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Review article

Tuftsin: On the 30-year anniversary of Victor Najjar's discovery

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Abstract

After a short description of the results of Victor Najjar's research on tuftsin and of the discoveries done by other authors in the early stage of tuftsin investigation, the current state of work on tuftsin is presented, based mainly on the literature published in the years 1984–1997. The presentation follows this order: the occurrence of tuftsin and retro-tuftsin sequences in proteins, their synthesis and biology, the antigenic properties of tuftsin, its influence on phagocytic cells, and other biologic activities of tuftsin, including antimicrobial, antiviral, antitumor and central effects, and the search for tuftsin superactive analogs.

Keywords: Tuftsin; Kentsin; Substance P; Tuftsin and retro-tuftsin sequences; Phagocytosis; Immunomodulation; Antinociceptive activity

1. Victor Najjar's work. A short history of early tuftsin studies

In 1970, Najjar and Nishioka published a paper evidencing that leucokinin, a leucophilic fraction of Ig G protein, splits under the action of a specific enzyme (leucokininase) located in the outer membrane of the neutrophils, a phagocytosis stimulating tetrapeptide. That tetrapeptide was subsequently named tuftsin (after Tufts University, Boston, MA) [213].

Such was the beginning of intensive studies on the chemistry and biology of this new natural peptide immunomodulator. Several important statements and observations concerning tuftsin resulted from Najjar's studies. Najjar's laboratory established the sequence of tuftsin as Thr-Lys-Pro-Arg [227]. The synthesis, subsequently performed in the same laboratory by solid phase method, confirmed this structure. The synthetic peptide, just like the natural one, stimulated the particle ingestion, bacteria and latex, and also stimulated the pinocytosis of ^{131}I-albumin by PMN leucocytes. In the presence of small concentrations of the peptide, PMN leucocytes migration was also stimulated. It was also discovered that a synthetic analog of tuftsin, a pentapeptide Thr-Lys-Pro-Pro-Arg, strongly inhibited such properties of tuftsin as the stimulation of phagocytosis and the stimulation of cell migration [233].

Tuftsin is a 289–292 sequence in the CH2 domain of the Fc fragment of leucokinin molecules. Najjar's group claimed that tuftsin is split from the protein carrier by the successive action of 2 enzymes: splenic tuftsin endocarboxypeptidase, that splits a Arg^{292}-Glu^{293} peptide bond of leucokinin, and phagocyte membrane enzyme—leucokininase, that clears Lys^{288}-Thr^{289} peptide bond [200,202]. It should be noted that the existence of the splenic enzyme was supported by circumstantial evidence only. Thus, in splenectomized dogs and humans, no phagocytosis stimulating activity of leucokinin was observed, although the tuftsin sequence was found to be present there [199,208].

The first phagocytosis assay for tuftsin was likewise developed in Najjar's laboratory. It consisted in a degree of stimulation of phagocytic activity in rabbit blood granulocytes as well as in rabbit lung macrophages, and in using a *Staphylococcus aureus* as the target particle [57,207]. With the use of this assay, the familial and induced tuftsin deficiency was studied [59,201].

A mutated tuftsin, a tetrapeptide Thr-Glu-Pro-Arg, was isolated by the Najjar's group from human leucokinin, obtained from a patient with tuftsin deficiency and so deprived of phagocytic activity [203]. Its solid-phase synthesis was performed by Konopińska, Najjar and Callery [144]. It was also shown by Najjar that canine tuftsin is characterized by $Arg^4 \rightarrow Lys^4$ exchange and has the sequence of Thr-Lys-Pro-Lys [264].

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In 1973, Constantopoulos and Najjar showed that treating of PMN-leucocytes with neurominidase, that splits the sialic acid from its polymeric carriers, abolishes the phagocytosis-stimulating activity of tuftsin. This result indicated that negatively charged fragments of cell surfaces play a role in tuftsin binding [58].

Pursuing the observation made by his earlier collaborator, Nishioka [224], who noticed that the tumoricidal activity of macrophages from tuftsin-treated mice is much higher than that of the control group, Najjar turned his attention to the antineoplastic effects of tuftsin. He showed that in the experiment with L1210 cell, tuftsinyltuftsin (an octapeptide Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg), is more active than tuftsin [210]. The same result was also obtained for B16 melanoma in synergic C57BL/6 mice [212]. Chemical synthesis of tuftsinyltuftsin has been described elsewhere [145].

In further studies, Najjar observed a significant decrease in mortality in DBA/2J mice infected with Friend leukemia virus after treatment with tuftsin [350]. He also ascertained that tuftsin induced tumor necrosis activity in mouse macrophages and in promyelocytic cells (HL60) [351] and that it stimulated growth of HL60 cells [39].

When investigating the binding of tuftsin and its analogs to specific receptors found on macrophages and granulocytes, Najjar's team observed that tuftsin receptors and tuftsin antibody showed identical binding properties [47, 48]. Tritium labeled [^{3}H-Pro^{3}]-tuftsin was used in these investigations as the principal ligand. The synthesis was described by Najjar et al. separately [211]. Bump and Najjar also found that the binding of tuftsin by specific receptors diminishes after prior treatment of the cells with dithiothreitol (DTT) [38]. Using [^{3}H-Arg^{4}]-tuftsin, they also showed that rabbit peritoneal granulocytes incorporate radioactivity by the covalent bonding to a membrane acceptor protein of 100 kDa [205].

Najjar's group's attempts to isolate tuftsin receptor from the solubilized rabbit peritoneal granulocyte membranes resulted in obtaining two tuftsin receptor subunits with molecular masses of 62 kDa and 52 kDa, respectively. An affinity chromatography with tuftsin pentapeptide analog Thr-Lys-Pro-Pro-Arg covalently bound to a solid support was used for this purpose (It was found that this pentapeptide binds to the receptor more than 4 times as avidly as tuftsin) [37]. Using the same method, 2 receptor subunits of approximately 66 kDa and 57 kDa, respectively, were isolated from HL60 cells. The dissociation constant of the receptor complex was determined to be 4.7×10^{-8} M with 5×10^{4} receptors per cell [40].

The interest in tuftsin chemistry and biology prompted by Najjar's discovery resulted in a number of important new results obtained shortly thereafter by other research groups. In 1975, Spirer et al. found that tuftsin increases the respiratory-burst of phagocytic cells, activating the hexosemonophosphate shunt. As a measure of this effect, the reduction of nitrous blue tetrazolium by normal human polymorphonuclear leucocytes was adopted [306]. An alternative way of the monitoring respiratory-burst increase in the phagocytic cells in the form of the $^{14}CO_2$ uptake from PMN-leucocytes was also used [83]. As a consequence of this activation, superoxide ions and hydroxyl radicals, that participate in the bacterial killing, may be formed. The paper in question provided the first piece of evidence concerning stimulation of bactericidal activity of phagocytic cells by tuftsin. The fact that tuftsin augments formation of O_2^- superoxide ions was confirmed recently by Tritsch and Niswander [324]. The stimulation of the release of oxygen radicals and thromboxane from guinea pig peritoneal macrophages was demonstrated by Hartung and Toyka [103].

In fact, Martinez et al. showed in 1977 by experiments performed in vitro with macrophages, as well as in vivo in mice, that tuftsin exhibits a marked antibacterial activity [176]. It was found that tuftsin enhances the clearing of blood of several bacterial species by mouse peritoneal macrophages and that it stimulates bactericidal activity in liver and spleen of mice [174]. It should also be noted that, according to Blok–Perkowska et al., at high concentration of about 60 μg/ml, tuftsin can destroy various microorganisms, inter alia of the Pneumococcus type [29].

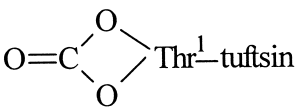
In 1977, Spirer et al. produced antibodies against tuftsin moiety, by using as the antigen a BSA conjugated with p-diazonium phenylacetyl-tuftsin. The antisera thus obtained were found to bind with ^{125}I-labeled p-aminophenylalanyl-tuftsin, providing grounds for radioimmunoassay for tuftsin concentration determination in trypsinized sera. Tuftsin concentrations in the sera from normal patients were determined by this procedure to be 278.47 ± 13.49 ng/ml, whereas those of patients who underwent traumatic and elective splenotomy contained 239.0 ± 47.68 ng/ml and 94.71 ± 22.49 ng/ml, respectively. The competition experiments performed with different tuftsin analogs showed that the main antigenic determinant recognized by tuftsin—antibodies was the sequence Lys-Pro-Arg [305]. A tritium-labeled tuftsin (Thr-Lys-Pro-[^{3}H]Arg) was synthesized by the same group, and the binding of the product to human PMN-leucocytes and to monocytes was studied. The dissociation constants of the binding process were determined to be approximately 130 and 125 nM, with the number of binding sites 50 000 and 100 000 per cell, respectively. No binding was observed for erythrocytes and a threshold binding only for lymphocytes [310]. Decreased serum tuftsin concentrations were found in sickle cell disease materials [304].

Binding of [^{3}H-Arg^{4}]-tuftsin to normal and thioglycolate-stimulated mouse peritoneal macrophages was studied using the same method. The dissociation constant was found to be 5.3×10^{-8} M, and the number of binding sites approximately 72 000 per cell. Two biologically inactive tuftsin analogs, i.e. [des-Arg^{4}]-tuftsin and *N*-acetyl-tuftsin, manifested no binding to tuftsin binding sites. However, some affinity to these sites was exhibited by [D-Arg^{4}]-tuftsin [17].

In the search for new study methods in tuftsin-tuftsin receptor interaction studies, Gottlieb et al. synthesized some new types of tuftsin analogs, prolonged on their C-terminus with the fragments bearing: (i) the aromatic azido moiety (2-nitro-4-azidophenyl sulfenyl group) for photoaffinity labeling, (ii) biotinyl residue—for affinity chromatography purpose, (iii) dansyl or rodamine fluorescent probes for receptor binding studies [95]. The fluorescent derivative, H-Thr-Lys-Pro-Arg-Gly-Lys-(N^{ϵ}-tetramethylrodamine)-OH, recognized specifically the tuftsin receptors and made them visible on the surfaces of mouse peritoneal macrophages [97].

Studies on tuftsin receptors employing ^{14}C- or ^{125}I-labeled tuftsin derivatives were also performed by Nair et al. [198]. Amoscato et al. used for this purpose fluorescein labeled tuftsin ([N^{α}-Thr^1-fluorescein]-tuftsin). It was reported that the compound was able to bind to human neutrophils even at temperature as low as 4°C [5,6].

In the first years of tuftsin research, numerous research groups paid much attention to synthesis of different tuftsin analogs. The aim of these studies was, in part, to clarify the relation between the chemical structure and the biologic activity of tuftsin. The results of these investigations were reviewed in 1981 by Siemion and Konopińska [288]. The general conclusion was that tuftsin is quite sensitive to structural changes such as extending the tuftsin peptide chain on its N-terminus, that leads to a distinct decrease in biologic activity, exchanging of N-terminal Thr residue for other amino acids, that produces analogs with reduced activity or inhibitory properties. Thus, [Ala^1]-tuftsin (and to a lower degree [Val^1]-tuftsin) were found to be inhibitors of tuftsin [85]. On the other hand, [Leu^1]-tuftsin was found to be even more potent than tuftsin in activation of phagocytosis [181]. The role of N-terminal group in the elucidation of biologic effects was uncertain: *N*-acetyl-tuftsin had a low inhibitory effect in phagocytosis [85], whereas [Pyro-Glu^1]-tuftsin [140] and 2-oxo-oxazolidine-tuftsin [309]



were active phagocytosis stimulators.

