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Second-generation peptidomimetic inhibitors of antigen presentation effectively treat autoimmune diseases in HLA-DR-transgenic mouse models

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Abstract

Peptidomimetic compounds that bind to major histocompatibility complex class II molecules and are resistant to cathepsins can competitively inhibit the presentation of processed protein antigens. Therefore, compounds that bind to autoimmune disease-associated class II molecules are expected to compete with autoantigens for presentation and thereby interrupt the disease process. The first generation of such competitors developed for rheumatoid arthritis-associated HLA-DR molecules, although resistant to cathepsins, has remained sensitive to plasma proteases, and was thus unlikely to be effective in vivo. We have therefore produced a second generation of compounds that is resistant to cathepsins and stable in plasma while maintaining binding affinity for HLA-DR molecules associated with rheumatoid arthritis and multiple sclerosis. Selected compounds of this series are shown to inhibit antigen presentation in vivo, as well as effectively treat collagen induced arthritis and experimental autoimmune encephalomyelitis in HLA-DR transgenic mouse models.

Keywords

autoimmunity; MHC class II; peptidomimetics; therapy

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1. Introduction

Major histocompatibility complex (MHC) class II proteins are heterodimers of α and β chains that bind peptide antigens in their peptide binding groove for presentation to helper T cells. Genes of the MHC are highly polymorphic, resulting in a large number of allelic variants of molecules in the population. It is well known that human autoimmune diseases are genetically associated with particular alleles of MHC class II (or class I) genes [1]. For example, the susceptibility to rheumatoid arthritis (RA) is associated with class II HLA-DR alleles *DRB1*0101*, *DRB1*0401*, *DRB1*0404*, and *DRB1*0405*, whereas multiple sclerosis is linked to *DRB1*1501* [2-6]. The amino acid sequences and crystal structures of disease-associated MHC molecules have revealed that, at least in certain autoimmune diseases, e.g., type I diabetes mellitus, and pemphigus vulgaris, MS, and possibly RA, the disease association can be narrowed down to a single polymorphic amino acid residue within the β chain of disease-linked class II molecules [7-11]. These polymorphic residues are located in pockets of the peptide binding site, and critically influence the specificity of peptide binding by imparting selectivity for certain side chains at defined peptide positions (e.g., via charge preference or size limitation), as also demonstrated in peptide binding studies [12,13]. Collectively, the available evidence favors a model that explains disease association with certain MHC alleles by selective binding of autoantigenic peptides in the binding site of the respective MHC molecules. These peptides in turn activate autoreactive T helper cells that trigger and/or maintain the pathological process [14, 15].

The binding of peptides to MHC class II molecules requires specific side chains at defined anchor positions of the peptide to be accommodated in pockets of the binding site. For HLA-DR molecules, the critical anchor residues are at peptide positions 1, 4, 6, 7, and 9 [16-19]. At non-anchor positions, including the amino- and carboxy-terminal regions beyond position 1 and 9, respectively, a large variation of side chains is permitted without influence on binding affinity. The side chains at non-anchor positions usually point out of the binding site, and contact the antigen receptor of T cells (Tcr). This binding mode implies that peptides sharing anchor residues but differing at non-anchor positions (i.e., in antigenicity for T cells) can compete with each other for presentation by the same class II binding site [20]. This mechanism of antigen-competition has been demonstrated to function also in vivo, by choosing two closely related peptides binding to the same class II molecule, one of which (the “competitor”) was not antigenic for T cells due to self tolerance [21].

A possible application of this principle is to construct ligands for autoimmune disease associated class II binding sites that would compete with autoantigenic peptides for presentation and thereby serve as MHC-selective immunosuppressants for treatment of the disease. Such competitors are expected to have the following characteristics: first, they should have sufficiently high binding affinity for the disease associated MHC molecule, second, they should not resemble the autoantigenic peptides from the Tcr point of view, third, they should be able to enter the antigen presenting cells (APC), and reach the endocytic compartment, where loading of the class II binding sites takes place [22,23], and fourth, they should be resistant to proteases, since the loading compartment is rich in cathepsins [22].

Previous efforts to develop pharmacologically useful competitors of antigen presentation have focused on RA-associated HLA-DR molecules [24]. First, the optimal residues at anchor positions 1, 2, 4, 6, and 7 were determined for RA-associated HLA-DR molecules by using random peptide phage display libraries [12,16,17]. Based on this information, a heptapeptide lead was constructed that carried residues accepted by the major RA associated molecules at all five anchor positions, and consequently bound to these molecules with high affinity [18]. Subsequently, guided by a crystal structure [25,26] based HLA-DR molecular model, the amino acid residues in the lead peptide were replaced stepwise with unnatural (mimetic) substituents without significant loss of binding affinity for the target molecules. Several of the resulting mimetic compounds were co-crystallized with HLA-DR4 (DRA1*/DRB1*0401), and were shown to assume a peptide-like conformation in the binding groove of this molecule [27]. In contrast to natural peptides, many of the mimetic compounds were resistant to cathepsins, the major proteases in the peptide loading compartment. Selected compounds were shown to inhibit very efficiently the presentation of processed protein antigens indicating that they were able to reach and survive the class II loading compartment [24]. The use of unnatural residues has also reduced the probability of cross-reaction with natural autoantigenic peptides, the identity of which may not be entirely clear, to an achievable minimum. However, subsequent investigations have revealed that this first-generation of compounds had low stability in plasma, and was thus unlikely to be effective in vivo. We therefore set out to investigate the basis for instability in plasma, and to produce a second generation of compounds that can resist cathepsins as well as rodent and human plasma enzymes, while maintaining binding affinity for the targeted HLA-DR molecules. The production and biological activity of these second generation compounds is reported here.

2. Materials and methods

2.1 Mice

Mice expressing the chimeric (human/mouse) DRA1*0101/DRB1*0101 or DRA1*0101/DRB1*0401 construct (referred to as DR1tg, DR4tg) were raised in the pathogen-free facility at VA Medical Center, (Memphis, TN). The chimeric constructs and the production of the transgenic mice have been described previously [28,29]. Both the α and β chains of the chimeric proteins contain DR α and DR β first domains (encoding the peptide binding site) followed by murine I-E α and I-E β second, transmembrane, and cytoplasmic domains. A distinct, chimeric class II transgenic strain with the DRA1*0101/DRB1*0401 binding site was generated and backcrossed to murine class II deficient mice to result in a strain expressing solely the chimeric class II molecule (referred to as DR4tg-ko) [30]. The latter strain was bred and maintained at Charles River Laboratories (Sulzfeld, Germany).

