

Review

Peptide-based approaches to treat lupus and other autoimmune diseases

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This paper is dedicated to Professor Pierre Youinou, as part of the journal series that recognizes outstanding immunologists. Professor Youinou is a long-time colleague and dear friend of two of us (SM and JPB). All these years, his enthusiasm for science was unfailing as his generosity for helping young researchers start playing in “the big league”. It is with a deep recognition that we dedicate this review to Pierre. Finally, we note that this issue is part of the long-term commitment by the Journal of Autoimmunity to recognize distinguished figures in autoimmunity, individuals previously recognized have included Ian Mackay, Chella David, Harry Moutsopoulos and Noel Rose [125–128].

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Altered peptide ligand

ABSTRACT

After a long period where the potential of therapeutic peptides was let into oblivion and even dismissed, there is a revival of interest in peptides as potential drug candidates. Novel strategies for limiting metabolism and improve their bioavailability, and alternative routes of administration have emerged. This resulted in a large number of peptide-based drugs that are now being marketed in different indications. Regarding autoimmunity, successful data have been reported in numerous mouse models of autoimmune inflammation, yet relatively few clinical trials based on synthetic peptides are currently underway. This review reports on peptides that show much promises in appropriate mouse models of autoimmunity and describes in more detail clinical trials based on peptides for treating autoimmune patients. A particular emphasis is given to the 21-mer peptide P140/Lupuzor that has completed successfully phase I, phase IIa and phase IIb clinical trials for systemic lupus erythematosus.

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1. Introduction

Current pharmacologic treatments for inflammatory and autoimmune diseases are largely palliative rather than curative. Most of them result in nonspecific immunosuppression, which can be associated with disruption of innate and induced immunity with significant, sometimes dramatic, adverse effects [1]. Among the novel strategies that are under development, targeting specific molecular pathways and cells to specifically modulate the immune system and restore normal tolerance mechanisms is central.

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The therapeutic peptides market emerged in the 1970s when Lypressin, a vasopressin analogue, was launched. Some 60 peptide drugs are currently on the market and hundreds are in the pipeline [2–4]. Technical developments in chromatographic equipment and price reductions for reagents and amino acid derivatives used in peptide synthesis contribute lowering the cost of synthesizing such peptides at up to multi-kilogram scale with full compliance with Good Manufacturing Practice rules.

Beside numerous advantages peptides have over other drug candidates (higher activity per unit mass, increased selectivity and specificity, greater stability at storage, weaker immunogenicity, better organ or tumor penetration), some limitations require attention. These limitations concern in particular their bioavailability and biodistribution. The extent of these drawbacks will depend on their sequence and structure. Some peptides display a rapid hepatic or renal clearance; some are rapidly degraded by

proteolytic enzymes. A number of original strategies have been developed to overcome these limitations. Introduction of non-natural residues or pseudo-peptide bonds as well as cyclisation approaches have been exploited with success to increase peptide stability without altering their bioactivity [5–9]. Lipopolysaccharides, fatty acids, and polyethylene glycol conjugates were used to enhance their bioavailability and membrane permeability. The intracellular delivery of peptides using membrane permeable carrier peptide vectors also showed some promises. Finally their formulation with nanocarriers, such as liposomes, nanoparticles or nanocapsules was found to be advantageous.

The route of peptide administration is also central. The way a peptide is given can dramatically modify its bioactivity. Among non-invasive delivery routes, oral delivery of therapeutic peptides remains problematic. Except for cyclosporine, very limited success has been achieved using this route of administration, the pulmonary and transcutaneous routes providing so far the most encouraging data for non-invasive peptide delivery [10–12].

Peptide-based immunotherapy has been evaluated with success in several appropriate experimental animal models, yet a few peptides are currently evaluated in clinical trials for the treatment of human chronic inflammatory diseases [13,14]. In a near future, such therapeutic peptides might find important applications in addition to other strategies, which are more commonly put forward, such as gene and cellular therapies or therapies based on monoclonal antibodies that currently represent one of the fastest growing areas of new drug development within the pharmaceutical industry [15].

2. Candidate peptides that target adverse inflammatory responses

Synthetic peptides targeting adverse inflammatory responses have been developed and introduced in different therapeutic strategies. For example some peptides have been used to advantageously replace cognate anti-inflammatory proteins, which are laborious to produce in pure form. This is the case of a peptide termed Ac2-26, spanning the first 24 amino acid residues of annexin 1, which mimics human recombinant annexin 1 for its ability to inhibit neutrophil extravasation in models of acute inflammation [16].

Peptides have also been used as immunogens in active vaccination strategies developed for neutralizing the interaction of proinflammatory cytokines to their receptor(s) [17]. For example, this strategy showed promises in a mouse model for rheumatoid arthritis (RA) in which vaccination with a biologically inactive but immunogenic human TNF α derivative [keyhole limpet hemocyanin (KLH)-human TNF α heterocomplex] led to the production of high titers of neutralizing antibodies and protection from spontaneous arthritis [18]. Based on the same active vaccination process, the peptide YCYISTSQAIEHKPVFLGC derived from murine IL-1 β used under a cyclic form, was shown to protect model animals against induction of collagen-induced arthritis [19] and an IL-2/IL-23 9-mer peptide conjugated to HBCAg ameliorated acute and chronic murine colitis [20]. Other successful vaccination data have been related in mouse models based on IL23p19 peptide in collagen-induced arthritis and asthma [21,22]. Clinical trials are in progress in RA, systemic lupus erythematosus (SLE), Crohn's disease and type 1 diabetes with entire cytokines administered under the form of KLH conjugates also called kinoids [23], yet to our knowledge, there is no clinical trial based on vaccination of patients with cytokine-issued peptides.