It was also established that lengthening of the peptide chain at the C-terminus provides a better way of presenting tuftsin-like activity than any changes performed on N-terminus. A very interesting result obtained in this field concerned substance P and its N-terminal tetrapeptide Arg-Pro-Lys-Pro that were found to be as active as tuftsin in phagocytosis stimulation test and competed with tuftsin for the same receptors [16]. A tetrapeptide Arg-Pro-Lys-Pro is in fact the retro sequence of tuftsin analog Pro-Lys-Pro-Arg. The result suggests that a tuftsin receptor can recognize not only a tuftsin-, but also a retro-tuftsin sequence. However, the data concerning the phagocytosis-stimulating activity of retro-tuftsin (Arg-Pro-Lys-Thr tetrapeptide) were inconsistent. Yasumura et al. reported for it the same activity as tuftsin [355], whereas other investigators could not observe any activity at all [111].

Among the results obtained in this field by the Siemion's group, 2 appear to be especially notable. In 1977, the group synthesized a series of tuftsin-like tetrapeptides—partial sequences of histones. Some of these peptides showed distinct phagocytosis-stimulating activity. To the best of our knowledge, this was the first paper that much attention was given to the tuftsin-like sequences present in the molecules of different proteins [147].

The same group synthesized a series of tuftsin analogs substiting different amino acid residues within the position 3 of peptide chain. The aim of the study was to prove a hypothesis concerning the existence of the functional conservatism in the coding of the tuftsin sequence. It was found that analogs constructed on the basis of one-point mutations in Pro^3 codons possess higher biologic activity than other analogs that do not conform to such a condition [148].

Numerous new observations on various biologic activities of tuftsin reported at the end of the 1970s and the beginning of 1980s, stimulated a growing interest in this particular area of peptide chemistry and biology. The most encouraging of those observations, made by Nishioka, showed that tuftsin demonstrates antitumor activity [224]. Nishioka's in vivo experiments were initiated with the L1210 leukemia systems and were subsequently extended by Nishioka et al. on other experimental tumor models [226]. In the authors' opinion, antitumor activity of tuftsin appears to be exerted through the activation of immunologic effector cells.

At approximately the same time, Florentin et al. stated that tuftsin is able to potentiate various types of immune response when injected into mice, and can be used as a potent activator of macrophages in cancer therapy [81]. The validity of such proposition was to same extent confirmed by the discovery that tuftsin-treated mouse peritoneal macrophages exert cytostatic activity for tumor cell proliferation [33].

In 1981, Kavai et al. conducted studies of the stimulation of chemotaxis of human monocytes by tuftsin and several of its analogs. They found that preincubation of monocytes with tuftsin enhances the locomotion in tests of random migration, as well as leucotaxis. The results, in the opinion of those authors, suggested that tuftsin may be useful in certain pathologic events connected to defect in chemotaxis and random migration of leucocytes [128]. In 1982, Caroll et al. reported the results of their studies on tuftsin ability to enhance human monocyte cytotoxic in vitro response. Tuftsin was found to enhance cytotoxic response against K562 tumor cell line at the doses of 5×10^{-2} to 5×10^{-1} μg/ml. The natural killing activity of lymphocytes against that particular cell line was not affected by tuftsin [42].

To evaluate the prospects of using tuftsin as an agent for treatment of human cancer, Catane et al. conducted correspondent experiments with animals (mice and dogs) as well as with human patients. It was established that tuftsin at doses ranging between 50 and 500 μg/kg of body weight enhances cytotoxic activity of mononuclear cells in mice and humans, and, in mice, also shows antitumor activity. In the course of those studies the acute lethal dose of tuftsin in mice was determined to be approximately 2.4 g/kg. The effects of tuftsin were accompanied by leucocytosis induction [44]. It was also stated that tuftsin significantly increases survival rates among Rauscher virus leukemia infected mice [138] and demonstrates antitumor activity against murine melanoma in vivo [236]. Konopińska et al. investigated the influence of tuftsin analogs possessing elongated peptide chains (canine tuftsinyltuftsin, tuftsin elongated at the N-terminus with canine tuftsin and Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln octapeptide derived of human serum γ-globulin EU) on the growth of murine sarcoma virus. The researchers found that those peptides had an inhibitory effect on MSV tumor development [143].

However, as in 1977 it was also reported by Luftig et al., the addition of 1–100 nmol per ml of tuftsin to cultured mouse cells infected with Rauscher murine leukemia virus increases the level of both virion-associated reverse transcriptase and that of building virus from mouse cells [170]. The authors paid a particular attention to the fact that in the molecule of p12 Rauscher leukemia virus protein, there appears at the N-terminus a tuftsin sequence, that may have a regulatory function in the viral protein expression. Enhancement of endogenous xenotropic virus expression after treatment of Balb/c mouse cells transformed with Kirsten sarcoma virus with tuftsin and Kentsin (a sequential isomer of tuftsin: Thr-Pro-Arg-Lys) was also reported by Suk and Long [314].

In 1978, Tzehoval et al. reported that tuftsin augments the antigen-specific macrophage dependent education of T lymphocytes [329]. They found that the most important moiety for that activity is the Pro-Arg dipeptide fragment of tuftsin. Taking into account the fact that this sequence appears in a number of regulatory peptide hormones, Tzehoval et al. formulated a hypothesis that the fragment plays the role of the dominant epitope of regulatory molecules "while the accompanying neighboring amino acids determine the affinity and specificity for the particular target cells." Tzehoval et al. found that there was no correlation between the phagocytic and immunogenic effects of tuftsin analogs.

In the same year, Horsmanheimo et al. investigated the influence of tuftsin on the migration of polymorphonuclear and mononuclear human leucocytes [113]. The effect of migration enhancement in human mononuclear cells (lymphocytes and monocytes) evoked by tuftsin was also studied at the same time by Nishioka [223], who found that tuftsin also abrogates the migration inhibition effect of human malignant membrane antigen.

Beretz et al. utilized in their experiments concerning chemotaxis of human monocytes tuftsin and tuftsin analogs conjugated with *N*-formyl-Met-Leu-Phe tripeptide, a known chemotaxis stimulator. Two such conjugates exhibited a somewhat higher activity than that expressed by *N*-formyl-Met-Leu-Phe peptide [22]. The stimulating activity of tuftsin and its analogs regarding the monocyte chemotaxis was also observed by Lukacs et al. in the case of monocytes derived from patients with systemic lupus erythematosus. Lupus erythematosus, a representative autoimmune disease, is accompanied by impaired monocyte chemotaxis, restored in the presence of tuftsin [128,171].

At the same time, Stabinsky et al., tried to incubate human PMN leucocytes, or, alternatively, thioglycollate-stimulated mouse peritoneal macrophages, with tuftsin. They found that tuftsin raises the levels of cyclic guanylate acid (cGMP) and depresses those of cyclic adenylic acid (cAMP). The peptide also significantly affected the release of exchangeable Ca^{+2} ions from the cells. On the ground of these observations, the authors submitted that the mechanism of phagocytosis stimulation by tuftsin may consist in its having an "effect on intracellular cyclic nucleotide levels, be it mediated via a primary effect on $^{45}Ca^{+2}$ association with cellular components or via another as yet undetermined mechanism" [308].

Using a fluorescent analog of tuftsin (obtained by the coupling of the peptide with tetramethyl rodamine isothiocyanate), that visualizes tuftsin receptors on the mice macrophage cells, Gottlieb et al. showed that the binding of the peptide to the receptors is followed by internalization of tuftsin by the cells with endocytosis mechanism [96]. As we noted above, similar results were also obtained by Amoscato et al. [6].

Independently from the studies on the role of tuftsin within the immunologic system, some other, new activities for this peptide were also found. Thus, Veretennikova et al. investigated the myotropic tuftsin activity and stated that it reduced such activity of angiotensin II by 20%. Isolated colon ascendens of rats were used in these experiments [336]. Paradowski et al. examined the influence of tuftsin on the circulatory system and found that after guinea pig and rats were injected with tuftsin, there was a distinct increase in both the systolic and also the diastolic blood pressure of the animals. A parallel effect, however, was not observed in rabbits [247].

The collaboration of Herman's and Siemion's groups resulted in the observation that tuftsin and its analogs produce analgesia after injection into the lateral brain ventricle of rats and mice [108–110]. The results suggested the existence of a link between the immunologic system and the central nervous system, and that result was a first piece of evidence for such possibility. The central effects of tuftsin will be discussed separately below.

The cumulative results of investigations in the tuftsin field were reviewed in several papers by Najjar [202–204, 206] and also by Fridkin and Gottlieb [83], as well as by

Siemion and Konopińska [288]. Of these 2 latter reviews, the first one was focused mostly on the biologic effects, whereas the other one was on the chemistry of tuftsin. A mini-review by Nishioka et al. published at the same time should also be noted [225].

On February 16–17, 1983, Najjar organized in New York a special international conference on the antineoplastic, immunogenic, and other effects of tuftsin. A volume of Annals of the New York Academy of Sciences, with the materials presented at that conference contains a veritable *summa tuftsinologiae* of that time [209]. Further progress of investigations in the tuftsin field was outlined in the excellent review by Fridkin and Najjar in 1989 [84]. The problem of tuftsin receptors was separately discussed in a review by Dagan [64]. The review by Nishioka et al., published very recently, is devoted mainly to the medicinal chemistry of tuftsin [231].

2. The research on tuftsin and retro-tuftsin sequences in proteins

The tuftsin circulating in blood is released from leucokinin fraction of Ig G proteins only. However, tuftsin sequences appear in all 4 types of Ig G's. The biologic role of these Ig G protein fragments remains unknown. It was reported, however, that the basic residues of tuftsin fragment play some role in the binding of the antigen—human Ig G complex to the first complement component, C1 [172]. The tuftsin motifs are also present in other proteins of immunologic system, e.g., in the peptide chain of (Ig A Tro.) II immunoglobulin (the sequence determined by Kratzin et al. [152]), where the following fragments occur:

Thr-Ser-Pro-Lys (123–126)
Thr-Gly-Leu-Arg (269–272)
Thr-Ile-Asp-Arg (447–450)

The same sequences appear in Ig A_1 immunoglobulin molecule [166].

In the molecules of human histocompatibility HLA-B7 antigen, there appear tuftsin-like fragments Ser-Arg-Pro, Ser-Pro-Arg, Glu-Pro-Arg, and Thr-Arg-Pro. A similar situation was observed in the case of HLA-A2 histocompatibility antigen: a tuftsin-like sequence Thr-Lys-His-Lys occurs there (for the actual sequences, see Ref. [241]).

Some evidence of the presence of tuftsin and tuftsin-like sequences within the protein molecules was collected in another paper by Najjar [204], who pointed out that tuftsin sequence occupies the position 289–292 of guinea pig G_2 protein and that in mouse G_1 protein a tuftsin mutant (Thr-Gln-Pro-Arg) appears. Najjar also indicated the tuftsin sequences of viral proteins, that of Rauscher leukemia virus p12 phosphoprotein (position 9–12) (noticed also by Luftig et al. [170]), and the tetrapeptide Thr-Arg-Pro-Lys, that constitutes the 214–217 fragment of influenza virus hemagglutinin. The Thr-Arg-Pro-Arg tetrapeptide fragment of biologically active pancreatic polypeptide was also examined. Because the probability of accidental appearance of tuftsin sequences in different proteins appeared to be very low, Najjar suggested that the sequence could have some functional significance.