2.2. Compounds

α -N-Fmoc protected ChaOH, Arg(Pmc)OH, MetOH and NleOH were purchased from Merck-Bioscience (see *Abbreviations* for full chemical names). FmocDiscOH and FmocTicOH were purchased from Neosystems. FmocGpg(Pmc)OH was purchased from RSP-Aminoacids, H[S(*t*Bu) Ψ (oxaz)L]NMe₂ was synthesized as described [31]. Fmoc β PhProOH was synthesized from known N-acetyl-trans-3(*R*)-phenyl-(*S*)-proline-1-(*S*)-

phenylethyl amide [32] as outlined in Figure 1. All other reagents and solvents were purchased from Sigma-Aldrich (Taufkirchen, Germany) or VWR International (Ismaning, Germany).

Compounds p1, p3, p12-1, p31, p66-1, p67 and p31 were synthesized on Rink amide resin by standard solid phase peptide synthesis using Fmoc protection strategy and the aforementioned building blocks. Removal of the side chain protecting groups was performed simultaneously with detachment of the compound from the resin.

Compounds p33, p51, p53, p60 and p61-1 were prepared by solution-phase fragment condensation of the dipeptide mimetic H[S(*t*Bu)Ψ(oxaz)L]NMe₂ and the corresponding Pmc-protected pentapeptide mimetic acids, the latter being synthesized on 2-chlorotrityl chloride resin by solid phase synthesis. Subsequent deprotection of the side chains gave the desired compounds.

Compounds p68, p69, p74, p76-1 and P82 were synthesized by solution-phase coupling of *N*-Methyl-*N*-benzylamine or 2-phenylethylamine, respectively with the corresponding Pmc-protected tetrapeptide mimetic acids, the latter being synthesized on 2-chlorotrityl chloride resin by solid phase synthesis. The desired compounds were isolated after subsequent deprotection of the side chains.

All compounds were purified by preparative HPLC (purity > 90%) and analysed by analytical HPLC and mass spectrometry. The TFA salts were used for all experiments described. Compounds containing Disc were prepared using racemic FmocDiscOH and the resulting diastereomers were separated during purification by preparative HPLC. The diastereomers eluting first showed significantly higher in vitro binding affinity in all cases and were therefore assumed to contain (*S*)-Disc.

2.3 HLA-DR peptide binding assays

HLA-DR molecules were purified from homozygous lymphoblastoid cell lines LG-2 (DRA1*0101, DRB1*0101), PRIESS (DRA1*0101, DRB1*0401), BIN-40 (DRA1*0101, DRB1*0404) and E4181324 (DRA1*0101, DRB1*150201) by affinity chromatography [33] using the murine anti-HLA-DR monoclonal antibody (mab) LB3.1 [34]. The DRB1*150201 chain differs from the MS-associated DRB1*1501 chain only at position 86 (Val vs. Gly). This residue is located in pocket 1 accommodating the side chain of position 1 in the compounds. The consensus residue Cha used in these compounds is accepted by both 86-Val and 86-Gly pocket 1 versions equally well [24].

The previously described competitive binding assay [35] was adapted to a non-radioactive, time-resolved fluorescence readout. The indicator peptide was 6-(biotinamido)-hexanoyl-TyrAlaPheArgAlaAlaAlaSerAlaLysAlaAlaAla-NH₂ designed to bind to many different HLA-DR molecules equally well [35]. Compound dilutions (4 nM-400 μM) and compound-MHC interactions were set up in 96 well polypropylen plates blocked with 1% bovine serum albumine (BSA) in phosphate-buffered saline (PBS) for 1h at room temperature (rt). The interaction mixtures contained 20 μl compound dilution, 10 μl 0.8 μM indicator peptide, 10 μl MHC (15-60 μg/ml, titrated to achieve comparable signal-noise ratios) and 40 μl 2x

binding buffer (2xPBS containing detergents and protease inhibitors as described [30]). Wells without compound and without compound and MHC, respectively, were used as positive and negative controls. Interaction mixtures set up in duplicate were incubated for 16h at rt. The capture plates were high binding capacity black FIA plates coated with 100 μ l LB3.1 (10 μ g/ml) overnight at 4°C, blocked with BSA/PBS as above, and washed with PBS before use. Sixty μ l interaction mixtures were transferred to capture plates and incubated for 2h at 4°C. The wells were then washed 6x with cold DELFIA wash, incubated with cold Europium-streptavidine conjugate (diluted 1:1000 in DELFIA assay buffer) for 30 min at 4°C, washed again 6x, and finally incubated with cold DELFIA enhancement solution for 1h at rt. Time resolved fluorescence was read at λ_{ex} 340 nm and λ_{em} 613 (615) nm in a Wallac Victor² multilabel counter (all DELFIA reagents and equipments were from Wallac-ADL-GmbH, Freiburg, Germany). Fluorescence counts per second were plotted against log₁₀ compound concentration and IC₅₀ values were determined. Mean IC₅₀ values from at least three experiments are reported.

2.4 Assays for protease cleavage of peptidomimetics

Stability of compounds in mouse, rat (Charles River, Sulzfeld, Germany) and human plasma (Bayerisches Rotes Kreuz, Munich, Germany) was determined as described [36].

Compounds were diluted into plasma at 5 μ M final concentrations and incubated at 37°C. Samples were drawn at 0, 6, and 24h, proteins were precipitated with acetonitril, and removed by centrifugation. Supernatants were dried and the residue reconstituted in 40 μ l DMSO and 360 μ l 50% acetonitril+ 0.1% TFA in water. After filtration (0.2 μ m) the solutions were analysed by RP-HPLC. Recovery of compounds at 6h and 24h was calculated from the relative peak area compared to the start (0h) value.

Cathepsins B, C,D (Sigma, Taufkirchen, Germany) and L (Calbiochem, Schwalbach, Germany) were dissolved in assay buffer containing 100 mM NaOAc (cathepsin D), plus 1 mM EDTA and 1 mM DTT (cathepsins B,C,L). Compounds were incubated with cathepsins at a 0.4 U/ μ mol ratio at 37°C for 4h. The reaction was stopped by 50% aqueous TFA. Samples were then centrifuged at 13000 rpm and the supernatants analysed by RP-HPLC. Compound recovery after 4h was calculated by comparing the peak area with the start (0h) value.

2.5 Assays of competition for antigen presentation in vitro and in vivo

Two different assays were used to assess competition for antigen presentation in vitro: first, hen egg lysozyme (HEL)-specific proliferation of T cells from DR4tg-ko mice [30], and second, antigen induced IL-2 production of an ovalbumine (OVA)-specific T cell hybridoma from the same mouse strain.