Peptides derived from the complementary determining regions (CDRs) of autoantibody VH domains have also shown remarkable therapeutic efficacy in lupus mice. They are for example the

so-called 15-mer pCons peptide (FIEWNKLRFQGLEW), a consensus of sequences derived from the VH region (CDR1 and second framework FR2) of several different DNA-reactive autoantibodies from (NZBxNZW)F1 (NZB/W) mice [24,25] and the 19-mer peptide GYYWSWIRQPPGKGEWIG issued from the CDR1 sequence of a monoclonal anti-DNA autoantibody that bears the common idiotype 16/6Id (hCDR1/edratide/TV-4710) [26–29]. Initial clinical investigations in patients with SLE showed that pCons activated T cells secrete cytokines at levels that were higher than in healthy individuals [30] and that the tolerogenic hCDR1 peptide down-regulated pathogenic cytokines and up-regulated immunosuppressive molecules and regulatory T cells in peripheral mononuclear cells (PBMCs) [31]. Disappointedly, however, hCDR1 that was safe and well-tolerated in human did not give expected results in a randomized, double-blind, placebo-controlled phase II clinical trial in 340 SLE patients (PRELUDE trial).

The paradoxical use of self-antigens and more particularly of peptides derived from autoantigens hold important promises to treat autoimmune and inflammatory diseases. Thus, numerous peptides have been successfully tested in induced and spontaneous murine models of autoimmunity. This is the case, for example, of peptides derived from histones H4, H3 and H1' [32–37] or of the so-called 21-mer peptide VRT102 (KEGYKVRDLNLTLEFRITTSK) of laminin α -chain administered to lupus-prone mice and shown to prevent antibody deposition in the kidneys, ameliorate renal disease and markedly increase longevity of treated mice [38]. Insulin B chain peptide 9–23 (B: 9–23; SHLVEALYLVCGERG) and analogues of this sequence were also used to protect nonobese diabetic (NOD) mice from diabetes [39–41]. Thus Eisenbarth and coll. demonstrated that intranasal administration of an analogue of insulin B: 9–23 peptide (the CTL sequence was truncated) to NOD mice (20 μ g/administration starting at 4 weeks of age, and administered at days 1–5, 8 and then weekly until 10 weeks of age) resulted in the elimination of spontaneous insulin autoantibodies, a significant inhibition of insulinitis and a remission from hyperglycemia, and prevented the progression to diabetes [42]. To our knowledge, however, none of these promising peptides were evaluated clinically in patients.

Tolerization with peptides of heat shock proteins (Hsps) have also been attempted. For example, assays have been conducted with peptides of Hsp60 for modulating experimental Sjögren's syndrome (SS) in the NOD mouse model [43–46]. At 21 weeks of age, nondiabetic mice that received the Hsp60 peptide 437–460 (VLGGGVALLRVIPALDSLTPANED; also called P277) in incomplete Freund's adjuvant showed a significant reduction of SS-related histopathologic features. A randomized, double-blind, phase I/II study therapy with the hsp60 diaPep277TM in recent onset type 1 diabetic patients demonstrated the safety of the tandem repeated peptide construction. Phase II trials with DiaPep277 that was elaborated by I. Cohen and his team at the Weizmann Institute of Science showed a significant preservation of β -cell function in adult patients (but not children) with type 1 diabetes. Unfortunately, however, compared to placebo, no differences were seen in treated patient in the glycosylated haemoglobin levels and daily insulin requirement [47–49]. Two large Phase III trials are currently engaged and should give important information [50].

Lehner and coll. evaluated another Hsp60 peptide for preventing relapses of uveitis in Bechet's disease [51]. A phase I/II clinical trial was undertaken with the Hsp60 peptide 336–351 (CQPHDLGKVGIVTKD) linked to recombinant cholera toxin B subunit, and administered orally. The peptide was shown to be safe and withdrawal of the immunosuppressive drugs showed no relapse of uveitis in 5 of 8 treated patients. Further investigation on a larger scale is warranted.

A 15-mer synthetic peptide called dnaJP1 derived from dnaJ/Hsp40 (QKRAAYDQYGHAAFE) and given orally to patients with active RA gave promising results in a pilot double-blind, placebo-controlled phase II clinical trial [52]. Patients received 25 mg of peptide (or placebo) daily during 6 months. The peptide which was safe and well-tolerated led to a significant reduction in the percentage of T cells producing TNF α and an increase percentage of IL10-secreting T cells. Differences between clinical response in treated and placebo-treated groups were found and this trend was also observed when dnaJP1 peptide was given together with hydroxychloroquine. This peptide is remarkable since in addition to encompass an Hsp-derived sequence, it mimics a so-called “share epitope” sequence common to HLA alleles associated to RA.

Some strategies used altered peptide ligands (APL) for the T cell receptor (TCR) to modulate deleterious immune responses. APLs are peptide analogues derived from antigenic sequences that encompass amino acid substitutions or backbone modifications altering TCR contact residues [53–62]. Following APL binding in the MHC context and TCR engagement, normal T cell functions are generally impaired. Some of these APLs (antagonists) are able to specifically antagonize and inhibit T cell activation. Others called partial agonists deviate T cell signaling and can therefore change their Th1/Th2 phenotype and cytokine secretion. Impressive results were described in different experimental models based on the use of APLs [for reviews, see [63–65]]. Oral administration of a retro-inverso analogue of encephalitogenic myelin basic protein (MBP) peptide 87–99 was found to significantly decrease severity and delay the onset of autoimmune encephalomyelitis in treated mice [66]. In retro-inverso analogues, also referred to as retro-all-D peptides, the amino acid side chains are oriented in the same way as in the original sequence, while the direction of the CO–NH bond in the backbone is reversed [67,68]. Such peptidomimetics display a much higher stability to proteases and represent highly valuable tools for therapeutic strategies. Successful treatments of model mice for experimental autoimmune encephalomyelitis [69,70], diabetes [41,42] and arthritis [71] have been described with APLs.