The list of tuftsin and tuftsin- like fragments in proteins was enlarged by Segal et al. [275]. They pointed out that a sequence Thr-Lys-Glu-Lys appears in the polypeptide hormone, thymopoietin (res. 11–14), whereas the sequences Thr-Gln-Pro-Arg and Thr-Arg-Pro-Ala appear in Ig E heavy chain and in the heavy chain of HLA-B7, respectively. These authors also noted the fact that in procollagen type I, there occurs a sequence Thr-Gly-Pro-Arg, whereas partial tuftsin sequences Lys-Pro-Arg and Thr-Lys-Pro occur in the C-reactive protein.

We have noted above that in 1977 Konopińska et al. [147] indicated the presence of tuftsin- like sequences in the molecules of $F_{2a}1$, F_{2b}, and F_3 histones. Henderson et al., who performed a comparison of the sequences of p12 protein of murine leukemia viruses with those of H5 histone, isolated from goose and chicken red blood cells, showed what appears to be a homology between these proteins in the region of tuftsin sequence. Positions correspondent to p12 tuftsin are occupied in H5 peptide chain by Ala-Lys-Pro-Lys sequence; the same sequence also appears in p12 protein of the Moloney murine leukemia virus [105]. The presence of tuftsin-like sequences in different proteins has also been discussed in 1986 by Siemion [286].

The most intriguing result, with regard to the tuftsin sequences in proteins, was obtained in 1991 by Audhya et al. [12]. Those authors submitted that bovine probursin contains the amino acid sequences of somatostatin, tuftsin, and bursin, coupled together. Probursin was isolated from bone marrow and liver. In that particular tetradecapeptide, the residues 1–5 correspond to the active site of somatostatin, 5–8 to tuftsin, and 9–11 to bursin, an indicator of B-cell differentiation. Bursin is a tripeptide Lys-His-Gly-NH_2, found in avian and mammalian bone marrow and in other tissues.

Probursin sequence:

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Phe-Phe-Trp-Lys-|-Thr-Lys-Pro-Arg-|-Lys-His-Gly-|-Gly-Arg-Arg

In probursin the tuftsin sequence is flanked by basic amino acids, and therefore the release of this peptide from the precursor molecule by the action of trypsin-like enzyme appears possible.

Bar–Shavit et al. underlined the fact that tridecapeptide neurotensin, that exhibits a broad spectrum of activities both in the central nervous system and in peripheral tissues and modulates, inter alia, the phagocytic capability of thiogly-

collate-elicited mouse peritoneal macrophages, also contains tuftsin-like sequence Asn-Lys-Pro-Arg [18].

The list of such tuftsin and tuftsin-like sequences that constitute fragments of proteins and natural peptides could be now greatly expanded, especially when not only tuftsin, but also tuftsin-like sequences are taken into account, for instance, a sequence Thr-Arg-Leu-Arg resides in porcine vasoactive intestinal polypeptide (VIP) (res. 11–14) [191]. Exactly the same sequence is also to be found in the peptide chain of *Aplysia* neuropeptide precursor [273].

This latter polypeptide shows a homology to the N-terminal part of basic attacin, an antibacterial immune protein of Cecropia moth (*Hyalophora cecropia*). In this protein, the discussed sequence is then transformed into sequence Ser-Arg-Val-Arg [139]. The Gln-Arg-Pro-Arg sequence, present in parathyroid hormone shows even more similarity to tuftsin [260]. Another such sequence, [Tyr^2]-tuftsin (Thr-Tyr-Pro-Arg), appears in salmon calcitonin [261].

A tuftsin-like motif, consisting of dicarboxylic amino acid residue and two basic residues inserted by leucine, exists in the helix 3 segment of the growth hormone molecule of mammals (fragment Glu-Lys-Leu-Lys) [1]. The N-terminal fragments of prolyl and leucyl aminopeptidases (variously isolated from pig intestine and kidney, human liver and bovine lens) likewise contain a tuftsin-like sequence Ser-Lys-Glu-Lys [180].

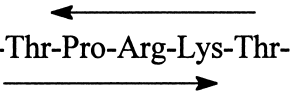
In the sequence of p21 protein deduced from p21 cDNA (this protein is an universal inhibitor of cyclin kinases of mammals) a tuftsin-like fragment Thr-Gly-Pro-Arg is apparent [353]. Another interesting acidic analog of tuftsin (Thr-Glu-Pro-Glu), appears in the molecule of bovine neurophysin (neurohypophysial hormone precursor) [162].

Many proteins contain fragments that are sequential isomers of tuftsin. For instance, in the immunosuppressive pentadecapeptide loop of lactoferrin a Thr-Arg-Lys-Pro fragment appears [296]. The Arg-Thr-Pro-Lys moiety composes a part of 94–98 loop of human transforming growth factor β3 (TGF β3) molecule, endowed with immunostimulatory activity [346].

A similar situation obtains in the case of Kentsin (Thr-Pro-Arg-Lys), a contraceptive tetrapeptide found by Kent in the oviductal lumen of 2-day progravid hamsters [130]. It should be noted here that in the conserved region of human γ-tubulin, a very similar sequence, Thr-Asp-Arg-Lys, appears [119].

A sequential isomer of canine tuftsin (Thr-Pro-Lys-Lys) occurs in the protein product of rum 1^+ gene, that regulates the progression through the G1 phase of the cell cycle. This sequence constitutes a putative phosphorylation site of the protein [190].

A very interesting situation occurs in the V1a arginine vasopressin receptors of rats [189]. The receptor contains a Arg-Thr-Pro-Arg-Lys-Thr sequence in that both the sequence of a sequential isomer of tuftsin and the retro-sequence of other tuftsin sequential isomer can be easily recognized:



In the human oxytocin receptor molecule, there appears a tuftsin-like sequence Thr-Arg-Gln-Lys [135].

Those sequences, that are sequential isomers of tuftsin, can play some role in protein—DNA recognition processes. It was reported that sequences representing the general formula [Ser, Thr]-Pro-[Lys, Arg]-[Lys, Arg] might function as a DNA-binding region in histone termini. The fragment Thr-Pro-Lys-Arg-Pro-Arg-Gly-Arg-Pro-Lys-Lys of non-histone chromosomal protein HMG-I was found to bind to AT-rich DNA, whereas Lys-Pro-Arg-Gly-Arg-Pro-Lys peptide protected the AT-rich sites in hydroxyl-radical footprinting experiments [54].

Frequently, shortened tuftsin sequences (with deletion of one of tuftsin amino acid residues), appear in proteins, but enlarged ones are also known to appear, with the insertion of an additional residue to the peptide chain. Thus, in the A-chain of human relaxin, the [des-Pro^3]-tuftsin sequence appears (Thr-Lys-Arg) [34]. Such sequences exist also in ω-conotoxin, a polypeptide toxin from fish-eating mollusc [240]. In the polypeptide chain of PH-30β protein involved in sperm/egg fusion, a tripeptide fragment Thr-Lys-Pro ([des-Arg^4]-tuftsin) is present alongside the fragment Thr-Lys-Ser-Arg, a tuftsin analog [28]. The same tripeptide fragment also appears in the polypeptide chain of elafin, the elastase inhibitor, isolated from human skin [349].

Finally, Thr-Arg-Pro tripeptide moiety is observable in the active center of urease whereas Thr-Pro-Lys moiety can be seen in B-chain of insulin. On the other hand, the canine tuftsin sequence inserted by His (Thr-Lys-His-Pro-Lys) is apparent in the P_2 fragment (res. 49–149) of staphylococcal nuclease.

There also appear within proteins many retro-tuftsin sequences, some complete and some incomplete. In the arginine vasopressin receptor there are, apart from the fragment Pro-Arg-Lys-Thr (see above), the sequences Arg-Gly-Lys-Thr and Arg-Lys-Thr [189].

A tuftsin retro-sequence Arg-Glu-Pro-Lys-Thr containing the inserted Glu residue occurs within the molecule of TBP protein, a central component of the eucaryotic transcriptional apparatus [222].

The comparison of the sequences of certain plant storage proteins, as performed by Sebastiani et al., evidences the presence in these proteins of a common motif of Lys-Pro-(Asn, His)-Thr type, that is a retro-sequence for Thr-Asn-Pro-Lys tuftsin analog [274].

Many tuftsin-like fragments (Thr-Lys-Pro, res. 23–25; Lys-Pro-Lys, res. 37–39; Thr-Arg-Gly-Lys, res. 169–72;

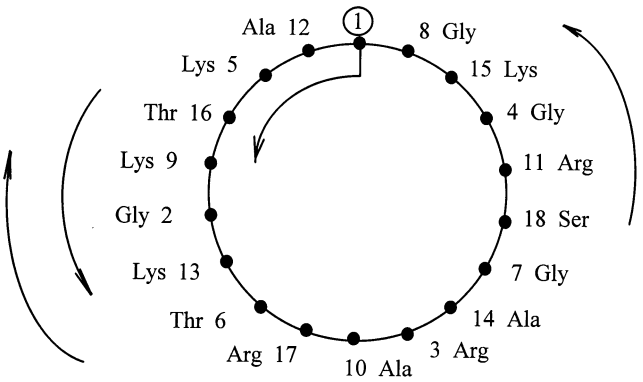


Fig. 1. Tuftsin and retro-tuftsin motifs in histone IIb_1. The sequence is plotted in the form of the helical wheel.

Thr-Lys-Thr-Lys, res. 212–215), appear in the polypeptide chain of diptheria toxin beside the retro-tuftsin-like sequence Arg-Pro-Lys-Ser (res. 472–475).

A peculiar way to create tuftsin motifs in proteins consists in bringing together the respective, sequentially distant amino acid residues by proper conformational changes. When, for example, the N-terminal sequence of histone IIb_1 from trout testis is plotted in the form of the helical wheel, 2 such tuftsin-like motifs on one of the sides of this wheel, and an additional one, situated on the other side of the wheel, can be recognized (see Fig. 1). (The idea of such plotting of histone IIb_1 sequence was adopted by us from [168]).

A systematic examination of sequential data derived from 8 different data bases (http://prowl.rockefeller.edu) enabled us to establish that, to date, during the search for tuftsin sequences, about 470 proteins of very different biologic origins, all containing tuftsin Thr-Lys-Pro-Arg fragment, have been collected. The search for retro-tuftsin fragments containing proteins resulted in locating about 500 such proteins. This shows that tuftsin- and retro-tuftsin containing proteins are fairly common in the structure of living cells.

A careful review of these data shows that those proteins routinely play important roles in cells, belonging to different groups of regulatory and defense biopolymers. During this search 13 tuftsin and 20 retro-tuftsin sequences were found within the molecules of human proteins. Apart from Ig G molecules, tuftsin sequences appear, for example, in leptin receptor, rapamycin target protein, pyruvate kinase and protoporphyrinogen oxidase. The retro-tuftsin sequences are present, inter alia, in such important proteins as serotonin receptor, Na/K dependent ATPase, ubiquitin-activating enzyme, and tyrosine-protein kinase receptor TIE-1 precursor.

The partial sequences of proteins, containing tuftsin and tuftsin-like motifs can be used as models for the synthesis of new 'natural' analogs of tuftsin with interesting biologic properties.

