-84-80T_2	YRLATRLNER-RENLRTALRY	2565.0	2565.2

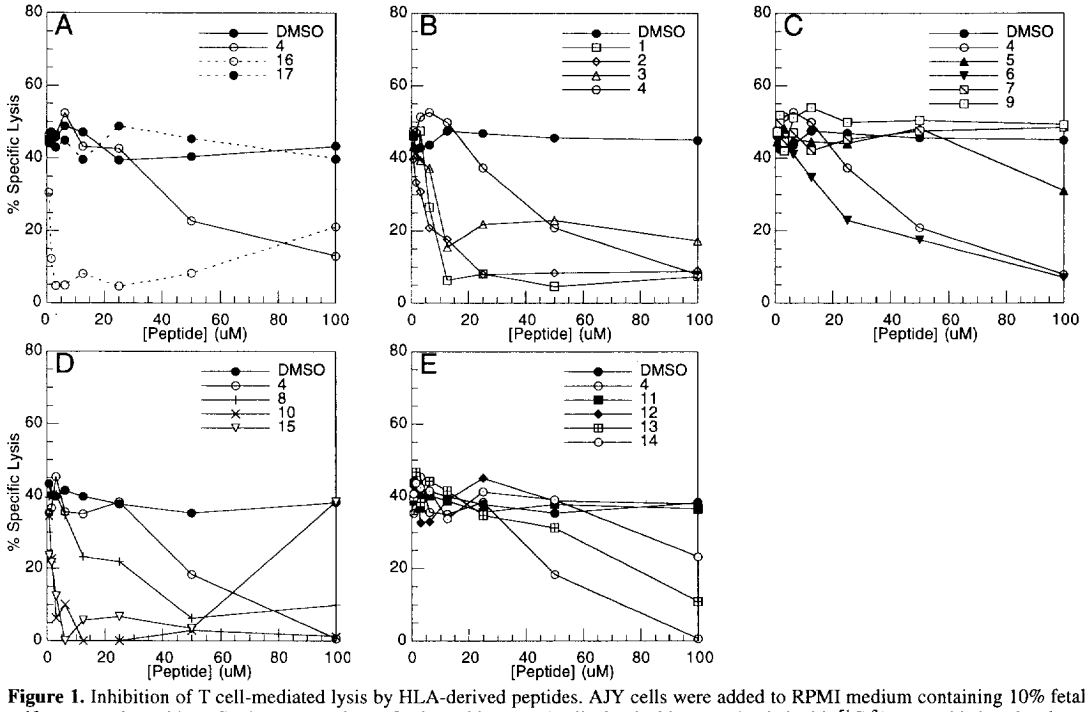

Figure 1. Inhibition of T cell-mediated lysis by HLA-derived peptides. AJY cells were added to RPMI medium containing 10% fetal calf serum and peptide at final concentrations of .78 to 100 μM. JY cells that had been preloaded with $^{51}Cr^{3+}$ were added to the above mixture and after incubation at 37 °C for 4 h the cells were pelleted and the amount of $^{51}Cr^{3+}$ released into the supernatant was determined by direct γ-counting. Percent specific lysis was calculated using the formula 100(x - s)/(t - s), where x is the experimentally determined value, s is the value for spontaneous lysis of JY in the absence of AJY, and t is the value for the total lysis of JY when incubated in triton X. A: Comparison of the C-N/N-C dodecapeptide dimer with the active and inactive C-N/N-C eicosapeptide dimers. B: Effects of orientation in HLA-derived dodecapeptide dimers. C: Serine substitutions which maintain or lead to apparent decreases in the activity of the C-N/N-C dimer **3**. D: Serine substitutions which lead to apparent increases in activity. E: Substitutions for isoleucine at B2702 position 80.

cell lysis whereas substitution for arginine at position 83 (**6**) had little effect (Figure 1C). In contrast, substitution of serine for alanine at position 81 (**8**) led to a small increase in potency, and serine-for-arginine substitution at position 79 (**10**) afforded a large increase in potency (Figure 1D). Furthermore, substitution of threonine for isoleucine in both dodecapeptide and eicosapeptide C-N/N-C dimers (**11** and **17**, respectively) abolished the ability to inhibit T cell-mediated cell lysis. Replacing the isoleucine residues of peptide **4** with the very similar amino acids valine (**13**), leucine (**14**), or the isoleucine isostere *O*-methyl threonine (**12**)[6] also diminished or, in the last case, entirely eliminated activity (Figure 1E). Lastly, a dodecapeptide in which phenylalanine had been substituted for tyrosine at position 84 (**15**) suppressed lysis of JY target cells by AJY effectors at concentrations up to 40 μM; however, no suppression was observed at 100 μM **15** (Figure 1D).