A phase II clinical trial based on an APL of the cognate MBP peptide 83–99 showed disappointing results in patients with multiple sclerosis (MS). The peptide was poorly tolerated and for the short period of treatment that was rendered possible, no clinical or magnetic resonance imaging parameters improvement could be demonstrated [72]. In another phase II placebo-controlled clinical trial also based on an APL of MBP peptide 83–99, a shift of the MBP-specific T cells response favouring Th2 cytokines over Th1 cytokine production was noted [73]. At lower doses reduction in brain lesions was detected while, at higher doses, exacerbation of the disease was observed. Unfortunately, 9% of treated patients developed immune-mediated hypersensitivity and a safety board suspended the trial. The immunomodulatory decapeptide RDP58 (delmitide), an all-D peptide of sequence arg–nle–nle–nle–arg–nle–nle–nle–gly–tyr–CONH₂, was found to be effective when given orally to patients with mild-to-moderate ulcerative colitis [74]. This rationally-designed peptide derived from the heavy chain of HLA class I molecules was also evaluated in different mouse models (e.g. experimental autoimmune encephalomyelitis, interstitial cystitis) with promising results. It inhibits the synthesis of pro-inflammatory cytokines (TNF α , IFN γ , IL12 and IL2) by disrupting cell signaling at the pre-MAPK MyD88-IRAK4-TRAF6 protein complex. To date approximately 400 patients and volunteers have received RDP58, which is safe and well tolerated.

Glatiramer acetate (also known as Cop-1, Copaxone) is a random amino acid copolymer [poly (YEAK)_n] [75]. It immunologically cross-reacts with MBP both at the antibody and T-cell level and suppresses experimental autoimmune encephalomyelitis (EAE) disease in several animal species. Glatiramer acetate

slows-down the progression of disability and reduces relapse rate in exacerbating-remitting patients with MS. It is not immunosuppressive and not toxic. The product was approved by the Food and Drug Administration in 1995, and is used today by tens of thousands of MS patients [76,77]. The mechanism of action by which glatiramer acetate is beneficial in central nervous system autoimmune disease is thought to be mediated through a preferential Th2 deviation of myelin-specific T cells [78,79]. Glatiramer acetate also prevents graft-vs-host disease and transplant rejection and may also be effective in models of other autoimmune diseases such as experimental uveoretinitis and inflammatory bowel disease. It is noticeable that glatiramer acetate has no effect in NZB/W lupus mice [80]. Interestingly glatiramer acetate, which can be considered as an APL in EAE (and possibly MS), is among the first random complex mixture of polypeptides to be used as therapeutic immunomodulator based on the striking property of T cell recognition to harbor a certain degree of degeneracy.

3. A brief history of peptide P140

It is in studying a series of overlapping peptides designed to identify T cell epitopes of the spliceosomal U1-70K snRNP protein (U1-70K) that a sequence recognized by mouse CD4⁺ T lymphocytes was characterized in residues 131–151 (RIHMYYSKRSGKPRGYAFIEY) of the protein. The U1-70K snRNP protein was initially chosen for this study because this protein, in particular the RNA recognition motif (RRM), represents an important target for both autoantibodies (found in 30% of patients) and CD4⁺ T cells from mice and patients with lupus [81–83]. The RRM sequence 131–151, which encompasses a conserved motif involved in the binding of the protein to U1 RNA within the small ribonucleoparticle (snRNP) U1, was found to be recognized early during the progression of the disease by IgG antibodies and CD4⁺ lymph node (LN) T cells from H-2^k MRL/lpr and H-2^{d/z} NZB/W lupus-prone mice [84,85]. It was discovered later that a peptide analogue phosphorylated on Ser¹⁴⁰ (named P140) was strongly recognized by LN and peripheral MRL/lpr CD4⁺ T cells [86]. The peptide analogue synthesized with a phosphoSer¹³⁷ residue was also recognized by MRL/lpr T cells but left behind because of its poor solubility. In contrast, peptide analogues containing acetylated lysine residues in positions 138 and 142 were unable *ex vivo* to induce the proliferation of MRL/lpr T cells and IL-2 secretion. Consistent with the above-mentioned experiments with MRL/lpr and NZB/W T cells, P140 peptide was found to behave as a promiscuous epitope with regard to murine MHC molecules as well as to human HLA molecules [86,87]. As in the mouse model, peptide 131–151 of the U1-70K protein induced *ex vivo* proliferation of CD4⁺ T cells from lupus patients. Unexpectedly, however, it was observed that phosphorylation of Ser¹⁴⁰ prevented human T cell proliferation. This did not affect the production of regulatory cytokine IL-10 in the cell culture when lupus patient's peripheral CD4⁺ T cells – but not T cells from patients with other systemic autoimmune diseases – were incubated in the presence of the P140 analogue (IL-4 and IFN γ were not detected in the cultures) [87]. In a protection protocol set-up in MRL/lpr mice, the non-phosphorylated peptide 131–151 was found ineffective. However, the phosphorylated peptide administered four times intravenously in saline significantly prolonged survival of treated mice (Fig. 1), diminished their proteinuria, the extent of dermatitis and vasculitis (with less perivascular inflammatory infiltrates) and serum anti-native DNA antibody levels [86,88].

To better understand the mechanism explaining the efficacy of peptide P140 to change the course of the lupus disease in mouse models, the effect of P140 peptide on lymphocytes proliferation occurring in MRL/lpr mice was examined by monitoring cell counts in the blood and lymphoid tissues from P140-treated mice. P140

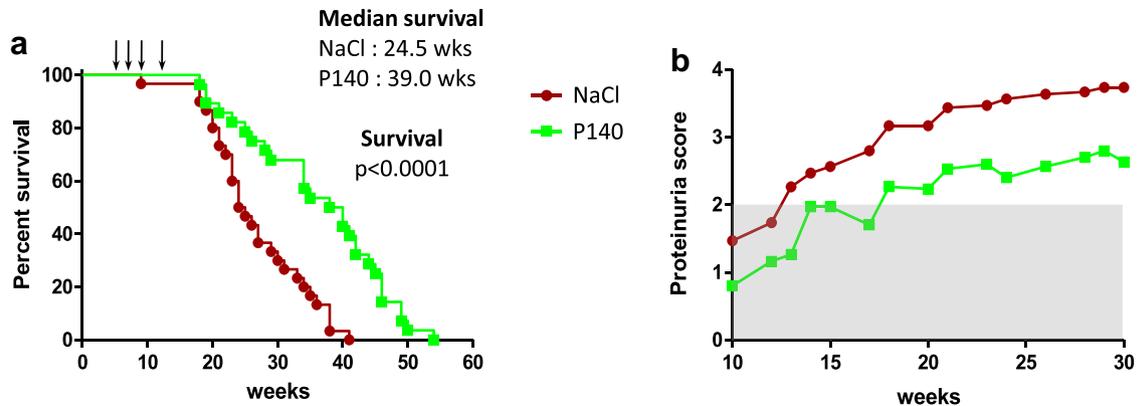


Fig. 1. Treatment of lupus mice with P140 peptide. Five week-old MRL/lpr mice ($n = 30$) were administered i.v. with P140 peptide in saline or with saline only. They were then subjected to three further administrations (weeks 7, 9, 12) in the same conditions, as indicated by the arrows. Their viability and the mean proteinuria score are shown in (a) and (b), respectively. Survival of control and P140 analogue-treated female MRL/lpr mice was analyzed by the Kaplan–Meier method, and the significance of differences was determined by the log-rank test. The grey zone corresponds to the normal zone (score ≤ 2).