To elucidate the relationship between the Fc receptor and tuftsin receptor, Gottlieb et al. synthesized in 1983 a series of peptides corresponding to the sequences of the Fc part of Ig G surrounding and containing a tuftsin fragment. The series was composed of the following peptides:

Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr (res. 289–296)
His-Asn-Ala-Lys-Thr-Lys-Pro-Arg (res. 285–292)
His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr (res. 285–296)

The elongated Ig G peptide segments encompassing tuftsin were shown to be able to specifically bind the tuftsin receptors on mouse peritoneal macrophages. The same peptides were also found to be potent phagocytosis stimulants. However, the tetrapeptide Glu-Gln-Gln-Tyr, according to the results of that research group, was also active in phagocytosis stimulation test [98].

At the same time, Martinez et al. synthesized Ig G decapeptide fragment Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg (res. 335–344) containing the C terminal rigin moiety and evidenced that fragment also possesses phagocytosis stimulating potency [175].

On the other hand, Konopińska et al., who synthesized a 287–294 fragment of human serum γ-globulin EU (Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln), found that this particular peptide, similarly as the canine tuftsinyltuftsin, is ineffective as a phagocytosis stimulant [143].

Same years ago, Biondi et al. obtained 289–299 Ig G undecapeptide fragment, containing *N*-glycosylated Asn^{297} residue [26]. Investigating the inhibition of the binding of rheumatoid factor to Ig G - Sepharose by different Ig G partial sequences, Stanworth found that tridecapeptide Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-Asp-Ser-Thr-Thr-Arg is a very active inhibitor of this reaction. The 407–416 fragment of human Ig G1, Tyr-Ser-Lys-Leu-Thr-Val-Asp-Lys-Ser-Arg also showed some reactivity and proved capable of inhibiting the binding of radiolabeled human Ig G to heterologous mouse macrophages. It is worth noting that the C-terminal tetrapeptide of that sequence manifests a remarkable similarity to tuftsin [311].

Lukas et al. estimated the ability of the peptides from the 282–292 region of Ig G to inhibit C1 mediated immune hemolysis. They found that the 282–292 undecapeptide with a tuftsin sequence on its C-terminus effectively inhibits this reaction. Tuftsin itself was found to be about four times less active as a hemolysis inhibitor. Besides those peptides, Lukas et al. examined the inhibitory activity of their dimeric forms, obtained by coupling the N-termini of the peptides to terephthaloyl-bis-(iminodiacetic acid). The dimeric peptides were twice as active on the molar basis as the corresponding monomers [172].

Kaurov et al. confirmed the results reported for undecapeptide. They also investigated a series of peptides of the types Xxx-Asn-Ala-Lys-Thr-Lys-Pro-Arg and concluded that the character of the N-terminal Xxx residue is an important factor in the inhibitory properties of a substance. Peptides substituted in this position with His and Arg were

found to be active, whereas those with Gly and Trp were inactive [127].

The fact that tuftsin inhibits the binding of first component C1q of human complement to sensitized sheep erythrocytes was also established by Kozlov et al. [150] and by Takada et al. [320].

As we note above, sequences related to tuftsin appear in the molecules of C-reactive protein (CRP). CRP is an acute phase reactant, whose level increases rapidly following an inflammation or other tissue injury. CRP contains Lys-Pro-Arg, Thr-Lys-Pro, and Thr-Lys-Arg tuftsin fragments. It is known that Lys-Pro-Arg tripeptide inhibits tuftsin action [85]. It has also been proposed that the Lys-Pro-Arg moiety acts as a signal sequence in a number of natural peptides [329]. Thr-Lys-Pro tripeptide, that occurs repeatedly in CRP, also inhibits the macrophage migration and generation and secretion of O_2^- by stimulated macrophages [14].

Buchta et al. [35], who investigated a series of such CRP fragments, found that 2 of those, Ile-Pro-Ser-Tyr-Ala-Thr-Lys-Arg and Thr-Lys-Pro-Gln-Leu-Trp-Pro, enhanced phagocytosis in mouse macrophages to an extent similar to that of tuftsin. However, the fragments showed no binding to the cells and differed from tuftsin as far as the influence on metabolic activity of neutrophils was concerned.

Robey et al. assayed three CRP tetrapeptides (Thr-Lys-Pro-Leu, Gly-Lys-Pro-Arg, and Thr-Lys-Pro-Gln) and found that they stimulate phagocytic leucocytes to chemotaxis and to superoxide production, as well as induce mononuclear cells to produce interleukin-1 (IL-1) in vitro when used at concentrations similar to those required for tuftsin to induce the same phenomena [265].

Fiedel investigated the influence of tuftsin- related fragments of human CRP on ADP- induced platelet aggregation. He found that Phe-Trp-Val-Asp-Gly-Lys-Pro-Arg CRP-octapeptide (res. 109–116) inhibits platelet aggregation and secretion, whereas the peptide Phe-Thr-Lys-Pro-Gln-Leu-Trp-Pro (res. 199–206) augments platelet activation when soluble and inhibits platelet activation when immobilized [77].

A number of small peptides produced during the incubation of CRP with neutrophils were identified by Shephard et al. [282]. Synthetic samples of those peptides were screened for their ability to inhibit neutrophil superoxide production and chemotaxis. It was found that the peptide Lys-Pro-Gln-Leu-Trp-Pro containing a part of tuftsin-like fragment of CRP demonstrates a pronounced activity in those tests. However, different results were reported by Robey et al., who found that the Thr-Lys-Pro-Gln CRP tetrapeptide (res. 200–203) enhanced phagocyte superoxide production and chemotaxis [265].

The influence of tuftsin-like peptides derived from CRP on oxygen metabolism in human blood phagocytes (neutrophils and monocytes) was also studied by Polevshchikov and Nazarov [255]. The same authors also studied the influence of these peptides on the proliferation of intact and mitogen stimulated peripheral blood lymphocytes. They found that the peptides did not increase the [^{3}H]-thymidine incorporation into the resting lymphocytes but accelerated it after mitogen stimulation [256].

Table 1
Phagocytosis stimulation and inhibitory activity against tuftsin of peptides derived from viral proteins

Peptide	Source	Phagocytosis stimulation	Inhibitory activity
Thr-Lys-Pro-Thr	HBV	+	−
Thr-Lys-Pro-Thr-Asp	HBV	−	+
Thr-Lys-Pro-Thr-Asp-Gly	HBV	−	+
Thr-Lys-Ala-Lys	HIV-1	−	+
Thr-Lys-Ala-Lys-Arg	HIV-1	+	−
Pro-Thr-Lys-Ala-Lys-Arg	HIV-1	+	−
Thr-Lys-Glu-Lys	HIV-2	−	+
Thr-Lys-Glu-Lys-Arg	HIV-2	+	−
Pro-Thr-Lys-Glu-Lys-Arg	HIV-2	−	−
Thr-Val-Pro-Pro-Arg	ad 2	−	+
Thr-Arg-Pro-Pro-Arg	ad 2	−−	++
Thr-Gly-Pro-Pro-Thr	ad 2	+	+
Pro-Arg-Pro-Pro-Arg	ad 2	−	++
Phe-Val-Pro-Pro-Arg	ad 2	−	+
Ala-Arg-Pro-Pro-Ala	ad 2	−	+
Tyr-Gly-Pro-Pro-Lys	ad 2	−−	++

The tuftsin- and tuftsin-like sequences are present in the coat proteins of many viruses. Our examination of the existing data as collected in protein data banks showed that the tuftsin sequence Thr-Lys-Pro-Arg is present in the molecules of bovine leukemia virus cell receptor precursor, Southern bean mosaic virus coat protein precursor, avian adenovirus GAL1 DNA-binding protein, human adenovirus type 7 large T-antigen, simian immunodeficiency virus polyprotein, Friend murine leukemia virus core polyprotein, and Epstain–Barr virus helicase. Retro-tuftsin sequences (Arg-Pro-Lys-Thr) for instance are observable in herpes simplex virus alkaline exonuclease, in Varicella–Zoster virus transcriptional regulator homolog, grapevine fanleaf virus, tomato black ring virus polyproteins, feline calcivirus non-structural polyprotein, and in Kennedya yellow mosaic virus replicase polyprotein. It was suggested that such fragments of the viral polypeptide chains may be involved in the inhibition of the defense mechanisms of the host organism through the competition between tuftsin and viral proteins for the tuftsin receptors on the leucocyte membrane [292].

To prove this hypothesis, Siemion et al. synthesized a series of respective peptides derived from: (i) main antigenic determinant of S-protein of HBV virus [292], (ii) gp120 protein of 2 subtypes (HIV-1 and HIV-2) of the human immunodeficiency virus [294], and (iii) III, IIIa, IV, V, and VII proteins of adenovirus type 2 [295]. The synthesized peptides were examined for their ability to inhibit the phagocytosis stimulation effect exerted by tuftsin. The results of these investigations are shown in Table 1.

Interestingly, the sequences derived from adenovirus proteins and possessing the largest inhibitory potency against phagocytosis stimulation exerted by tuftsin, closely

resemble the sequence of the pentapeptide inhibitor of tuftsin, Thr-Lys-Pro-Pro-Arg, mentioned above.

The same research group investigated a possible role of a retro-tuftsin- like sequence Ile-Pro-Lys-Thr (res. 210–213 of the precursor form) of interleukin-1α (IL-1α) peptide chain in the competition between tuftsin and IL-1α for the same cellular receptor. That such a competition actually takes place was established on the ground of the observed influence of tuftsin on leucocytosis evoked by IL-1α as well as on the ground of effects exerted by IL-1α in autologous rosette forming cell (ARFC) assay. Tuftsin, administered to mice together with IL-1α, inhibits the increase in leucocyte number, exerted by IL-1α. It also cancels the decrease in the ARFC number evoked by IL-1α. Moreover, when applied alone, tuftsin does not influence the ARFC number [293]. The effect of tuftsin in the ARFC test was also compared with similar effects evoked by tuftsin's sequential isomers: tetrapeptides Thr-Pro-Arg-Lys (Kentsin), Thr-Arg-Lys-Pro, and Thr-Lys-Arg-Pro. It was found that whereas tuftsin itself does not influence the ARFC number, the sequential isomers listed here do increase it. A corresponding difference was also found in the phagocytosis stimulation test. Unlike tuftsin, its isomers caused a decrease in phagocytic indices [347].

The competition between tuftsin and IL-1α was explained by the presence of the retro-tuftsin Ile-Pro-Lys-Thr sequence in mature IL-1α. It was also suggested that another tuftsin-like motif, Ile-Lys-Pro-Arg (res. 109–112 of the precursor form), itself absent from IL-1β, may be responsible for the fact that, unlike IL-1β, the precursor form of IL-1α is entirely active [293].

The competition between tuftsin and IL-1α is also confirmed by the observation that tuftsin inhibits the humoral immune response, decreasing plaque forming cell (PFC) number in mice [347]. The humoral immune response depends mainly on IL-1 action. Wieczorek et al. observed that tuftsin similarly inhibits in a dose dependent manner IL-1-induced IL-2 production [345]. Their observation supports the hypothesis that tuftsin can actually block IL-1 cellular receptors. The pentapeptide Glu-Ile-Pro-Lys-Thr and tetrapeptide Ile-Lys-Pro-Arg, derived from IL-1α peptide chain, produce the same effect, in keeping with the hypothesis that the presence of retro-tuftsin- and tuftsin-like sequences in IL-1α may be responsible for the phenomenon of tuftsin - IL-1α competition [287]. The inhibition of IL-1-dependent IL-2 production (established as the diminishing growth of CTLL-2 cell line in the presence of supernatant of LBRM-33–1A5 cell line) has also been observed for IL-1β fragment Asp-Lys-Pro-Thr-Leu (res. 76–80). This latter peptide resembles the retro-sequence of the Arg-Thr-Pro-Lys-Val (res. 94–98) of transforming growth factor β3 (TGF β3) polypeptide chain, that fragment stimulates the humoral and cellular immune response (determined by plaque forming cell (PFC) and delayed type hypersensitivity (DTH) tests, respectively). Another tuftsin-like fragment Lys-Thr-Pro-Lys-Ile, that appears in the same loop in TGF β2 molecule, suppresses both of those responses [346].