For the proliferation assay, mice were immunized at the tail base with 100 μ g (3.5 nmoles) HEL emulsified at 1:1 vol/vol with complete Freund's adjuvant (CFA H37 Ra, Difco, Detroit, USA) with two-fold Mycobacterium content. Nine days later inguinal and paraaortic lymph nodes were removed, cell suspensions prepared and the proliferation assay was set up as described [20] in flat bottom 96-well plates in serum-free HL-1 medium (BioWittaker, Walkersville, MD, USA). HEL was added to the cells at half-maximal

stimulatory concentration determined from dose-response curves previously. Three fold serial dilutions of compounds covering 3 log range (0.2-200 μ M) were then added and cells were incubated for 3 days. Controls had 0.1% DMSO instead of compounds, with or without antigen. A reference inhibitory compound was included in each assay to provide a measure of inter-assay comparability. Proliferation was determined by 3 H-thymidine incorporation during the last 8 hours of culture. The overall proliferative capacity of cells was assessed by their response to the purified protein derivative of tuberculin (PPD, 50 μ g/ml final concentration; Statens Serum Institute, Copenhagen, Denmark). Each datapoint represents the mean cpm of triplicate cultures with standard deviation (usually not exceeding $\pm$ 15% of the mean).

For assays of antigen-specific IL-2 production, a T cell hybridoma THyb-1 was generated by fusion of OVA-immune lymph node cells from DR4tg-ko mice with the T cell receptor negative variant of the BW5147 thymoma using standard procedures [37]. Erythrocyte-free spleen cells from DR4tg-ko mice served as APC (1.5×10^5 /well). APC were preincubated with 400 μ g/ml OVA at 37°C for 30 min and subsequently irradiated (5000 R). Peptidomimetics were added at different concentrations and incubated with APC plus antigen for 1h before adding THyb-1 (2×10^5 /well). Cultures were set up as triplicates in flat bottom 96-well plates. After 24h at 37°C, supernatants were collected and assayed for IL-2 by the OptiEIA mouse IL-2 ELISA kit (Pharmingen, San Diego, CA, USA). Each supernatant was assayed up to 10 times, and the average value was used to determine IL-2 concentration by means of IL-2 standard curves. The data are presented as mean IL-2 concentrations of triplicate cultures.

For in vivo inhibition of T cell priming DR4tg-ko mice were co-immunized with 100 μ g HEL and 210 nmoles of compound (=60 fold molar excess of compound over antigen) emulsified in CFA. Nine days later proliferation assay was set up as described above with lymph node cells from at least 3 mice/compound (both individual and pooled cell suspensions were assayed). Dose-response curves for HEL were established for each group, and compared to the dose response curve of cells from mice immunized with HEL only. Inhibition of priming was reflected in parallel curves with lower plateau responses from the co-immunized than from HEL-immunized mice. Per cent inhibition was calculated as the average % reduction of response at the three highest HEL concentrations compared to the control group (immunized with HEL only). Responses to PPD (see above) served as positive/specificity control.

2.6 Autoimmune disease models

For the induction of autoimmune arthritis, DR1tg and DR4 tg mice were immunized with an emulsion composed of equal parts of CFA containing 4 mg/ml of heat-killed *M. tuberculosis* (Central Veterinary Laboratory, Surrey, UK) and bovine type II collagen (bCII) solubilized at 4 mg/ml in 50 mM acetic acid. Native bovine type II collagen was purified from bovine articular cartilage as previously described [38]. Mice were immunized with 50 μ l of the emulsion (100 μ g of bCII) at the base of the tail. The DR4tg mice received a booster immunization of 100 μ g of bCII emulsified in incomplete Freund's adjuvant 21 days later. The development of arthritis was evaluated by grading each paw on a scale of one to four,

with four signifying the most severe arthritis [38]. Mice were examined for incidence and severity of arthritis 2 to 3 times per week. Treatment of mice with peptidomimetics started after disease onset, and continued for three weeks, using a dose of 250 nmoles, once a day, 5x/wk s.c. This dose was chosen on the basis of preliminary toxicity studies in mice that have demonstrated acute, transient sedative (probably CNS-related) effects of these compounds starting at a dose of 500 nmoles. Half of this dose (chosen in these studies) thus does not cause any acute sedation symptoms, and it corresponds to ~1/10 of the maximal tolerated dose.

Experimental autoimmune encephalitis (EAE) was induced in DR4tg-ko mice by 0.1 mg of myelin oligodendrocyte glycoprotein (MOG) emulsified in CFA in a total volume of 100 μ l, 50 μ l of which injected into each hind footpad. Simultaneously, 400 ng of pertussis toxin (List Biological Labs., Campbell, CA, USA) was injected i.p. For the disease induction mice were anesthetized with Xylazin (10 mg/kg) and Ketamin (100 mg/kg) i.p., and the eyes were protected with Bepanthen drops. Recombinant MOG corresponding to the N-terminal domain of the rat protein, was expressed in *E. coli*, and was purified as described [39]. The disease was scored as follows: score 1: limp tail or hind limb weakness; score 2: limp tail together with hind limb weakness; score 3: partial hind limb paralysis; score 4: total hind limb paralysis; score 5: moribund (sacrificed for ethical reasons). Clinical observations were performed daily from day 7 on, or on days of therapy. Immediately after disease onset mice were treated with 250 nmoles of peptidomimetics s.c. daily for 8 days, and subsequently 5x/wk. The total treatment period was 3 wks.

2.7 Statistical analysis of the data from disease models

The arthritis incidence data, because of their categorical (yes/no) nature, were analysed by Chi-square statistics. In contrast, the severity scores of CIA and EAE are ordered quantities and are not normally distributed. Thus the Wilcoxon rank sum test was the appropriate statistics to analyse these data. In both statistics, *p* values <0.05 are considered significant.

3. Results

3.1. Optimization of peptidomimetic compounds for plasma stability

First-generation HLA-DR antagonists that were potent inhibitors of antigen presentation, in vitro [24], were tested for stability in rodent and human plasma. As exemplified by compound pR1784 (Table 1), these compounds exhibited insufficient plasma stability, and thus were unlikely to be effective in vivo. We selected compound p33 for investigating the basis for instability in plasma. Mass spectroscopy analysis of p33 incubated with rat plasma has shown several metabolites, including a full length compound with the methionine residue oxidized, and several fragments corresponding to positions 2-7, 3-7, or 4-7 of the compound, with or without methionine oxidation (data not shown). These experiments identified the stretch from position 2 to 4 as the primary site of metabolic instability. To investigate the metabolically labile part of the compounds more closely, we synthesized a series of tetramer analogs corresponding to positions 1-4 of the heptapeptide. We used the HLA-DR-peptide model to select a number of substituents for positions 2, 3, and 4 that would not interfere with the binding of compounds to the target molecule. Nevertheless, most substituents

proved to have at least some negative effect on binding affinity and/or did not improve the serum stability compounds (data not shown). At position 2, as shown by selected tetramers in Table 1 (p1, p67, p12-1, p66-1), the only favorable substitution was that of Arg to the mimetic, Gpg, which resulted in a substantial improvement in plasma stability, and also in a slight increase of binding affinity. The improved plasma stability was probably due to the elimination of Arg, whose side chain can serve as a recognition site for tryptic peptidases. In fact, compounds with Arg at position 2 were sensitive, whereas those with Gpg were resistant to both trypsin and chymotrypsin (data not shown). At position 3, Tic and Disc were acceptable residues, the latter conferring somewhat improved plasma stability and binding affinity (p1 vs p12-1, and p67 vs p66-1 in Table 1). The Arg to Gpg substitution improved plasma stability also in the heptamer compound series (p31 through p61-1 in Table 1). At position 4, the substitution of Met with Nle also contributed to serum stability (p33 vs p51). The results also indicated an influence of compound length on stability. Thus, the underlying causes for instability in plasma turned out to be complex, nevertheless appropriate substitutions at positions 2, 3, and 4 resulted in tetramer, and heptamer compounds with full stability in rodent and human plasma, resistance to cathepsins, and sufficient binding affinity for the target molecules (e.g., p53, p61-1, p66-1).