administration markedly reduced peripheral blood cell counts, notably $\alpha\beta\text{TCR}^+/\text{CD3}^+/\text{CD4}^-/\text{CD8}^-/\text{B220}^+$ double-negative T cells that accumulate with age in MRL/lpr mice and activated T cells and B lymphocytes, via a mechanism of granzyme B- and caspase-dependent apoptosis. Apoptosis was not detectable in $\gamma\delta$ T cells-depleted mice [89]. Of first importance, remaining peripheral cells were potent at proliferating normally ex vivo in the presence of B or T cell mitogens [88]. In contrast, proliferation of B and T cells from MRL/lpr mice treated with a glucocorticoid (prednisolone) or an immunosuppressant (cyclosporine A) was significantly reduced. This result associated to the observation that P140-treated mice respond normally to a viral infection [90] led to the conclusion that P140 peptide does not behave as an immunosuppressant but rather as an immunomodulator of the autoimmune response.

In this process, the phosphoSer¹⁴⁰ residue appears central. This modification was found to exist naturally in early apoptotic Jurkat cells [91]. It was discovered that in the early stages of apoptosis the basal phosphorylation of the Ser¹⁴⁰ residue increases very significantly and in parallel, a caspase-dependent, PP1-mediated dephosphorylation of other serine residues occurs in a subset of U1-70K proteins, which are heavily phosphorylated [92]. We found that the hypo-phosphorylated U1-70K protein carrying a phosphoSer residue at position 140 is then clustered in heterogeneous ectopic RNP-derived structures, which are finally extruded in apoptotic bodies. It is not yet known whether this material displays particular antigenic and/or immunogenic properties and if the same pathway is activated in vivo in lupus cells.

4. P140 peptide, a ligand for more than one single receptor

P140 peptide is a promiscuous MHC binder that is recognized in this context by the TCR of CD4⁺ T cells [86,87]. Using real time biodistribution experiments, we observed that fluorescence of fluorochrome-labeled P140 peptide especially accumulate in the lungs and the spleen of MRL/lpr mice [88]. In the spleen, P140 fluorescence was mainly observed in the lymphoid white pulp mostly in the mantle and marginal zones. P140 homing was thus essentially detectable in organs enriched in activated B cells and more generally in antigen-presenting cells (APCs).

Pull-down experiments performed at room temperature and 4 °C with splenocytes from CBA/J normal mice and MRL/lpr mice revealed that peptide P140 also binds a unique cell surface receptor, the constitutively-expressed chaperone HSC70/Hsp73/HSP 70-8 protein [88,89]. This heat shock protein (Hsp) of the large Hsp70 family is abundant in the cell cytoplasm and nucleus (1–3% of total

cellular protein) and is highly conserved and expressed constitutively in most tissues [93–96]. It was found to be more abundant at the surface of MRL/lpr immune cells [88]. Interestingly, HSC70 (but not Hsp70) was found to be over-expressed in rheumatoid arthritis synovial tissue [97]. HSC70 plays important roles in the presentation of peptides to MHC molecules, both as chaperone of MHC molecules [98,99] and in the autophagy process, notably in chaperone-mediated autophagy (CMA) [100–105].

Cytosolic CMA substrates characterized by a targeting signal corresponding to the degenerated pentapeptide KFERQ are recognized by HSC70. The complex composed of protein substrates, HSC70 and co-chaperones, notably Hsp90, Hsp40, Bcl-2 associate athanogene 2 (Bag-1), hsc70–hsp90 organizing protein (Hop), and hsc70-interacting protein (Hip), is delivered to the surface of lysosomal membrane where it interacts with the lysosome-associated membrane protein type 2a (LAMP-2a) protein. Associated to this translocation complex, substrate proteins undergo complete unfolding and cross the lysosomal membrane with the assistance of the luminal form of HSC70 protein. In the lumen they are rapidly degraded by the resident proteases. The possible involvement of CMA in autoimmune diseases has been discussed. Since CMA as well as macroautophagy is implicated in the delivery of endogenous antigens to lysosomes for their eventual presentation by MHC class II (MHCII) molecules [102,106,107], it might play a decisive role in the maintenance of tolerance and its possible breakdown [108–111]. Consequently also, it is expected that any component interacting with HSC70 can potentially affect this central process and ultimately alter MHC presentation of antigenic peptides to CD4⁺ T cells [112].

Remarkably, it was effectively found that P140 peptide alters autophagic processes in MRL/lpr B cells [88]. The peptide provokes the accumulation of the autophagy markers p62/SQSTM1 and LC3-II, consistent with a down-regulated lysosomal degradation, probably at the autolysosome stage during autophagic flux (a stage that is also affected by E64d and pepstatin A, and by the anti-malarial drug hydroxychloroquine used in lupus treatment [113]). In vitro, P140 peptide was also shown to hamper refolding properties of HSC70 molecule and to induce destabilization of HLA-DR dimers in human lymphoma B cells (Raji cells). Interestingly, P140 led to a significant decrease of MHCII expression at the surface of spleen B cells from MRL/lpr mice that received P140 peptide intravenously, a result that might explain the much weaker reactivity of peripheral T cells from P140-treated MRL/lpr mice with peptides encompassing CD4⁺ T cell epitopes [114]. Although further investigations are required to consolidate this mechanism, it seems plausible that

Table 1
In vitro and in vivo characteristics of P140 and P140 analogues.