3. Antigenic properties of tuftsin

As we note above, in 1977 Spirer et al. made an attempt to produce antituftsin antisera using bovine serum albumin (BSA) - tuftsin conjugate for immunization of rabbits. They used p-diazonium phenylacetyl residue, located at tuftsin N-terminus, for the coupling of the peptide to the protein [305]. Najjar et al. used for the same purpose BSA and p-diazonium phenylacetylglycylglycyl-tuftsin [206]. Naim et al., however, did not succeed in their attempt to generate antituftsin antibodies by using as the antigens any of the following substances: (i) tuftsin coupled to methylated BSA by carbodiimide method, (ii) tuftsin polymerized by means of glutaraldehyde, and (iii) tuftsinylcysteine, conjugated to BSA, keyhole limpet hemocyanin (KLH), and purified protein derivative from *M. bovis*.

One possible reason for the lack of antigenicity in tuftsin could result, in the opinion of the authors, from a lack of 'foreigness' of tuftsin in mammalian species [195].

Further experiments of Naim and van Oss confirmed the conclusion that tuftsin is weakly antigenic. However, specific antituftsin antibodies formation in mice was observed by these authors for cysteinyl-tuftsin coupled to KLH [197]. The antituftsin antiserum thus obtained inhibited the phagocytosis of bacteria by human neutrophils, both with and without exogenous tuftsin [196].

However, Dagan et al. observed that tuftsin, when covalently linked to KLH and BSA, potentiates the immunogenic capacity of macrophages, transforming some very weak immunogens (like BSA) into potent immunogens, for example, in vivo BSA - tuftsin conjugate augments antibody production, whereas BSA alone or BSA administered together with tuftsin have no such immunogenic effect. In these experiments, the peptide was coupled to the protein either via p-diazonium phenylacetyltuftsin (conjugation through N-terminus) or via tuftsinyl-(p-diazonium phenylethyl)-amide (conjugation through C-terminus) [66].

According to Dagan et al., the effect of tuftsin conjugates applied to macrophages consists in the signaling de novo synthesis of IL-1 mRNA, and the induction of IL-1 secretion from the cells. They also found that free tuftsin can augment IL-1 production only in the presence of an antigen; when applied alone, free tuftsin does not induce either production or secretion of IL-1 [67].

Trudelle et al., who obtained a tuftsin polymer of M.W. 11 700, and found that the antibody response to it was very weak, used polytuftsin as a carrier molecule for vaccine preparation. Their coupling of two haptenic sequences of the pre-S region of hepatitis B virus antigen (res. 10–26 and 39–55) to polytuftsin via glutaraldehyde resulted in significant improvement of the anti-peptide antibody titer [325]. The same authors also performed a study on the influence of

different cross-linking reagents on the overall quality of conjugation of peptides to the polytuftsin carrier [69].

Polytuftsin of 35–40 - unit repeat was also used as a carrier for the preparation of synthetic immunogens in malaria vaccines against *Plasmodium falciparum* [249]. It was found that the polytuftsin-malarial peptide conjugate selectively enhances MHC class II molecules on antigen-presenting cells and may therefore stimulate better antigen presentation and proliferation of T cells [71]. Polytuftsin carrier was also used by Ivanov's research group to increase immunogenicity of the $(Asn\text{-}Ala\text{-}Asn\text{-}Pro)_3$ peptide from *Plasmodium falciparum* circumsporozoite protein [115] as well as of the peptide fragments 136–152 and 205–213 derived from the capsid protein of foot-and-mouth disease virus [116]. The authors concluded that when non-immunogenic peptides are synthesized in tandem with dituftsin, or are conjugated to polytuftsin, a significant response to that synthetic product can be elicited.

It was also shown by Surkis et al. that polytuftsin is digested by trypsin as well as by normal human serum to free tuftsin, and can be considered a potential precursor for the slow release of this peptide [315].

$[Ala^1]$-tuftsin coupled with 'ferritin' 14-mer was used to produce antiferritin antibody in rabbits [155].

4. Tuftsin and phagocytic cells

The primary effect of tuftsin, after it binds to specific cell surface receptors, consists in the stimulation of the functions of macrophages and polymorphonuclear (PMN) cells. Specific binding sites for tuftsin are also localized on human monocytes. No binding of tuftsin was observed in the case of human lymphocytes, erythrocytes, and mouse embryo fibroblasts.

The progress of the studies on tuftsin receptors was outlined in 1986 by Dagan et al. [64]. Notably, some years ago one of two macrophage receptors that bind neurotensin (a lower affinity receptor with K_d equal about 10^{-7} M) was identified as the tuftsin receptor [187].

After binding to the receptors, the tuftsin-receptor complexes are internalized. Using two independent techniques (fluorescence microscopy and autoradiography) Wagle et al. showed that human PMNs and monocytes differ in their mode of interaction with internalized tuftsin. Although in PMNs the internalized peptide remains in the cytoplasmic compartment, in monocytes it is translocated to the nucleus. This would suggest that there exists a possibility of a direct interaction of tuftsin with DNA. A large increase in DNA melting point observed in the presence of tuftsin appears to confirm such a possibility [341]. The authors paid attention to the structural similarity existing between tuftsin and the partial sequence of EIA oncogene product (Lys-Arg-Pro-Arg-Pro) required for translocation of this protein into the nucleus.

That tuftsin may be translocated to monocyte nuclei suggested that it can also modulate the growth of monocytes. In conformity with this premise it was found that tuftsin increases the $[^3H]$-thymidine incorporation by highly purified monocytes [234].

Tuftsin administered to organisms and to cell cultures stimulates the production of some cytokines. IP injection of tuftsin increases the production of TNF in serum and supernatants of cultured splenic and peritoneal cells [351]. In 1987, Robey et al. showed that tuftsin as well as its analogs, $[Gly^1]$-tuftsin, $[Leu^4]$-tuftsin, and $[Gln^4]$-tuftsin (all being fragments of human C-reactive protein), induce monocytes to produce IL-1 [265]. Spirer et al. showed that tuftsin stimulates the liberation of IL-1 from human peripheral adherent cells from splenoctomized subjects, as well as from human and mouse spleen cells [307]. On the other hand, Thr-Lys-Pro tripeptide inhibits IL-1 production by rat macrophages [13]. Recently, it was also found that treatment of mouse peritoneal macrophages with tuftsin or tuftsin-THF-γ2 chimeras in the presence of antigen also augments the IL-6 production [99]. In this way, tuftsin may perform its immunoregulatory functions and may influence inflammatory processes (by enhancing the IL-2 formation induced by IL-1).

It was pointed out by Florentin et al. that at the time that macrophage activities reach their maximal potential, lymphocyte functions, such as proliferative response to mitogen, T-cell mediated cytotoxicity, IL-2 and γ-interferon production, are all depressed. In the opinion of Florentin et al., this suggests that tuftsin also stimulates the negative regulatory functions of these cells [82]. Such a statement corresponds well with the finding of Siemion et al. that tuftsin competes with IL-1 for the same cellular receptor, thus realizing a feedback control in IL-1 action [293].

Tuftsin also potentiates antibody levels following a simultaneous injection with a T-cell -dependent antigen to mice with a genetically controlled defect in affinity maturation, but remains to have no effect on antibody affinity in the same mice [112].

It was originally found that tuftsin stimulates phagocytosis after binding to PMNs. Subsequently, the phagocytosis stimulating activity of tuftsin in monocyte-macrophages was also shown. The role of tuftsin in the regulation of macrophage phagocytosis was reviewed in 1986 by Coleman [56].

Nishioka et al. recently introduced a new assay for the study of phagocytosis stimulation exerted by tuftsin. The assay consists in the use of fluorescent microspheres and the flow cytometric procedure [235]. Tabata and Ikada used biodegradable microspheres prepared from L-lactic acid, D,L-lactic acid, or glycolic acid homopolymers and copolymers to study the phagocytosis in mouse peritoneal macrophages. The study showed that tuftsin enhances phagocytosis [319].

Gamalei et al. concluded that the kinetics of change in the fluorescence intensity of macrophages in the medium with fluorescein diacetate may serve to monitor early stages

of macrophage activation [88]. The fluorescein anions, formed in macrophages by the hydrolysis of fluorescein diacetate, are released into extracellular medium. The authors recorded separately the diacetate hydrolysis reaction and the fluorescein anion efflux. Tuftsin affects the first of these 2 processes only, whereas Lys-Pro-Arg tripeptide decreases both [89]. The authors also found that the decrease of fluorescence is much smaller in the medium containing catalase and superoxide dismutase, which suggests that the oxidative burst products may serve as regulators of the feedback control during macrophage activation [90]. The same research group stated that stimulation of macrophages with tuftsin results in a biphasic change in membrane potential in the form of depolarization followed by hyperpolarization of cell membrane, that is accompanied by an increase in cytoplasmic Ca^{+2} concentration. In the opinion of the authors, the effects of tuftsin are similar to those of calcium ionophore [91].

A number of papers was devoted in the last years to applying phagocytosis stimulative activity of tuftsin in diseases and microbial infection. Taking into account the fact that peritonitis caused by *Candida albicans* is a major complication of continuous ambulatory peritoneal dialysis (CAPD), Kain et al. studied the activation by tuftsin of human peritoneal macrophages from CAPD patient. They suggested that tuftsin may be considered as a potential therapeutic drug in that disease [121]. Kazanowska et al. studied the influence of tuftsin on the function of granulocytes of children with acute lymphoblastic leukemia [129]. Iyer et al. examined in vitro the influence of tuftsin on the phagocytic activity of human peripheral blood monocytes/macrophages derived from patients of tuberculoid leprosy (BT/TT) and lepromatous leprosy (BL/LL). A serious differences in the response to tuftsin in both these sorts of macrophages were found. The BL/LL macrophages exhibited premature inability to undergo tuftsin stimulation, that ability, according to authors, may be reversed by serial dosage of the peptide [117,118]. Price et al. studied the effect of tuftsin in permissive bovine mammary macrophages that were unable to control the intracellular replication of *Brucella abortus*. In the presence of tuftsin, the ability of the permissive macrophages to control the replication of *B. abortus* was definitely enhanced, with permissive macrophages being transformed into the restrictive ones. The Lys-Pro-Arg tripeptide (tuftsin inhibitor) abolished that effect of tuftsin [262].

Sorokin et al. investigated the influence of tuftsin on the phagocytosis of 'premedullary' lung macrophages. It was found that pretreatment of macrophages with tuftsin would enhance the uptake of native yeast cells by 100% in hamsters and 200% in rats. The macrophage-inhibitory peptide Thr-Lys-Pro inhibited phagocytosis in hamsters by 60%, but had no such effect in the case of the cultures of rat lung cells [303].