This latter result indicated that peptide **15** may cause nonspecific disruption of both JY and AJY cells. This led us to examine the propensity of HLA-derived peptides to damage cellular membranes, as such activity would complicate interpretation of or mask effects on T cells. Membrane disruption was analyzed using human red blood cells, a convenient and sensitive test for nonspecific cell lysis through release of hemoglobin.[7] Peptides **2**, **10**, and **15** caused significant disruption of red blood cells at concentrations similar to those at which they inhibit T cell-mediated cell lysis (Figure 2). Thus, the biological activity of these peptides may be due to T cell membrane disruption. This disruption need not lyse T cells to block their effector functions. Previous studies indicate that agents which cause calcium influx into T cells in the absence of costimulatory signals, including those that operate via membrane permeablization, lead to cellular anergy.[8]

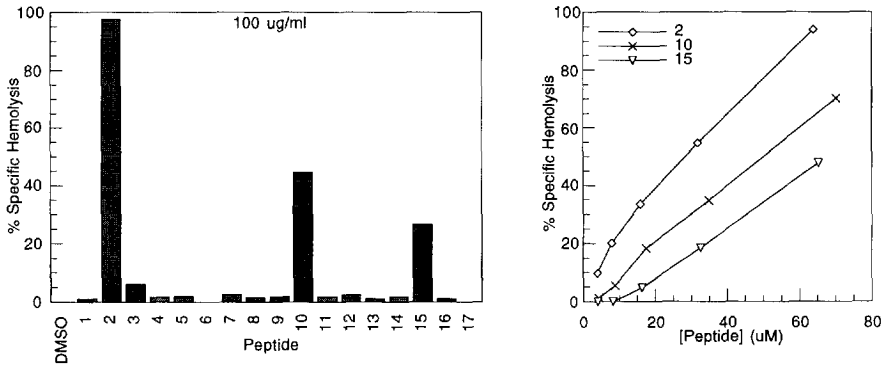

Figure 2. Lysis of human red blood cells (RBC) by HLA-derived peptides. Left: RBC (5-5.5 x 10^7) were incubated in 2 mL of sodium phosphate buffered saline for 20 minutes at 37 °C with peptides **1-17** at a concentration of approximately 64 μM (38 μM for eicosapeptides). Intact cells were pelleted by centrifugation, and the extent of hemoglobin release was determined by measuring absorbance of the supernatant at 413 nm. Right: Concentration dependence of RBC lysis by selected peptides.

Peptides **1**, **3**, **4**, **6**, **8**, **13**, and **16** inhibit T cell-mediated lysis but do not cause observable disruption of red blood cells at 64 μM, or in the last case 38 μM. This suggests that these peptides inhibit CTL function through a specific mechanism(s), such as binding to surface or intracellular receptors. Our results are consistent with the notion that HLA-derived peptides exert their effects through interaction with HSP70 proteins.[1] The presence of large hydrophobic side chains has been reported to be a prerequisite for high affinity binding of peptides members of the HSP70 family.[9] Serine substitutions for residues containing large hydrophobic side chains, as in peptides **5**, **7** and **9**, lead to decreased activity. Similarly, substitution of the position 80 isoleucine by the relatively more hydrophilic residues threonine and *O*-methyl threonine resulted in lower potency. Replacement

at this same position by the amino acids valine and leucine also decreased activity, albeit not as drastically as more hydrophilic substitutions.

Further delineation of the mechanism(s) through which HLA-derived peptides exert their CTL inhibitory effects will increase our understanding of T cell biology and may aid in the development of novel forms of immunotherapy. Towards these ends, future efforts will be directed toward determining whether general correlations exist between heat shock protein binding affinity and CTL inhibitory potency of B2702-derived peptides. If heat shock proteins are the cellular targets for these peptides, their potency may be improved by substitution with amino acids bearing hydrophobic side chains rather than with serine; however, such substitutions would be expected to substantially decrease the water solubility of the peptides and to increase their ability to cause nonspecific damage to cellular membranes. We also plan to analyze and optimize the activity of the direct/direct repeat dodecapeptide **1**, which has intrinsically greater potency than **4**.

Acknowledgment. This work was supported by a Program Project Grant from the National Institutes of Health (1P01AI35125, A.M.K., Project Director). E.J.S. was supported in part by a Public Health Services Training Grant (5T32GM07365). J.E.G. was supported by a predoctoral fellowship from the Howard Hughes Medical Institute. Mass spectra were obtained at the UCSF Center for Mass Spectrometry (A.L. Burlingame, Director), supported by the Biomedical Research Technology Program of the NIH NCRR.

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(*Received in USA* 26 *August* 1996; *accepted* 25 *November* 1996)