	HSC70 binding		Binding MHC II molar excess for 50 % inhibition					Cellularity decrease		MRL/lpr proteinuria	
	Ka μM^{-1}	Kd μM	I-Ed	I-Ad	I-Ek	I-Ak	% vs NaCl	p value	p value	p value	
V Y G P I K R T H M V Y S K R S G K P R G Y A F I E Y E H E R N M											
131-151 P140	1.37	7.32	10.0	10.6	9.90	15.2	40.9	0.0010	0.0001		
Ac 131-151 P140	0.40	24.8	26.4	29.2	26.4	41.3	0.0			NT	
131-151 P140 (I132→V)	2.14	4.67	35.7	44.3	35.8	∅	0.0			0.3079	
131-151 P140 (M134→Nle)	1.74	5.74	10.2	7.80	7.90	11.8	9.4	0.4499	0.4804		
131-151 P140 D-Tyr	1.79	5.59	7.40	6.40	6.00	5.00	4.5	0.6793	NT		
125-151 P140	7.70	1.30	22.7	15.6	25.5	34.0	9.8	0.5120	0.3658		
128-151 P140	2.49	4.02	22.8	18.4	21.3	39.8	21.4	0.0558	0.7969		
132-151 P140	0.50	20.1	23.8	38.3	35.3	∅	43.1	0.0007	0.0902		
135-151 P140	∅	∅	∅	∅	∅	∅	NT	NT	0.8288		
139-151 P140	∅	∅	∅	∅	∅	∅	NT	NT	NT		
131-157 P140	∅	∅	∅	∅	∅	∅	NT	NT	NT		
131-152 P140	2.99	3.34	∅	∅	∅	∅	0.0			0.9633	
131-150 P140	0.58	17.2	27.1	30.6	19.6	27.4	0.0	0.8153	0.7121		
131-148 P140	0.60	17.7	4.70	3.60	7.00	4.50	5.4	0.6464	0.1246		
131-151 P140 Scrambled	∅	∅	∅	∅	∅	∅	1.4	0.8999	NT		
131-151	1.50	6.72	6.10	7.70	4.40	8.60	0.0	0.2629	0.8353		

The peptide P140, 13 phosphorylated analogues of the P140 sequence as well as the non-phosphorylated peptide and the scrambled P140 peptide (ScP140) were studied. The analogue 131–154 was synthesized but its poor solubility precluded its further use. The binding and kinetic parameters of peptide-HSC70 interaction were measured by surface plasmon resonance analysis. The binding of peptide analogues to MHCII molecules was evaluated in a bioassay using MHCII-transfected fibroblast and specific T-hybridomas. In vivo properties of P140 analogues in MRL/lpr mice were evaluated by the decrease of peripheral hypercellularity measured five days after a single intravenous injection of peptide ($n = 5/\text{peptide}$) and by their capacity to delay the protein level measured in the urines of MRL/lpr mice that received four intravenous injections of peptide ($n = 10$ for each peptide analogue and $n = 30$ for P140). The results were compared within the same experiments to mice that received saline only. Statistical significance in the measurement of cellularity was assessed using the Student's t -test. The two-way ANOVA test was used to analyze statistical significance of proteinuria differences between control and peptide-treated groups of mice. P values less than 0.05 were considered significant. NT = not tested; \emptyset = no binding.

P140 peptide acts by altering the autophagy pathway leading thus to a defect of endogenous (auto)antigen processing in MRL/lpr antigen-presenting B cells and a decrease of T cell priming and signaling.

Thus, several pathways of intervention seem to be involved in the mode of action of peptide P140. First, the latter might act as an APL of TCR [87,90,114]. The TCR would interpret subtle changes in its natural ligand (the non-phosphorylated sequence), resulting in

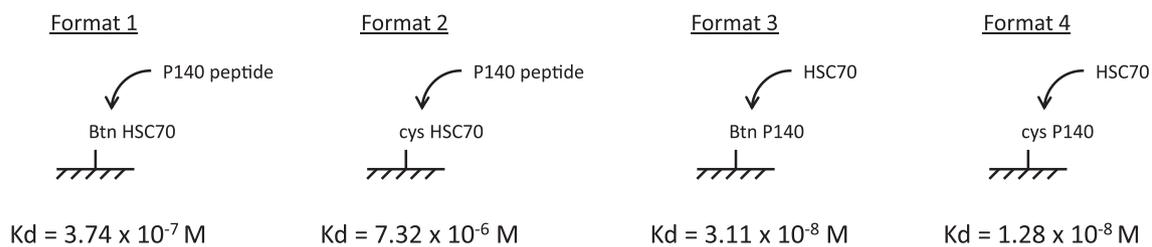


Fig. 2. Different test formats to measure the affinity of P140 peptide for HSC70 by label-free surface plasmon resonance-based technology. BiAcore 3000 system (Biacore AB) was used to evaluate the binding of P140 peptide to HSC70 protein in four different formats. In format 1, biotinylated recombinant bovine HSC70 (Stressgen) was immobilized on flow cells of a sensor chip and in format 2 (used in ref. [89]), HSC70 was immobilized through its thiol groups. In format 3 and 4, P140 peptide was immobilized on the chip either via a biotin- ϵNH_2 Lys moiety or via a thiol group of an additional cysteine residue added at its N-terminus. The direct binding measurement of P140 peptide to HSC70 was carried out at 25 °C with a constant flow rate of 20 $\mu\text{L}/\text{min}$. The kinetic parameters were calculated using the BiAeval 3.1 software and the dissociation equilibrium constant (K_d) values are indicated. Analysis was performed using the simple 1:1 Langmuir binding model, the fitting to each model was judged by the χ^2 value and randomness of residue distribution was compared to the theoretical model.

different phenotypic outcomes. A dramatic effect of APL stimulation with APCs is the induction of anergy as opposed to proliferation, a feature effectively observed when lupus patients T cells from the peripheral blood were incubated with P140 peptide [87]. Recently, the existence of specific CD4⁺ T cells was demonstrated using MHCII tetramers of I-E^k containing the peptide 131–151 or the peptide P140 (P.J. Utz, personal communication). A more frequent population of CD4⁺ T cells specific for the non-phosphorylated sequence was identified supporting the model.