The effect of tuftsin on decreased macrophage functional activity evoked by experimental neurosis was studied by Bulatova et al. Tuftsin did not restore the macrophage activity but led to additional rise of the adrenal glands weight and to granulocyte-monocytosis [36]. It was also found that tuftsin increases phagocytic activity in cultured Sertoli cells of rats [78], as well as in the cultures of murine Kupffer cells. The results obtained in the latter case indicate the presence of specific tuftsin receptors on the Kupffer cells [156].

The stimulation of phagocytosis by tuftsin was also observed for unicellular Tetrahymena. However, an even greater response was evoked by the threonine-free tuftsin tripeptide, placing the Tetrahymena response in a strong contrast to that of Mammalia [62].

Nishioka et al. performed a precise comparison between the phagocytosis stimulating activity of tuftsin and that of [Ala^1]-tuftsin. According to some earlier data (see above), an analog such as [Ala^1]-tuftsin should be more active than tuftsin itself. However, the results obtained indicate that this analog is not as active as tuftsin in phagocytosis stimulation [230].

Tuftsin was also used as the standard in the determination of macrophage stimulation by Quadrol (*N*,*N*,*N*',*N*'-tetrakis(2-hydroxyprolyl)-ethylenediamine) [24].

As we noted above, the role tuftsin plays in the stimulation of bactericidal activity of phagocytic cells stems mainly from its stimulation of the generation of reactive oxygen species like hydrogen peroxide and superoxide anion by the affected cells.

The study of Bielefeldt–Ohmann and Babiuk showed that PMNs are much more active in the reactive oxygen species generation than bovine blood monocytes or alveolar macrophages. In addition, the bovine phagocytes were found to lack tuftsin receptors and remained unresponsive to tuftsin [25].

For the induction of a respiratory burst in peritoneal exudate cells of mice, Singh et al. used tuftsin amide substituted in the amide group by a long hydrophobic moiety -NH-$(CH_2)_2$-$NHCOC_{15}H_{31}$. The macrophages exhibited enhanced levels of reactive oxygen species; enhanced activity of NADPH oxidase and myeloperoxidase was also observed. The activity of superoxide dismutase, catalase and glutathione peroxidase, all remained unchanged [298]. It is worth noting that in 1984, Singhal showed that the incorporation of that amide (as well as the amide Thr-Lys-Pro-Arg-$NHC_{18}H_{37}$) into egg phosphatidylcholine/cholesterol liposomes significantly enhances the binding of those liposomes to PMNs. No such enhanced binding takes place in contact with erythrocytes and lymphocytes, that would indicate that the incorporation of tuftsin motif into liposome bilayer specifically addresses the liposome to PMN leucocytes [301]. It was also found that phagocytosis-associated respiratory burst of PMNs may be inhibited by the N-terminal tetrapeptide of cystatin C (Lys-Pro-Pro-Arg), that is in fact a sequential isomer of N-terminal peptide of substance P (Arg-Pro-Lys-Pro), a phagocytosis stimulant (see above) [160].

Martinez Cairo Cueto et al. investigated the influence of tuftsin on the oxidative capacity of PMNs of low-birth weight newborns and concluded that the low oxidative capacity observed for such PMNs did not depend on any intrinsic cellular defects [173].

Khare et al. previously observed that macrophages of BL/LL leprosy patients exhibit premature inability to undergo the tuftsin-stimulated phagocytosis and microbicidal activity. The authors subsequently examined the influence of tuftsin on the production of reactive oxygen species and adenosine deaminase activity in the cultures of lepromatous macrophages. It was found that, after day one, the lepromatous macrophages were unable to undergo the tuftsin-mediated stimulation of these cell activities [132].

It was also reported by Cillari et al. that tuftsin activates in a dose-dependent manner the murine macrophages to express nitric oxide synthase and to produce nitric oxide. This effect may be in part responsible for the microbicidal activity of the cells; the authors found that murine peritoneal macrophages, when activated by tuftsin, kill in vitro the amastigotes of protozoan parasite *Leishmania major* [55].

The phagocytosis process is accompanied by chemiluminescence of the phagocytic cells. According to Lever et al., however, tuftsin stimulates the phagocytosis of *Haemophilus influenze* isolated from sputum of patients with hypogammaglobulinaemia without triggering chemiluminescence of neutrophils [161]. According to Phillips et al., also in vitro tuftsin has no effect on the chemiluminescence response of bovine PMNs [251]. Cornaglia–Ferraris et al. established that tuftsin and substance P elicit a strong burst of luminol-enhanced chemiluminescence of human PMNs; the preincubation of PMNs with L-arginine abolishes or diminishes the effect [61]. Tuftsin also exhibits a significant stimulation of lucigenin-amplified chemiluminescence of neutrophilic granulocytes [151].

Some further progress in the investigation connected to tuftsin chemotaxis phenomena must be noted as well. Zetter et al. have shown (by using a new in vivo elaborated assay) that tuftsin acts as a chemoattractant with respect to monocytes but not with respect to eosinophils and mast cells [357]. Pohajdak et al. investigated the chemotaxis of large granular lymphocytes and established that tuftsin in this case is inactive as chemoattractant [254]. Wiedermann et al. found that tuftsin inhibits the migration of human neutrophils towards the gradient of formylmethionyl-leucyl-phenylalanine or recombinant human IL-8. Such effect, however, may be partly reversed by [Pro^1]-tuftsin, a tuftsin receptor antagonist [348].

According to Shishova et al., tuftsin stimulates the activity of glucose-6-phosphate dehydrogenase and lowers the activity of lactate dehydrogenase in phagocytic rat peritoneal macrophages. No such effects, however, were observed in non-phagocytic cells [284].

Tuftsin also influences the activity of tyrosine hydroxylase of peritoneal macrophages of rats exposed to chronic stress [327], as well as the activity of 5-nucleotidase in macrophages of some strains of mice [183].

Arginine-specific ADP-ribosyl transferase, that is released from chicken PMN leucocytes, modifies tuftsin as its preferential substrate, producing ADP-ribosylated peptide. The modified peptide suppresses the receptor-binding capacity of tuftsin to murine peritoneal macrophages and phagocytosis-stimulating activity. This result suggests that ADP-ribosylation of tuftsin may be involved in the regulation of inflammatory processes [321].

Some studies have also been conducted on the influence tuftsin has on the intracellular pH of mouse peritoneal macrophages [328].

5. Antimicrobial, antiviral, and antitumor activities of tuftsin and its therapeutic effects

Further progress should be noted in the investigation of tuftsin antimicrobial effects. Fridkin and Najjar in their review [84] underline the importance of results obtained by Smith et al., who used Gentamicin combined with tuftsin in the treatment of experimental keratitis caused by *Pseudomonas aeruginosa* [302]. Fridkin and Najjar also discuss the results obtained by Nishioka et al. in connection with the prophylaxis by tuftsin of *Candida albicans* infections [229].

Potential use of tuftsin in treatment of *Candida peritonis* infections (in a murine model) was evidenced by Levy et al. [163]. The application of tuftsin derivative Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-$NHCOC_{15}H_{31}$ for protection of mice against *Plasmodium berghei* infection was reported by Gupta et al. [100]. Guru et al. found that tuftsin-bearing liposomes are active against *Leishmania donovani* infections [101]. Fatma et al. investigated the potentiating role of tuftsin in the chemotherapy of experimental filariasis by Ivermectin [75].

Another series of papers describe the stimulation of microbicidal activity of blood monocyte-macrophages of leprosy patients by tuftsin. The cultures of monocyte/macrophages from tuberculoid leprosy (BT/TT) patients as well as lepromatous leprosy (BL/LL) patients were found to be unable to kill intracellular *Mycobacterium leprae* after a single stimulation with tuftsin; this inability was reversed by a daily administration of the peptide [118]. It was also found that tuftsin concentration was significantly lowered in the sera of BL/LL patients, and only slightly so in the sera BT/TT patients [126]. Khare et al. also showed that the BT/TT cultures of monocyte-macrophages undergo a change in the number of tuftsin receptors and affinities in the maturation process, whereas the cultures of BL/LL monocyte/macrophages display a significantly lower number of receptors. Accordingly, the biologic response of BT/TT monocytes gradually increases with the age of the culture [133]. The same authors showed that tuftsin activates BL/LL macrophages to produce high levels of nitric oxide and that this production decreases with the age of the

culture. In normal macrophages, however, tuftsin would not exert similar effects [134].

It should also be noted that, according to the results obtained by Vishnevetskii et al., tuftsin stimulates *M. leprae* multiplication in the foot pads of CBA mice and prolongs *M. leprae* survival in the culture of macrophage-like cell line P.388 [339].

In this context it is worth noting that in different proteins of *M. leprae* (inter alia rRNA methylase, cytochrom C oxidase, Mg and Ca transport protein etc.) 8 tuftsin sequences as well as one retro-tuftsin sequence could be found.

Tuftsin also enhances the action of rifampin-bearing liposomes in the treatment of tuberculosis in mice [2], and that amphotericin B -bearing liposomes in the treatment of human aspergillosis in mice [242].

Corazza et al. established that the tuftsin activity and splenic functions in the patients with AIDS and AIDS-related complex are significantly lowered as compared to healthy volunteers. The researchers supposed that tuftsin deficiency may contribute to the risk of bacterial infection in HIV-positive individuals [60]. Szkaradkiewicz showed that decreased phagocytosis and decreased bactericidal activity in human monocytes of patients with AIDS-related complex or with persistent generalized lymphadenopathy are normalized by tuftsin [318]. According to Mathe et al., tuftsin potentiates the action of bestatin during immunotherapy of HIV1 patients [179]. Bestatin is an *N*-(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanyl-L-leucine dipeptide extracted from *Streptococcus olivoreticuli* and is a leucine aminopeptidase and aminopeptidase B inhibitor. The bestatin induced inhibition of aminopeptidase B leads to the decrease in tuftsin tripeptide formation (that tripeptide is an antagonist of tuftsin itself) [177,178].

The antitumor activities of tuftsin were reviewed in a paper by Fridkin and Najjar [84] and in one by Nishioka et al. [231].

Still, in 1983, Catane et al. showed during the preliminary clinical testing of tuftsin that the peptide, when administered i.v. in a dose of 0.96 mg/kg, had no acute or chronic toxic effects. Pronounce leucocytosis was observed, however, in more than 50% of patients at 4–5 days post treatment. Antitumor in vivo activity of tuftsin in the murine fibrosarcoma model was observed as well [44]. The authors performed a broad phase II study of tuftsin, administered i.v., using a target group of patients with various advanced malignant diseases, and found that the peptide has only a limited effect in patients with advanced malignancies [45]. Also investigated was a possible application of combined immunotherapy and chemotherapy consisting in the use of tuftsin plus cyclophosphamide in the treatment of non-small-cell carcinoma in the lung [343].

Schantz et al. showed that tuftsin restores the natural killer cells activity diminished after curative surgery of patients with head and neck carcinoma [272]. Tuftsin also prolongs the survival time of mice with 3-methylcholanthrene-induced primary fibrosarcoma and Lewis lung carcinoma [15] and delays murine peritoneal carcinomatosis [53]. The data reported in the latter paper show that antitumor effect of tuftsin is mediated through the increased cytotoxic properties of primed macrophages.

Hart investigated the effect of tuftsin in mice bearing the B16 melanoma or treated with *Corynebacterium parvum*. He found that the tuftsin treatment inhibits tumor progression but only if the treatment commences at the time of tumor transplantation. Tuftsin inhibited the development of splenomegaly in mice with tumors, but did not influence plasma proteinase activity of the animals [102].