In an effort to decrease the molecular mass of compounds, we produced a series of tetramers with carboxy terminal hydrophobic extensions. Tetramers without a hydrophobic tail have proven to be poor competitors of antigen presentation either because their small size does not allow the formation of stable complexes with HLA-DR molecules [35], or they may penetrate poorly into the APC due to their low logP (hydrophobicity) value[40]. The rationale of adding a hydrophobic tail was thus to achieve a moderate increase in size together with a substantial increase of hydrophobicity. As shown in Table 1, the logP values of these compounds could be increased to become comparable to those of heptamers. By using Gpg at position 2 and Nle at position 4, the desired stability in mouse and human plasma could also be achieved in this compound series (e.g., p69, p74). The binding affinity of these compounds for DRB1*0101, and DRB1*150201 was comparable to that of the heptamers, however, they had approximately 10 fold higher IC₅₀s for DRB1*0401 and 0404 than the heptamers.

3.2. In vitro competition of peptidomimetics with processed protein antigens

Two different assay systems were used to test the ability of the new generation of peptidomimetic compounds to interfere with T cell stimulation. First, antigen specific proliferation of HEL-primed lymph node T cells from DR4tg-ko mice, and second, antigen induced IL-2 production of an OVA-specific T cell hybridoma from the same mice. Responses to half-maximal stimulatory antigen concentration were measured in the presence (and absence) of serially diluted peptidomimetic compounds. As shown in Fig.2, both responses were fully dependent on the expression of the chimeric class II molecule, as monoclonal antibodies to HLA-DR first domains (1D09C3, L243, 1-1C4) [41,42] completely inhibited the responses, whereas an HLA-DR second domain-specific antibody (10F12) [42] as well as an isotype control had no effect. Peptidomimetic compounds also inhibited the entire response, although at higher molar concentrations than the antibodies (Fig.2). The entire second-generation of compounds was screened in these two assays, and

the ratio of IC₅₀ and maximal % inhibition, which we termed inhibitory profile index (IPI), was used for ranking of the compounds. Results for selected, informative compounds are shown in Table 2. As expected on the basis of previous studies [35], tetramers, despite good binding affinity for the target molecule were poor inhibitors of antigen presentation. Heptamers were, in general, good inhibitors as was also the case for the first compound generation [24]. Also some tetramers with a hydrophobic tail, despite their inferior binding affinity, proved to be as potent inhibitors as the heptamers. Their potency was parallel to their logP (hydrophobicity) value (see in Table 1). It is thus possible that the size and hydrophobicity of these compounds provide an advantage in cellular uptake by APC.

All compounds were screened for cellular toxicity on T cell hybridoma, and spleen and lymph node cells from DR4tg-ko mice (sources of APC). Within the concentration range used in these experiments (up to 200 μ M), no cellular toxicity was detectable (data not shown).

3.3. In vivo inhibition of T-cell priming by peptidomimetic compounds

Compounds with the three best IPI values and for comparison, additional compounds with position 3 substitutions and tetramers with different hydrophobic tails were tested for in vivo inhibitory activity. The HLA-DR-tg-ko mice used also above were primed with HEL or with a mixture of HEL and a 60 fold molar excess of peptidomimetic in CFA. The proliferative response of primed T cells was tested nine days later, ex vivo, to a range of HEL concentrations. As shown in Fig. 3, the dose response-curves of T cells primed with HEL, or with HEL+inhibitor ran parallel, but the latter had lower plateau responses at identical antigen concentrations than the former. The difference between the response level of HEL-primed and HEL+inhibitor-primed cells was attributed to the effect of the inhibitor on T cell priming. Surprisingly, this difference was fairly constant over a range of HEL concentrations, and this fact permitted the characterization of inhibitory activity by a single % inhibition value (being the average % inhibition at the three highest antigen concentrations; see in Table 3). These studies identified two good inhibitors (p53, p69), and two mediocre ones (p61-1, p74). The first generation compound pR1886, although performed very well in vitro, had only marginal effect in this assay (Fig. 3) probably also due to low plasma stability.

In principle the co-immunization protocol used in these experiments could give rise to both HEL-specific and competitor-specific T cell responses. We therefore tested these primed cell populations for anti-peptidomimetic responses. As shown in Fig. 3, no significant competitor-specific responses were detectable, suggesting that the observed inhibition did not involve any regulatory or competitive interaction at the T cell level, and thus can be attributed to competitive inhibition at the level of antigen presentation. The in vivo inhibition data for the most informative second-generation compounds are summarized in Table 3. The response of the same cell populations to the purified protein derivative (PPD) of Mycobacteria (in CFA) served as an internal control for specificity. The latter is a mitogenic response also comprising class II-independent components, and thus, it is not expected to be inhibited to the same extent as the anti-HEL response. Clearly, compounds p53, p69, and p74 showed the highest levels of inhibition that was also specific, as the PPD

response was only marginally affected. Two additional compounds, p68 and p76-1 failed to inhibit either response, and this fact correlated with their poor plasma stability. Compound p61-1 with Disc instead of Tic at position 3, was not completely stable in mouse serum, and showed a weak inhibition of both responses.

3.4. Treatment of collagen induced arthritis with peptidomimetic compounds

Compounds p53, p69, and p74 that showed the best performance in the in vivo inhibition assay were used for treatment of autoimmune diseases in HLA-DR transgenic animal models. Collagen induced arthritis (CIA), a model for RA, was induced in the DR1tg and DR4tg strains. The DRB1*0101 and DRB1*0401 genes expressed in these strains are known to be associated with susceptibility to RA [1-4]. Although these transgenic strains also express murine class II molecules (IA^f of strain B10.M), these molecules do not appear to participate in the disease, since T cell responses to the eliciting antigen, type II collagen, are inhibited by anti-HLA-DR antibodies, and the wild type strain fails to develop arthritis [28,29]. Treatment of mice with peptidomimetics commenced after disease onset, and continued for three weeks, using a 5x/wk s.c. administration schedule.