Second, another P140 mode of action might also operate when considering our findings that the peptide selectively interacts with HSC70. Downstream of this interaction, the binding of HSC70 to other chaperones and ligands could be disrupted. This might

greatly alter the endogenous (auto)antigen processing, which occurs via a mechanism involving lysosomal degradation pathway, in which HSC70 is central.

5. Attempts to generate an optimized P140-derived sequence with higher protective efficacy

To identify a putative P140 analogue that could display higher reactivity, a series of peptide analogues were synthesized, encompassing all a phosphorylated Ser¹⁴⁰ residue but differing either by their length or by punctual changes in their sequences (Table 1). The ability of P140 analogues to bind HSC70 was measured by surface plasmon resonance using recombinant HSC70 immobilized

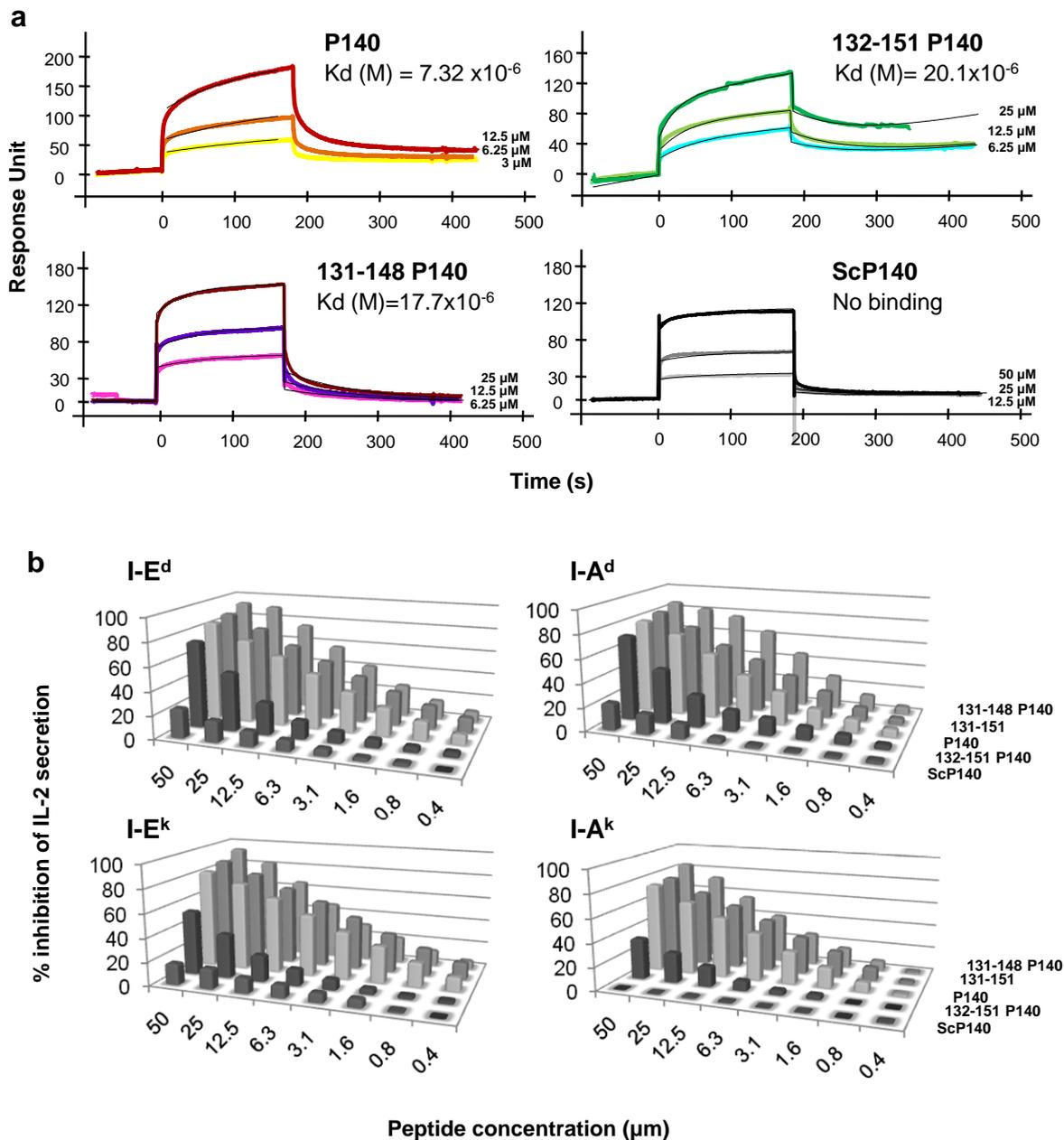


Fig. 3. Binding of P140 and P140 analogues to HSC70 and MHCII molecules. (a) Direct binding to recombinant HSC70 protein of P140 and P140 analogues used at increasing concentrations, as measured by surface plasmon resonance experiments (format 2). The Kd values of P140 peptide, the ScP140 peptide used as control and two analogues shown as example are indicated. (b) Binding assay of P140 and a selected set of P140 analogues to MHCII molecules using mouse L fibroblasts transfected with either I-A^d, I-E^d, I-A^k, or I-E^k molecules as APCs. The capacity of a peptide analogue to bind MHCII molecule is measured by its ability to inhibit IL2 secreted by a specific T cell hybridoma that recognizes a test peptide in the context of the said-MHCII molecule. Data show the mean of 3 independent experiments in duplicate.

onto the sensor chip via the thiol groups of its four cysteine residues. This test format was chosen for screening this large series of peptide analogues although absolute Kd values measuring the affinity of peptides for HSC70 protein were lower compared to other formats (Fig. 2). In the test format 2, P140 binding to HSC70 was not affected by conservative replacements such as Ile¹³² → Val or Met¹³⁴ → NLe, or by modifications such as acetylation of the peptide N-terminus (α NH₂ charge blocked) (Table 1). At the C-terminus, a D-Tyr residue could be introduced instead of the L-Tyr residue without effect. Phosphorylation of Ser¹⁴⁰ had no influence on HSC70 binding. At the N-terminal end, P140 could be shortened by one residue at most (sequence 132–151) while at the C-terminal end it could be shortened by three residues (sequence 131–148, shortest sequence that bound HSC70) (Table 1, Fig. 3a). Compared to the cognate peptide P140, some analogues to which additional residues of the U1-70K protein were added either at the N- or at the C-terminus bound HSC70 with similar affinities (i.e. 125–151, 128–151, 131–152). Of note, the longest fragment 131–157 did not bind HSC70, suggesting that HSC70-P140 interaction depends on conformation adopted by the peptide or on the size of the pocket accommodating the P140 peptide.