Therapeutic uses of tuftsin and other immunostimulants were discussed by Diezel et al. [72], whereas the usefulness of tuftsin for the restoration of secondary immunodeficiencies caused by immunosuppressive drugs was discussed by Caspritz and Hadden [43].

The role of tuftsin and spleen in promoting liver cirrhosis in rats was investigated by Wang et al. [342], whereas that of prolonged action of tuftsin in penicillin epilepsy in rats was studied by Popova et al. [257].

In 1985, Chu et al. investigated the effects of tuftsin on postsplenectomy sepsis in mice and concluded that tuftsin administration protects splenectomized animals against pneumococcal septic death [52]. The problem of tuftsin deficiency after splenectomy was also studied on mice by Tsunoda and Shibusawa [326] and on human patients by He [104]. Szendroi et al., who suggested splenic autotransplantation as a method of preserving splenic function, showed that the determination of tuftsin level adds support to the usefulness of this method [316,317]. Zoli et al. found that in comparison with healthy control group, decreased tuftsin activity can be observed in patients who had undergone splenectomy for trauma or elective splenectomy. That decrease was found to be greater after elective than after emergency removal [358].

6. Central effects of tuftsin and its analogs

In 1980, Herman et al. found that tuftsin administered intraventricularly to rats elicits analgesia of 20 min duration in the hot plate test. [D-Arg^3]-tuftsin was found to be even more active. The authors submitted that there may exist some link between immunobiological system and the central nervous system, because tuftsin, when continuously released to stimulate phagocytes, could also affect the central nervous system [109]. The idea was completely new at that time, but now the role of immunoregulators in the nervous system has gained a much wider recognition (for a review, see Refs. [252,253]).

The effect of tuftsin is not altered by Naloxone. Among different tuftsin analogs [D-Arg^4]-tuftsin appeared to be the most potent analgesic agent. Its antinociceptive effect persisted 150 min after injection into the lateral brain ventricle of rats. At a dose of 200 μg, the action of this peptide,

however, is accompanied by a high toxicity. No parallel result was observed between the analgesic potency and the phagocytic properties of the peptides investigated [110].

Among the di- and tripeptide partial sequences of tuftsin, only the dipeptide Pro-Arg revealed any evident analgesic action in both hot-plate and tail-flick immersion tests. Lys-Pro dipeptide was active in hot-plate procedure only, whereas other partial sequences of tuftsin were inactive in both tests [107].

Subsequent experiments showed that bradykinin injected ICV at the dose of 3 μg and 2 μg 10 min before the administration of tuftsin to rats and mice, respectively, decreases the antinociceptive action of this peptide. It was also found that tuftsin increases the blood pressure in rats in a dose-dependent manner and affects the exploratory and locomotor activity of rats in a biphasic manner. After a short period of depression, stimulation of the behavior was observed [108].

Neurotensin, an agonist of tuftsin in induction of analgesia, antagonized antinociceptive effect of enkephalins, that may suggest that it modulates in an opposite way the function of the enkephalinergic neurons and the central action of tuftsin [106]. Further studies using tuftsin analogs confirmed the role of the Pro-Arg fragment in generating analgesic activity [214].

The Pro-Arg dipeptide was found to possess a much stronger analgesic potency than the C-terminal dipeptide Phe-Arg of bradykinin, whereas the tripeptides Pro-Phe-Arg and D-Pro-Phe-Arg are deprived of any analgesic activity [289]. Some analgesic effect, but of a magnitude lower than that evoked by tuftsin, was also elicited by the N-terminal tetrapeptide of substance P (Arg-Pro-Lys-Pro). However, the tuftsin analog Thr-Arg-Pro-Arg was found to be inactive. Among the potential tuftsin inhibitors, peptides related to the main epitope of hepatitis B virus S-protein, Thr-Lys-Pro-Thr and Thr-Lys-Pro-Thr-Asp are weakly active analgetics, whereas pentapeptide Thr-Lys-Pro-Pro-Arg and Thr-Lys-Pro-Gly-Arg (an analog of the known tuftsin inhibitor) produced a weak hyperalgesia 30 min after ICV injection into the rat brain [291].

Changes in the configuration of particular amino acid residues in Pro-Arg dipeptide produce active compounds with markedly long duration of the analgesic effect (the exception being the Pro-D-Arg dipeptide). Tuftsin n-hexyl- and n-heptylamides, on the other hand, are toxic and deprived of antinociceptive effect [216].

Unexpectedly, some antinociceptive activity was also found in the pentapeptide tuftsin inhibitor Thr-Lys-Pro-Pro-Arg. The analgesic activity of 7 tuftsin-like fragments of human adenovirus type 2 (ad2) and of another 2 such fragments of human immunodeficiency viruses HIV1 and HIV2 was also tested. Very strong analgesia was observed for ad2 fragments: Thr-Arg-Pro-Pro-Arg, Pro-Arg-Pro-Pro-Thr, and Phe-Val-Pro-Pro-Arg [238].

Central effects of tuftsin have also been investigated by other research groups. Thus, Aronowski et al. showed that tuftsin and [Lys^4]-tuftsinyltuftsin attenuate the withdrawal behavior in morphine-dependent rats [9]. Galasik–Bartoszek et al. reported that ICV injection of hydroxyproline analog of tuftsin ([Hyp^3]-tuftsin) at a dose of 100 nmol induces the antinociceptive effect for about 2 h. The effect was blocked by IP injection of Naloxone (1 mg/kg). The [Hyp^3]-tuftsin also evoked an increased diuretic effect, whereas tuftsin itself did not produce any such effect [87].

Nicolaides et al. examined the analgesic activity of tuftsin (administered ICV) against acetic acid-induced writhing in mice. They found that tuftsin, Pro-Arg, and Pro-Arg-Pro peptides were only weakly active in this test whereas the Lys-Pro-Arg tuftsin fragment was essentially inactive [217].

A series of studies on the central effects of tuftsin were performed by Russian scientists. In 1981, Valdman et al. produced enhanced locomotor activity, aggressiveness, and diminution of the acquisition of the passive avoidance reaction during a single reinforcement after IP administration of tuftsin at a dose of 500 mg/kg. In vitro experiments revealed a direct inhibitory effect tuftsin has on the reaction of brain tyrosine hydroxylase activity, that, in the opinion of those authors, indicated a relationship between the central effects of tuftsin and the changes in brain catecholaminergic processes [333]. Subsequently, the same group observed that tuftsin and [D-Arg^4]-tuftsin (20–250 mg/kg, IP) decrease immobility of mice under the experimental conditions of the despair test and increase exploratory activity in rats [332].

Valdman et al. also investigated the influence of tuftsin and some of its analogs on dopamine-dependent rotational behavior and on tyrosine hydroxylase activity in the brains of rats, and found that tuftsin and [Leu^1]-tuftsin have no effect on postsynaptic dopaminergic receptors and decrease the activity of the enzyme, whereas [D-Arg^4]-tuftsin decreases rotational behavior and increases the enzyme activity, probably by modulating postsynaptic dopaminergic receptors [331]. The influence of tuftsin on the locomotor activity of animals was also investigated by Lavretskaya et al. [158].

Semenova et al. found that IP injection (300 μg/kg) of tuftsin to rats induces an increase in memory traces stability during a 30 days period [278] and affects the learning and exploratory behavior in rats [279].

The temporal characteristics of the behavioral effects of tuftsin on rats were studied by Kamenskii et al. [122]. The same group found that tuftsin at the doses adapted to its physiological concentration in blood partially restored to normal the locomotor activity and orientation behavior previously altered by drugs affecting aminergic brain system of rats [11]. Kamenskii et al. also showed that tuftsin selectively affects the central nervous system when administered intranasally [124].

Lovedova et al. studied the effects tuftsin has on the activity of acetylcholinesterase and monoaminooxidase in individual brain structures. It was found that tuftsin alters

the evoked potentials to a signal light stimulus in the cortical and subcortical brain structures [169].

The effect of tuftsin on the bioelectrical activity of brain structures was studied also by Veskov et al. [337] and Popova et al. [259]. Experiments performed with dogs, cats, rabbits and rats all showed that tuftsin modifies the configuration of the evoked potentials, especially in the visual cortex, as well as augments catecholaminergic and suppresses serotonin activity in the sensorimotor cortex and caudate nucleus. In the opinion of those authors, the results of tuftsin administration may be interpreted as an intervention into the processes of visual perception. Morphochemical changes in the visual and sensorimotor cortical neurons of rats exposed to tuftsin were studied by Chebotareva [49,50].

Gershtein et al. found that a single administration of tuftsin distinctly altered the synthesis and degradation of monoamines, acetyl choline, and proteins in the cortex-subcortex structures of the brain locomotory system [94].

Dovedova found that tuftsin (when administered IP) evokes changes of the monoaminooxidase and acetylcholinesterase activity in the synaptosomal and cellular mitochondrial subfractions from the rabbit sensorimotor cortex and nucleus caudatus [73]. No change, however, was registered by Sergutina by quantitative cytochemical methods in the activity of these enzymes after tuftsin administration in the brains of rats [281].

Dovedova and Monakov found that tuftsin normalized to some extent the levels of dopamine, noradrenaline, serotonin, and 5-hydroxy-indolyl acid. It also caused a decrease in the rate of depolarization dependent Ca^{2+} capture in the synaptosomes of the rat brain cortex and neostriatum, when altered by a sedative drug Haloperidol [74].

The catecholamine content in striatum and hypothalamus was also examined during the investigation of the influence of tuftsin on immobilization stress-induced alterations in the neuroendocrine and immune systems [137].

Enhancement of electrical activity in the visual and motor cortex and in the nucleus caudatus of Haloperidol-treated rats after tuftsin administration was also reported [258]. Possible uses of tuftsin as regulatory drug, e.g. to correct the disturbances evoked by chronic Haloperidol treatment were discussed recently by Gershtein et al. [93].

The relation between the brain monoaminergic systems and the effects of tuftsin on animal emotional behavior was also studied by Semenova et al. [277]. It was found that intracutaneous administration of tuftsin or its heptapeptide analog (Thr-Lys-Pro-Arg-Pro-Gly-Pro) to rats neonatally treated with 5,7-dihydroxytryptamine results in a weakened perception of stress situations, an increase in stability of investigation behavior, and a normalization of serotonin level in the brain [280]. The heptapeptide tuftsin analog (at intranasal application) was also effective as a stimulant for dogs in early stages of learning and in restoration of their lost skills [250].

Nader and Barrett have examined the effects of ICV administration of a corticotropin-releasing factor, tuftsin, and dermorphin on the behavior of squirrel monkeys. They found that tuftsin selectively increases responses maintained by food presentation at the doses that decreased shock-maintained responding [192].

The influence of tuftsin and its derivatives on the metabolism of various structures of the brain was also investigated. It was shown that tuftsin increases the intensity of cell respiration and enhances intracellular redox processes, raising the content of nicotinamide coenzymes at the expense of the oxidized forms of NAD and activating cytochrome C -oxidase and dehydrogenases (largely in the limbic structures) [20,21].

Tuftsin's influence on the activity of a number of different dehydrogenases and oxidases in the microstructures of the neocortex, hippocampus and hypothalamus, as well as in the structures of the limbic system, was also investigated. An increase in succinate dehydrogenase and malate dehydrogenase activity in neurons and glycocytes of all hypothalamic formations, as well as an increase in monoaminooxidase activity in the nerve terminals of hypothalamic neurons and in the preterminal fibers of the neocortex, were noticed [114].