As shown in Fig. 4, the disease incidence in DR1tg mice was >80% in the control (solvent treated) group. In the p69-treated group, disease incidence was <30%, but it increased to the control level after cessation of the treatment. Compound p53 had an intermediate effect on disease incidence, however, the effect lasted after the treatment throughout the observation period. The effect of p74 was only marginal. The peptidomimetics also affected disease severity. The p53 compound was the most effective in reducing the severity of arthritis, while p69 was effective only during the treatment period, and again, p74 was the least effective compound. The difference between controls and treated groups reached statistical significance at several timepoints of treatment with p53 and p69, but it was barely significant in the case of p74 (Table 4).

Compounds p53 and p69 also affected CIA elicited in DR4tg mice (Fig. 4). The disease incidence in this model was 50%. Compound p53 affected this parameter similarly to p69 in the DR1tg model, ie., its effect was pronounced, but lasted only until after the cessation of treatment. In contrast, p69 had a moderate but long-lasting effect on disease incidence. Methotrexate was included in this experiment as a positive control (a standard treatment in RA), and it performed comparably to the peptidomimetics. Disease severity decreased in all three treated groups, p69 and methotrexate seemed to be equally potent, whereas p53 was somewhat less effective. Because of the overall lower disease incidence in the DR4tg model, statistical significance was not reached in this experiment, but the trends observed were similar to those seen in the DR1tg model. Indeed, in a recent study with larger number of mice statistical significance was reached also in this model (data not shown). Taken together, two of the selected peptidomimetics, p53 and p69, exhibited a therapeutic effect in both CIA models.

3.5. Treatment of experimental autoimmune encephalitis with peptidomimetic compounds

Compounds p53, p69 and p74 were also used to treat EAE elicited with MOG in the DR4tg-ko mouse strain [30]. Although the HLA-DR transgene in these animals is not the one

strongly associated with MS (DRB1*0401 instead of DRB1*1501), DRB1*0401 is associated with MS in a distinct subgroup of patients [43,44]. In this transgenic strain, MOG elicited the disease in virtually 100% of mice. The occasional (1-2%) failure of disease induction was considered technical, and thus, such animals were excluded from the analysis. Importantly, all animals, treated and untreated, showed at least mild symptoms of EAE, excluding thus the possibility that a failure of induction would be evaluated as a therapeutic effect. The disease in this model assumes two different forms, either severe relapsing, or less severe monophasic. The two forms are induced by the same immunization regime, and probably environmental factors decide as to which disease form will manifest. The effect of peptidomimetics on both disease forms are shown in Fig. 5. Daily treatment with 250 nmoles of compounds s.c. started after disease onset, and continued for 8 days. Subsequently a 5x/wk schedule was applied. The total treatment period was 3 wks.

The monophasic disease in control animals peaked around day 20 after induction with ~2.5 mean severity, and persisted at lower severity level (~1.5) until the end of the observation period. Treatment with p53 did not abolish the early disease peak, but mice in this group recovered faster than the controls, and showed only mild symptoms thereafter. Treatment with p69 completely eliminated the early disease peak and rendered the later, stationary phase of the disease also milder. In this experiment, p69 performed equivalent, if not superior to the standard MS drug, β -interferon. Compound p74 showed, similarly to the CIA study, only a marginal therapeutic effect in the EAE model. Statistical analysis of the data showed significant differences of the p69 and β -interferon treated groups versus control at many timepoints, whereas the treatments with p53 and p74 were significant only at three timepoints (Table 5).

In the relapsing disease shown in Fig. 5, a severe early peak occurred on day 20 after elicitation, with a mean severity score approaching 3.5, and with 50% lethality. The first, mild relapse occurred between days 40 and 45, and a second, more pronounced relapse after day 60. Treatment with p53 reduced the first disease peak very significantly, although did not completely abolish it. Interestingly, although the treatment ended on day 30, its effect was clearly visible on both subsequent relapses. All p53-treated animals survived until the end of the observation period. The difference between the solvent and the p53 treated groups was highly significant during the first disease episode, and also reached significance during both relapses (Table 5).

5. Discussion

Blockade of the MHC peptide binding site was recognized as an immunosuppressive principle decades ago [20,21], but its translation into pharmacologically useful compounds was hampered by several difficulties. First, natural peptides proved to be inappropriate for this purpose, because of poor bioavailability and pharmacokinetics [45]. Second, the structure-based design of non-peptidic MHC class II binding compounds had to wait more than a decade until the necessary crystallographic and structure-activity relationship data became available for the molecules of interest [8,9,16-19,25,26]. Third, the structure-based design of non-peptidic MHC blockers resulted in compounds that were active in vitro [24], but proved to be sensitive to plasma proteases. Therefore, the compounds reported herein

represent the first set of non-peptidic MHC class II blockers with a potential pharmacological utility.

The present compound generation was aimed at achieving stability in plasma while maintaining binding affinity for the three major RA-associated HLA-DR molecules. Binding to the MS-associated HLA-DR molecule was initially not incorporated into the design, however, covering the three RA-associated binding sites required to build in a sufficiently broad cross-reactivity that serendipitously included also the MS-associated molecule (see binding data in Table 1). The critical step in reaching the goal for this compound series was the replacement of the Arg at position 2 with the mimetic Gpg. Since the Arg residue might have served as part of a recognition site for triptic peptidases, its replacement, together with the replacement of Met at position 4 with Nle, resulted in compounds with very good plasma stability.

In addition to the previously explored heptamer compound class [24], we have also produced a novel series of tetramers with carboxy-terminal hydrophobic extensions. Tetramers without the carboxy-terminal tail were also made previously, but these compounds, despite having sufficient binding affinity, were ineffective as competitive antagonists of antigen presentation. A possible explanation for this finding is that short compounds may be preferentially “edited away” from HLA-DR binding sites by HLA-DM molecules in the loading compartment [35]. Another possibility is that the hydrophobicity of these compounds is not sufficient for effective crossing of cell membranes [40], and thus, they do not reach sufficient concentration in the MHC class II loading compartment of APC. Our finding that the novel tetramers exhibit improved inhibitory potential parallel to high logP values underscores the importance of hydrophobicity. A formal proof, however, that these compounds are indeed taken up by APC more effectively remains to be provided.

When tested for inhibition of antigen presentation *in vitro*, the new compounds, both heptamers and tetramers, performed comparably to the previous compound generation. The best compounds inhibited 50% of T cell responses at <10 fold molar excess over antigen. This finding was expected, since all compounds were sufficiently stable against cathepsins. However, a dramatic improvement in the new generation of compounds was seen in inhibiting T cell priming *in vivo*. In this assay the inhibitory potency correlated with plasma stability.