The ability of P140 analogues to bind murine MHC molecules was evaluated using a well-established assay based on mouse L-fibroblasts stably transfected with MHCII molecules I-A^d, I-E^d, I-A^k and I-E^k as APCs, and T cell hybridomas recognizing a test peptide in the context of each MHC molecule [84,115,116]. With the only exception of peptide 131–152, we found that all peptide analogues that bind HSC70 also recognize cells transfected with at least three of four tested murine MHCII molecules (Table 1, Fig. 3b). None of the analogues bind MHCII transfected cells only and not HSC70. This result likely reflects the fact that targeting particular sites of HSC70 affects the stable presentation of MHCII molecules that requires chaperoning to be functional [99,117,118].

P140 analogues were also tested *in vivo* for their capacity to lower peripheral hypercellularity in MRL/lpr mice as P140 peptide does [88], and this property was analyzed in comparison to their ability to protect MRL/lpr mice from lupus disease. Although 11 of 14 phosphorylated peptides readily bind HSC70 and 10 of 14 analogues bind MHCII molecules, none of them, apart from P140 peptide and peptide 132–151, was able to induce a minimum 30% decrease of peripheral blood cells (Table 1, Fig. 4). Overall, P140 peptide was the only one able to significantly decrease proteinuria (Table 1; Fig. 1). Thus, in an acute murine model of lupus, none of the peptide analogues, including longer peptides encompassing the P140 sequence, was able to decrease peripheral hypercellularity and delay biological and clinical signs of the disease with an efficacy at least similar to that of peptide P140.

6. Evaluation of P140 peptide/Lupuzor™ in clinical trials

In a phase I and IIa clinical trial conducted by ImmuPharma (Mulhouse, France), the P140 peptide (IPP-201101, Lupuzor™) the sequence of which is totally conserved between the mouse and human, was found to be safe and well tolerated. It led to a significant decrease of marker anti-native DNA antibodies and significantly improved the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and biological status of lupus patients who received three subcutaneous doses of 200 µg peptide given two weeks apart [119]. A higher peptide dose (3 × 1000 µg) was less effective than the lower dose (3 × 200 µg). P140 peptide evaluated in a phase IIb, double-blind, placebo-controlled, dose-ranging study in Europe and Latin America lately showed that the 200 µg dose of Lupuzor administered subcutaneously every four weeks was statistically superior to placebo. Patients enrolled in this study achieved a score ≥4 of the American College of Rheumatology

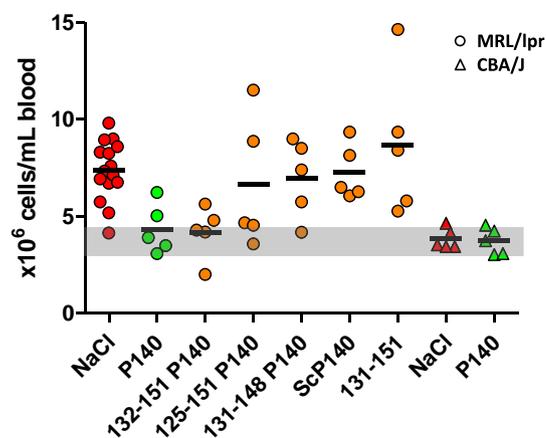


Fig. 4. *In vivo* effect of P140 peptide and P140 peptide analogues on peripheral hypercellularity. Measurements were performed five days after a single injection into 11–13 week-old MRL/lpr or CBA/J mice (100 µg/mouse). A selected set of analogues are shown as example, saline only was administered into control mice (untreated). The grey zone corresponds to the normal counting zone (mean ± SD for CBA/J mice). Each symbol corresponds to one individual mouse. Horizontal bars represent the respective average cell count values. Statistical significance was assessed using the Student's *t*-test.

criteria for SLE, had a clinical SLEDAI ≥ 6, and had no BILAG A (the highest) score (scoring in the British Isles Lupus Assessment Group index is based upon the physician's intention to treat, ITT; [120]). Patients were randomly assigned to receive subcutaneous injections of Lupuzor 200 µg every 4 weeks (group 1; *n* = 49), Lupuzor 200 µg every 2 weeks (group 2; *n* = 52) or placebo (group 3; *n* = 49) in addition to standard of care. Interim analyses were carried out with 125 patients (group 1: *n* = 40; group 2: *n* = 42; group 3: *n* = 43). One hundred fifteen patients completed the 12-week treatment period and 10 patients were withdrawn from the study, i.e. 1 patient in group 1, 1 patient in group 2, and 8 patients in group 3 (placebo). Withdrawn patients were considered as treatment failures. Overall, 23 patients (57.5%) in group 1 (*p* = 0.012), 20 patients (47.6%) in group 2 (*p* = 0.079) and 13 patients (30%) in group 3 achieved an SLE Responder Index (SRI) combined score response. The legal disclosure of the interim data influenced the overall ITT analysis. Ninety percent of the ITT population met the new inclusion criteria (clinical SLEDAI ≥ 6). Group 1 (Lupuzor 200 µg every 4 weeks) of this subpopulation achieved a significantly higher SRI score (62%) when compared to placebo (38.6%; *p* = 0.016). In the ITT group the percentage of SRI responders was respectively 53.1 (group 1), 45.1 (group 2) and 36.2 (group 3), indicating, however, that Lupuzor administered once a month was significantly more efficient than placebo (*p* = 0.048). The efficacy was maintained after the 24-week follow-up period. Lupuzor was generally well-tolerated with no significant drug-related adverse events recorded. The most frequently reported adverse events were urinary tract infection and injection-site erythema. Lupuzor has recently been granted approval by the FDA to commence phase III and has also been designated "Fast Track".