A somewhat unexpected finding was that the peptidomimetic compounds, despite their high binding affinity for class II molecules and competitive inhibitory potential *in vivo*, failed to induce measurable T cell responses, when administered in a typical immunizing schedule (emulsified in CFA). A possible explanation for this favourable property is that five residues in the heptamers (positions 1, 2, 4, 6, 7) and three in the tetramers (positions 1, 2, 4) interact with the presenting molecule, and the remaining, solvent exposed side chains (two in the heptamers and one in the tetramers) may not represent sufficient information for triggering T cells via their antigen receptors. We have previously made similar observations using natural hexa- and heptapeptides with four and five anchoring residues, respectively, that bind to HLA-DR with high affinity. In DR4tg-ko transgenic mice [30], the hexapeptides were not

immunogenic at all, the heptapeptides immunized poorly, whereas longer peptides with the same core sequence induced normal T cell responses (Falcioni and Nagy, unpublished data).

The most important finding of this study is a significant therapeutic effect of compounds of the new series in two different types of autoimmune disease models, CIA and EAE in HLA-DR transgenic mouse strains. The transgenes expressed by these strains are chimeric, consisting of human exon 2 sequences encoding the peptide binding sites, whereas the remaining sequences are of the murine IE genes. The resulting chimeric molecules allow optimal interaction with murine CD4, while presenting peptides capable of binding to HLA-DR [28,29, 30]. These mice are perfectly immunocompetent in terms of T cell responsiveness, and can develop autoimmune diseases often in the absence of a “permissive” genetic background that is required for disease induction in wild type animals. Although the DR1tg and DR4tg transgenic strains used in the CIA studies also express murine class II molecules (I^A^f of strain B10.M), these molecules do not appear to participate in the disease, as the vast majority of T cell responses to the eliciting antigen, type II collagen, are HLA-DR restricted, and the wild type strain fails to develop arthritis [28,29]. In the EAE studies, a possible influence of murine class II is excluded, since the strain used does not express any class II molecule other than the one encoded by the chimeric transgene [30]. Thus the therapeutic effects observed in these models can be attributed to the interaction of compounds with the transgenic HLA-DR binding sites.

The new compounds seem to have sufficient therapeutic efficacy, as they can treat ongoing disease to a comparable extent to methotrexate and β -interferon, the “golden standards” for treatment of RA and MS, respectively. Toxic side-effects were not observed during the treatment period at the doses studied (250 nmoles $\sim$ 10 mg/kg). It remains to be investigated whether the compounds show a therapeutic effect at lower or less frequent doses.

Although the disease models used here are widely accepted, their relevance for human disease is still a matter of debate. Thus a final proof for the therapeutic effect of the peptidomimetic compounds must come from clinical studies. In this context it is interesting to note that Cop 1, a random copolymer of Ala, Lys, Glu and Tyr that has shown clinical efficacy in relapsing-remitting MS [46,47], can bind to several different HLA-DR molecules including the MS-associated one, and compete with the binding of other peptides [48]. The promiscuous HLA-DR binding property of Cop1 is well explained by specific structural features of HLA-DR peptide binding sites [8,9,47,49]. It is thus possible that Cop1 has a similar mode of action to that of the compounds described here, and therefore its clinical efficacy may be regarded as a proof of principle of MHC blockade in human autoimmune disease.

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Glossary

Abbreviations:

Ac	acetyl
APC	antigen presenting cell
bCII	bovine type II collagen
Bn	benzyl
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
Cha	(S)-cyclohexylalanine
CIA	collagen induced arthritis
Disc	(1,3)-dihydro-2 <i>H</i> -isoindole-(S)-2-carboxylic acid
DMSO	dimethylsulfoxide
DR1tg	chimeric DRA1/DRB1*0101 transgenic
DR4tg	chimeric DRA1/DRB1*0401 transgenic
DR4tg-ko	chimeric DRA1/DRB1*0401 transgenic, murine class II deficient
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediamine-tetraacetate
Fmoc	9-fluorenylmethoxycarbonyl
Gpg	(S)- <i>N</i> -amidino-4-piperidinylglycine
Haic	5-amino-4-oxo-1,2,4,5,6,7-hexahydro-azepino[3,2,1- <i>hi</i>]indole-2-carboxylic acid
HEL	hen egg-white lysozyme
IC₅₀	50% inhibitory concentration
IL-2	interleukin 2
IPI	inhibitory profile index
mab	monoclonal antibody
Me	methyl
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
OVA	ovalbumin

PBS	phosphate buffered saline
Ph	phenyl
Pmc	2,2,5,7,8-pentamethylchromane-6-sulfonyl
PPD	purified protein derivative of tuberculin
RA	rheumatoid arthritis
RP-HPLC	reverse-phase high performance liquid chromatography
[SΨ(oxaz)L]	oxazole mimetic of the dipeptide Ser-Leu
<i>t</i>Bu	<i>tert.</i> -butyl
Tcr	T-cell antigen receptor
TFA	trifluoroacetic acid
Tic	tetrahydroisoquinoline-(<i>S</i>)-3-carboxylic acid
βPhPro	2-(<i>S</i>)-3-(<i>R</i>)-3-phenylproline

The three letter code is used for standard amino acids.

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Fig. 1.

A. Synthesis of FmocβPhProOH (through a two step synthesis as described in Materials and Methods). **B:** structure of Gpg, and **C:** structure of [SΨ(oxaz)L].

Fig. 2.

In vitro inhibition of antigen-specific T cell proliferation and IL-2 secretion by peptidomimetic compounds and HLA-DR-specific antibodies. HEL-primed lymph node cells from DR4-tg-ko mice were restimulated in vitro with 2.1 μ M HEL. Inhibitors were titrated into the cultures, and T cell proliferation was measured 3 days later. Thyb-1, a T cell hybridoma generated from the same tg mouse strain immunized with OVA, was tested in the presence of 2.4 μ M OVA, tg spleen cells as APC, and different inhibitors. IL-2 production was measured by ELISA after 24h. L243, 1-1C4, and 1D09C3 are HLA-DR first domain-specific mabs that inhibit T cell activation by antigen plus HLA-DR. 10F12 is a second domain-specific, non-inhibitory anti-HLA-DR mab, control IgG₄ is a polyclonal isotype control (for 1D09C3). Results represent the mean $\pm$ SD of triplicate cultures, except for 1D09C3-IgG₄ and control IgG₄ (single determinations; upper right box).

Fig. 3.

In vivo inhibition of T cell priming to HEL by co-immunization with peptidomimetic compounds. DR4-tg-ko mice were primed with 3.5 nmol HEL or with a mixture of 3.5 nmol HEL and 210 nmol inhibitor. Nine days later, T cell proliferation from primed lymph node cells was tested using a range of concentrations of HEL or inhibitor. The response of pooled cell suspensions from 3 mice per treatment group are shown as mean cpm $\pm$ SD of triplicate cultures.

Fig. 4.