In the preparation administered to patients, P140 peptide was conditioned in a 5.4% mannitol solution, a naturally occurring sugar alcohol classically used as excipient. In preliminary experiments run in our laboratory it was noticed in MRL/lpr mice that the choice of excipient, which should be pharmacologically inactive and used only as a carrier for the active compound, is not trivial in the case of peptide P140. As shown in Fig. 5a, using our validated assay ([88], Table 1), we found that P140 injected intravenously in saline or mannitol significantly reduced peripheral hypercellularity to levels that approach the one measured in

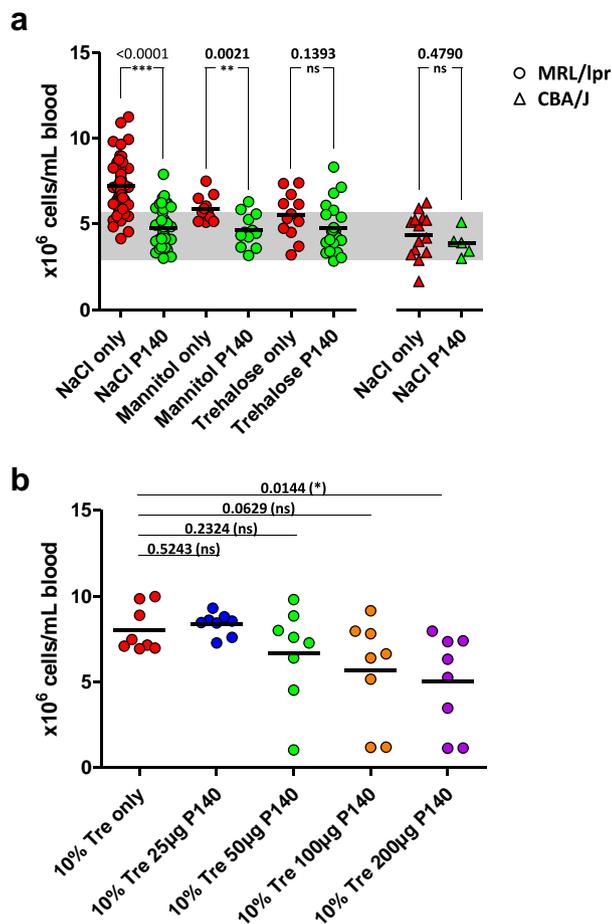


Fig. 5. In vivo effect of P140 peptide in different excipients on the peripheral hypercellularity. 11–13 week-old female MRL/lpr and CBA/J mice received a single administration of peptide P140 intravenously (100 µg/mouse in panel a, 25–200 µg/mouse in panel b) in different excipients, namely saline, 5.4% mannitol and 10% trehalose (Tre). The number of leukocytes/mL was evaluated by counting cells five days later. Each symbol represents one individual mouse (panel a: $n = 55$ and 44 without and with P140 in NaCl, $n = 11$ and 12 in mannitol and $n = 12$ and 22 in trehalose; panel b: 8 mice/group). Horizontal bars represent the respective average cell count values. The coefficient of variation on cell counts was 20.6, 12.8 and 24.3% in the groups of mice that received saline, mannitol and trehalose, respectively, and 23.2, 20.6 and 29.7% in the groups of mice that received P140 in saline, mannitol and trehalose, respectively. Statistical significance was assessed using the Student's *t*-test. ns = non-significant.

normal CBA/J mice and that significantly differ from the one measured with the respective excipients used as placebo (cell counts measured five days after peptide injection). In contrast, the same peptide given in 10% trehalose, a non-reducing very stable disaccharide also widely used as excipient, did not significantly reduce cell counts when compared to mice that received trehalose alone (Fig. 5a). To observe a significant effect of P140 peptide in trehalose the peptide dose had to be raised at least twice in mice (Fig. 5b). In addition to the highly disperse effect found in trehalose conditions, we observed a dramatic peripheral immunodepletion in certain MRL/lpr mice treated with P140 peptide in trehalose (Fig. 5b) that might result from an excessive death of pre-activated cells. These findings indicate that an excipient can dramatically influence the bioactivity of a therapeutic compound. Autophagic activity has been shown recently to be increased in certain T cell subsets both in lupus mice (NZB/W and MRL/lpr mice) and patients with a significant accumulation of autophagic vacuoles [121]. Knowing that trehalose has been reported to be an autophagy enhancer [122–124], this excipient should be discarded

in the case of lupus treatment, in particular with P140 peptide that reduces autophagic flux.

7. Epilogue

Peptide-based drug discovery provides a serious option for addressing new therapeutic challenges in multiple pathologies. After a period of excitement and then years of disfavor in the 90' due to their low metabolic stability and high clearance by the liver, synthetic peptides are again in an ascending phase of interest as potential drug candidates. Active research has introduced elements such as pseudo-peptide bonds that are stable to proteolytic degradation [9] or modifications that improve their bioavailability. Different carriers (nanoparticles, liposomes, peptide scaffolds) have also been developed to allow their delivery by non-invasive oral or nasal routes. The main advantage of peptides rely on the possibility to change the nature of amino acid side chains and/or peptide bonds, attach fatty acids for example or add fluorescent, radioactive, or any types of labels such as biotin or tags. These chemical modifications, if they do not affect the bioactivity of the peptide, can prove to be highly valuable to enhance their potency and to follow their uptake in organ and cells. In the case of P140 peptide/lupuzor, it is striking to observe that while the active dose to prolong the survival of lupus mice is around 2 mg/kg given intravenously, it is close to 3 µg/kg in patients administered subcutaneously. Since synthetic peptides are generally safe in human and devoid of proper immunogenicity, they are probably facing again a long avenue of general interest for therapeutic applications.

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