Treatment of collagen induced arthritis in DR1-tg and DR4-tg mice with peptidomimetic compounds. Treatment started immediately after disease onset. Mice (8/group) received 250nmol compound daily s.c., 5x/week for 3 weeks. Control animals received solvent ("DMSO"). Methothrexate (MTx), a standard RA treatment, was given at 1 mg/kg i.p., 3x/wk for 3 wks. Arrows indicate the starting and ending time points of the treatment. Mean severity score is the cumulative score of a group divided by the number of mice in the group. Asterisks indicate significant differences from the control.

Fig. 5.

Treatment of experimental autoimmune encephalomyelitis in DR4-tg-ko mice with peptidomimetic compounds. Treatment started immediately after disease onset. Mice received 250nmol compound s.c., daily for 8 days, and subsequently 5x/week. Total treatment time was 3 weeks. Control animals received solvent. Mouse interferon β , a standard treatment control, was given at a daily dose of 10 000 units using the same schedule. Arrows indicate the starting and ending time points of the treatment. The upper and middle panel depict the same study but different treatments. In the monophasic disease

experiment, the solvent and β -interferon groups comprised 8 mice each, and the remaining groups 6 mice each. The relapsing disease experiment comprised 12 solvent and 5 p53-treated animals. Mean disease score is the cumulative score of a group divided by the number of animals per group. Asterisks indicate significant differences from the control.

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Table 1

Optimization of HLA-DR binding peptidomimetic compounds for protease stability

Compound	Sequence ^a	Binding affinity (IC ₅₀ , μM) for HLA-DRB1 ^b				Protease stability (% remaining after incubation with)						logP ^c
		0401	0101	0404	150201	Rat plasma	Mouse plasma	Human plasma	Cath B	Cath D	Cath L	
p3	Ac-Cha-Arg-Ala-Met-Ala-Ser-Leu-NH ₂	0.16	0.23	0.06	0.13	2	2	0	8	100	0	0.20
pR1784 ^e	Ac-Cha-Arg-Tic-Haic-Ser-Met-Leu-NH ₂	0.28	.	.	.	5	0	73	37	.	.	.
p1	Ac-Cha-Arg-Tic-Met-NH ₂	1.6	0.26	2.3	.	11	0	5	87	100	100	0.68
p67	Ac-Cha-Gpg-Tic-Met-NH ₂	0.41	0.12	1.06	.	34	10	97	100	100	.	1.20
p12-1	Ac-Cha-Arg-Disc-Met-NH ₂	0.37	0.43	0.86	.	67	10	75	0	96	100	0.19
p66-1	Ac-Cha-Gpg-Disc-Met-NH ₂	0.29	0.29	0.59	.	100	81	100	87	100	100	0.71
p31	Ac-Cha-Arg-Tic-Met-βPhPro-NH ₂	0.28	0.29	0.85	0.26	42	5	93	86	100	95	3.69
p33	Ac-Cha-Arg-Tic-Met-βPhPro-[S(oxaz)L]-NMe ₂	0.3	0.19	0.22	0.29	78	40	97	94	98	100	3.88
p60	Ac-Cha-Gpg-Tic-Met-βPhPro-[S(oxaz)L]-NMe ₂	0.23	0.25	0.19	.	100	100	95	100	98	100	4.39
p51	Ac-Cha-Arg-Tic-Nle-βPhPro-[S(oxaz)L]-NMe ₂	0.67	0.4	0.21	0.3	81	60	100	100	100	100	4.77
p53	Ac-Cha-Gpg-Tic-Nle-βPhPro-[S(oxaz)L]-NMe ₂	0.41	0.34	0.18	0.36	100	100	100	100	97	99	5.29
p61-1	Ac-Cha-Gpg-Disc-Nle-βPhPro-[S(oxaz)L]-NMe ₂	0.26	0.22	0.09	.	100	100	84	100	100	100	4.80
p82	Ac-Cha-Arg-Tic-Nle-NHCH ₂ CH ₂ Ph	18	3.1	14	0.41	65	18	31	94	72	100	4.24
p69	Ac-Cha-Gpg-Tic-Nle-NHCH ₂ CH ₂ Ph	3.6	0.69	4.5	0.2	41	42	85	100	93	100	4.76
p68	Ac-Cha-Gpg-Tic-Nle-NHBn	2.1	0.5	2.0	.	7	0	49	13	85	52	4.27
p74	Ac-Cha-Gpg-Tic-Nle-NMeBn	3.4	0.36	2.2	0.3	91	71	97	100	100	100	4.48
p76-1	Ac-Cha-Gpg-Disc-Nle-NMeBn	2.3	0.55	1.4	.	80	84	93	16	93	100	3.99

^aFor the full names of building blocks see Abbreviations^bdesignations of the polymorphic β chain is given. All β chains are complexed with the monomorphic α chain DRA*0101.^cHydrophobicity^dNot tested^eExample of a first-generation compound with good in vitro cellular efficacy^fShaded groups of values represent the best protease stability profiles achieved

Table 2

Inhibition of T cell responses by peptidomimetic compounds, in vitro

Compound	Sequence	Inhibition of T cell proliferation ^a			Inhibition of IL-2 production ^a		
		IC ₅₀ (U/S) ^b	% max ^c	IP ₁ ^d	IC ₅₀ (U/S)	% max	IPI
p3	Ac-Cha-Arg-Ala-Met-Ala-Ser-Leu-NH ₂	>100	15	>6.7	1	>100	10
pR1886f	Ac-Cha-Arg-Tic-Met-βPhPro-Ser-MeLeu-NH ₂	9.5	99	0.096 ^g	1	5.0	92
p1	Ac-Cha-Arg-Tic-Met-NH ₂	40.0	41	0.976	1	>100	0
p67	Ac-Cha-Gpg-Tic-Met-NH ₂	26.2	25	1.048	1	47.9	33
p12-1	Ac-Cha-Arg-Disc-Met-NH ₂	>100	5	>20	1	<i>h</i>	.
p66-1	Ac-Cha-Gpg-Disc-Met-NH ₂	57.1	57	1.002	2	41.7	24
p31	Ac-Cha-Arg-Tic-Met-βPhPro-NH ₂	8.6	64	0.134	1	22.9	64
p33	Ac-Cha-Arg-Tic-Met-βPhPro-[S(oxaz)L]-NMe ₂	.	.	.	.	11.7	91
p60	Ac-Cha-Gpg-Tic-Met-βPhPro-[S(oxaz)L]-NMe ₂	13.8	97	0.142	1	12.5	97
p51	Ac-Cha-Arg-Tic-Nle-βPhPro-[S(oxaz)L]-NMe ₂	17.1	100	0.171	3	25.8	88
p53	Ac-Cha-Gpg-Tic-Nle-βPhPro-[S(oxaz)L]-NMe ₂	19.0	100	0.190	7	10.4	99
p61-1	Ac-Cha-Gpg-Disc-Nle-βPhPro-[S(oxaz)L]-NMe ₂	25.2	99	0.255	2	13.3	89
p82	Ac-Cha-Arg-Tic-Nle-NHCH ₂ CH ₂ Ph	12.8	100	0.128	1	32.5	100
p69	Ac-Cha-Gpg-Tic-Nle-NHCH ₂ CH ₂ Ph	9.0	100	0.090	5	17.5	96
p68	Ac-Cha-Gpg-Tic-Nle-NHBn	8.6	100	0.086	5	16.3	100
p74	Ac-Cha-Gpg-Tic-Nle-NMeBn	8.6	100	0.086	3	19.6	100
p76-1	Ac-Cha-Gpg-Disc-Nle-NMeBn	16.2	100	0.162	1	26.7	100

^a Values are from individual experiments or means from the indicated number of experiments^b Molar ratio between inhibitor and stimulator (antigen). Antigen concentrations used: HEL: 2.1 μM, OVA 2.4 μM^c % maximal inhibition achieved at the highest inhibitor concentration used (100 μM)^d Inhibitory profile index= IC₅₀%maximal inhibition^e number of independent experiments^f Example of a first generation compound with good in vitro cellular activity

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Top three inhibition values are shaded
 $I_{H,N}$
Not tested

Table 3

Selectivity of inhibition of T cell priming by peptidomimetic compounds, in vivo.

Compound	Sequence	Binding affinity (IC ₅₀ , μM) for HLA-DRB1*0401	%stability in mouse plasma		%reduction of response to ^a	
			4h	24h	HEL	PPD ^b
p53 ^c	Ac-Cha-Gpg-Tic-Nle-βPhPro-[S(oxaz)L]-NMe2	0.41	100	100	68.4	10.2
p61-1	Ac-Cha-Gpg-Disc-Nle-βPhPro-[S(oxaz)L]-NMe2	0.26	87	84	37.9	32.3
p69	Ac-Cha-Gpg-Tic-Nle-NHCH2CH2Ph	3.6	100	85	59	2
p68	Ac-Cha-Gpg-Tic-Nle-NHBn	2.1	49	13	-3.2	25.1
p74	Ac-Cha-Gpg-Tic-Nle-NMeBn	3.4	100	97	52.6	3.3
p76-1	Ac-Cha-Gpg-Disc-Nle-NMeBn	2.3	93	16	6.2	-28.6

^a Average reduction of proliferative response to 300, 100 and 33 μ g/ml of HEL of T cells from mice co-immunized with HEL₊-inhibitor, compared to mice immunized with HEL alone. Values were calculated from one (p53, 61-1, 68, 76-1) or two (p69, 74) experiments. Co-immunization of DR4 tg mice: 3.5 nmol HEL₊+210 nmol compound in CFA

^b Purified protein derivative of CFA used at 50 μ g/ml.

^c Compounds with the best selective inhibitory capacity and the respective inhibition values are shaded.

Table 4
Statistical analysis of CIA in DR1tg mice treated with peptidomimatic compounds

Day after CIA induction	Arthritis incidence p ^a			Arthritis severity p ^b		
	p53	p69	p74	p53	p69	p74
19	0.302	0.302	0.302	1.000	1.000	1.000
22	0.021 ^c	0.021	0.021	0.077	0.077	0.077
26	0.039	0.007	0.039	0.026	0.026	0.082
29	0.317	0.131	0.614	0.106	0.067	0.302
33	0.614	0.131	0.614	0.183	0.093	0.281
36	0.131	0.012	0.302	0.061	0.024	0.168
40	0.302	0.046	0.302	0.066	0.054	0.172
43	0.302	0.046	0.590	0.096	0.054	0.259
47	0.131	0.590	0.590	0.021	0.056	0.241
50	0.039	0.522	0.248	0.007	0.028	0.147
54	0.039	1.000	0.522	0.006	0.145	0.247
57	0.039	1.000	0.522	0.005	0.202	0.238
60	0.039	1.000	0.522	0.005	0.121	0.238
69				0.003	0.223	0.222

^aBased on the chi-square test (compared to the DMSO group)

^bBased on the Wilcoxon rank sum test (compared to the DMSO group)

^cSignificant (p<0.05) values are in bold face italics

Table 5

Statistical analysis of EAE data

Day after EAE induction	Monophasic EAE p ^a				Relapsing EAE p ^a	
	p53	p69	p74	IFB	p53	
12	1.000	1.000	1.000	1.000	1.000	
13	1.000	0.750	0.750	0.137	0.231	
14	0.334	1.000	0.198	0.259	0.071	
15	0.258	0.592	0.039 ^b	0.311	0.030	
16	0.072	0.131	0.111	0.508	0.013	
17	0.107	0.444	0.863	0.512	0.025	
18	0.691	0.227	0.895	0.083	0.019	
19	0.571	0.146	0.920	0.041	0.021	
20					0.022	
21					0.053	
22	0.186	0.004	0.404	0.063	0.023	
23	0.252	0.027	0.550	0.151		
24	0.103	0.022	0.579	0.109	0.015	
25	0.014	0.012	0.139	0.023		
26	0.154	0.060	0.199	0.065	0.029	
27					0.038	
28					0.010	
29	0.224	0.004	0.430	0.163	0.143	
30	0.070	0.008	0.213	0.017	0.143	
31	0.103	0.007	0.094	0.010		
32	0.047	0.093	0.136	0.010		
33	0.074	0.119	0.045	0.010	0.333	
34					0.333	
35					0.333	
36	0.238	0.027	0.502	0.341	0.333	
37	1.000	0.457	0.605	0.831	1.000	
38	0.599	0.060	0.289	0.163		

Day after EAE induction	Monophasic EAE p ^a			Relapsing EAE p ^a	
	p53	p69	p74	IFβ	p53
39	0.144	0.082	0.348	0.026	
40	0.781	0.597	0.967	0.133	0.448
41	0.214	0.095	0.874	0.020	1.000
42	0.444	0.015	0.391	0.400	0.143
43	0.755	0.021	0.973	0.309	0.048
44	1.000	0.008	0.679	0.272	0.143
45	0.504	0.009	0.552	0.135	0.143
46	0.152	0.022	0.019	0.028	
47	0.108	0.131	0.781	0.215	0.667
48					1.000
49	0.216	0.012	0.765	0.097	1.000
50	0.252	0.037	0.504	0.048	1.000
51	0.020	0.007	0.346	0.006	1.000
52	0.224	0.268	0.857	0.038	
53	0.112	0.084	1.000	0.115	
54	0.380	0.270	0.783	0.013	0.667
55					0.667
56					0.667
57					0.667
58					0.667
61					0.229
62					0.190
63					0.067
64					0.029
66					0.019
68					0.010
69					0.048
70					0.095

^a p-values based on the Wilcoxon rank sum test (compared to controls)

^b Significant (p<0.05) values are in bold face italics