Tuftsin also changes the activity of leucyl-arylamidase of the brain tissue [125]. The cytosolic aminopeptidase in the monkey brain was found to cleave amino acid sequentially from N-terminus of tuftsin [263].

The work of Klegeris and McGeer should be mentioned here as it produced some interesting results. Those two authors reported that the rat brain microglia and peritoneal macrophages show similar responses to various respiratory burst stimulants, including tuftsin [136].

As far as the antinociceptive effects of tuftsin are concerned, the results obtained by Ferreira et al. deserve particular attention. Namely, it was found that IL-1β produces hyperalgesia in rats. Its tripeptide fragment Lys-Pro-Thr (res. 193–195), a sequential isomer of retro-tuftsin tripeptide, was found to be weakly hyperalgesic and acting as a partial agonist of IL-1β. However, the analog Lys-D-Pro-Thr was found to inhibit IL-1β action in a dose-dependent manner. That effect is, however, not mediated centrally [76].

This last result prompted Caliendo et al. to synthesize a series of Lys-Pro-Thr analogs containing unconventional (nonproteinaceous) amino acids. According to these authors, Lys-D-Pro dipeptide showed a pronounced analgetic activity in the writhing test performed on mice [41].

The role of tuftsin and its tripeptide fragment Thr-Lys-Pro in the process of axonal regeneration should also be briefly discussed here. Single and repeated injections of tripeptide into the vitreous body of adult rats after transection of the optic nerve resulted in retardation of axotomy-induced ganglion degradation in the retina. Tuftsin injections produced effects opposite to those observed for tripeptide, i.e. tuftsin enhanced the degradation of ganglion cells. The effect depends on the suppression of microglia activity during the time following a transection of the optic nerve [322].

7. Other examples of the biologic activity of tuftsin

Devillier et al. showed that tuftsin is practically inactive in histamine-releasing test performed on rat mast cells [70]. However, Paradowski et al. found that tuftsin administered i.v. to rabbits and guinea pigs lowers the concentration of histamine in the lungs but elevates it in the kidneys and liver. The changes in histamine concentration in duodendum and blood were found to be insignificant [245]. Similar results were obtained upon the IP administration of tuftsin to guinea pigs [244].

Pro-Arg and Lys-Pro-Arg peptides as partial sequences of tuftsin influence histamine concentration in the tissues of guinea pigs and rats in the same manner as tuftsin itself. However, Thr-Lys and Thr-Lys-Pro peptides do not affect it. Some correspondence is apparent between analgesic effects produced by partial sequences of tuftsin in the central nervous system and the influence of those sequences on histamine concentration in tissues. The most active sequence in both these systems was found to be the Pro-Arg dipeptide [246].

However, when the influence of tuftsin's partial sequences on histamine concentration is compared with the results of the influence of those sequences on the arterial blood pressure, it appears that the Lys-Pro-Arg tripeptide, that otherwise influences the histamine concentration, does not influence the arterial blood pressure in guinea pigs [243], whereas tuftsin, Thr-Lys-Pro, Thr-Lys, and Pro-Arg peptides cause a protracted rise of blood pressure.

Tuftsin (injected at the dose of 0.3 mg/kg) also affects the heart rate and body temperature, producing a positive chronotropic effect, when administered in the state of low heart rate and a bradycardiac response at higher basal heart rates. Its influence on body temperature is biphasic: a transient hyperthermia is followed by a longer period of hypothermia [123].

Antiprocoagulatory properties of tuftsin were studied by Ashmarin et al. According to the results reported, tuftsin counteracts in vitro the polymerization of fibrin monomer; whereas in vivo it causes an increase in plasma clotting time and an increase in fibrinolysis [10].

The same research group obtained a complex of tuftsin with low-molecular weight heparin, and studied its fibrinolytic and anticoagulant activity in albino rats [157]. Tuftsin was also reported to have antithrombotic and thrombolytic activity [165].

However, Kornberg et al. found that tuftsin, when acting on mixed human mononuclear cells and monocytes, is a potent stimulant of procoagulant activity (PCA). A mild PCA was observed for lymphocytes and cell lines of monocytic origin, and no such activity in several lymphoid cell lines and HLA-60 cells. The PCA accelerates clotting through the extrinsic coagulation pathway. It can be inhibited by concavalin A and by monoclonal anti-tissue factor antibodies. Tuftsin was reported, moreover, to affect mainly the mature cells. The authors presume that the induction of PCA by tuftsin in mononuclear cells constitutes "an important link between mononuclear cells and the immune and coagulation systems" [149].

Tuftsin interacts weakly with BSA; their binding can be enhanced by introducing an apolar substituent at the C-terminus of the peptide [23].

At concentrations from 10 μg/ml to 10^{-6} μg/ml, tuftsin was found to stop the contraction of lymphatic microvascular walls and to produce a vasodilatation in rats [159].

Both tuftsin and the N-terminal substance P tetrapeptide inhibits the nicotine-evoked [^{3}H]-adrenaline outflow in rat adrenal gland slices (postsynaptic effect). However, only the SP^{1-4} peptide influences the presynaptic effect, as it inhibits the electrically stimulated [^{3}H]-acetylcholine outflow [218].

Mitsuma et al. found that i.v. injection of tuftsin to rats evokes marked changes in thyrotropin (THS) and thyrotropin-releasing hormone (TRH) levels in plasma and hypothalamus. The results suggest that tuftsin stimulates the TRH release by hypothalamus. The effect could be prevented by a pretreatment of the respective groups with 5-hydroxy-tryptophan or L-DOPA [184]. Tuftsin also stimulates thyrotropin secretion in rats [185].

Chan and Ng investigated the influence of various amino acid derivatives, peptides, and proteins on mouse embryonic development in vitro, and found that tuftsin has no effect on such development [46].

8. In search for tuftsin superactive analogs

Potential uses of tuftsin in cancer therapy and microbial infections made investigators focus their attention on such problems as the large-scale synthesis of tuftsin, its purification, enhancing its stability under storage, as well as its resistance to different proteolytic enzymes.

Large-scale solution synthesis of tuftsin was elaborated by Bonnaud et al. [32]. Some years ago the synthesis was reinvestigated: the acid-labile 4-methoxy-2,3,6-trimethylbenzene sulfonyl, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl protective groups were applied for arginine residues protection in tuftsin [86]. An attempt was also made to synthesize tuftsin by means of recombinant techniques. A hybrid β-lactamase gene with a synthetic tuftsin-coding DNA fragment was used for this purpose [356].

For the identification of tuftsin in serum, the method combining the reverse-phase HPLC and mass spectrometry was elaborated [194]. Fast atom bombardment (FAB) tandem mass spectrometry was also used in the analysis of the stability of retro-inverso synthetic isomer of tuftsin [68].

Single-step bulk purification of synthetic tuftsin by HPLC was described by Siddiqui et al. [285]. Ohta et al. demonstrated the utility of a column-switching HPLC system, consisting of an anhydrotrypsin-immobilized diol-silica precolumn and a reversed-phase analytical column for selective separation of tuftsin [239].

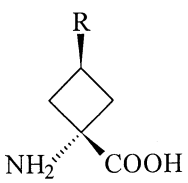
The stability of sterile saline solutions of tuftsin was investigated by Nishioka et al. The study showed that for the preservation of activity, the solution must be frozen [228]. Alekseenko and Orekhovich studied the action of proteolytic enzymes, such as the human erythrocyte prolyl endopeptidase II and pituitary gland Pro-D,L-Ala peptidylhydrolase, on tuftsin [3]. Lokshina et al. investigated the enzymatic degradation of tuftsin by cathepsin H from bovine spleen, and found that the enzyme splits the N-terminal amino acid from tuftsin, rendering the peptide inactive [167]. Tuftsin is also actively hydrolyzed by surface aminopeptidase N from human placenta [186]. Shibata and Watanabe have shown that arginine-specific carboxypeptidase from *Mycoplasma salivarium* selectively cleaves the C-terminal Arg residue of tuftsin [283].

It is known that partial sequences of tuftsin often contaminate commercial preparations of the peptide, causing a loss of the peptide activity [4].

Because biologic degradation of tuftsin may also lead to the formation of tuftsin inhibitors, the leucine aminopeptidase located on plasma membrane of human neutrophils may produce an efficient tuftsin inhibitor, the tripeptide Lys-Pro-Arg, by splitting of tuftsin [85]. The role of this enzyme in the degradation of tuftsin was studied by Nagaoka and Yamashita by using guinea pig polymorphonuclear neutrophils [193].

It was also shown by Auriault et al. that an alternative tuftsin tripeptide, Thr-Lys-Pro, inhibits various macrophage functions including IL-1 production, Ig E - specific receptor expression, IgE-dependent cytotoxicity, chemotaxis, chemiluminescence, and β-glucoronidase release. This peptide is often mentioned in literature as a macrophage inhibitory factor [13,14]. One such example is the suppression of the immune response to sheep red blood cells, evoked by the preincubation of the mixture of nonadhering and adhering spleen cells in tuftsin solution reported by Voevodin, that may depend, in the opinion of the author, on the macrophage suppressor formation [340].

To extend the in vivo lifetime of tuftsin analogs, Gershonov et al. synthesized a series of peptides containing derivatives of 1-aminocyclobutane-1-carboxylic acid (2,4-methano amino acid, MMA) in successive positions of the tuftsin peptide chain. For this purpose, the following analogs of Thr, Orn, Lys and Arg were constructed:



MMA

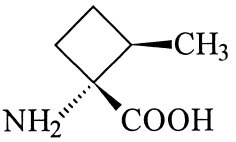
$R = CH_2OH$ (MThr)

$R = CH_2NH_2$ (MOrn)

$R = CH_2CH_2NH_2$ (MLys)

$R = CH_2NHC(NH)NH_2$ (MArg)

As a substitute for Pro, the following methyl derivative of MMA was applied:



MVal

The authors found that a substitution of amino acid residues in tuftsin by methano amino acids produces substances more resistant to enzymatic hydrolysis than tuftsin, that enhances their biologic activity. In the IL-6 assay (enhancement of the secretion of IL-6 by mouse peritoneal macrophages) [MThr[1]]-tuftsin and [MVal[3]]-tuftsin were found to be especially potent. The ELISA assays with antituftsin antibodies, as well as the CD studies, evidenced, however, that the solution conformation of the active "proline-related" [MVal[3]]-tuftsin is different from that of tuftsin [92].

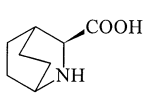
Another approach to obtaining tuftsin analogs with extended vitality was successfully attempted by Mezo et al. Increased resistance toward enzymatic degradation was obtained by the introduction of an isopeptide bond connecting the proper amino acid residue with ϵ-amino group of Lys. In this way, the following series of peptides were synthesized: H-Lys(Thr)-Pro-Arg-OH, H-Lys(Ala)-Pro-Arg-OH, H-Thr-Lys(Ala)-Pro-Arg-OH, H-Thr-Lys(Thr)-Pro-Arg-OH, and H-Ala-Lys(Ala)-Pro-Arg-OH. Some of those isopeptides showed a higher potency of IL-1 secretion activation by monocytes than it was observed for [Ala[1]]-tuftsin [182].

Promising results were also obtained for partially modified retro-inverso-tuftsin analog, that the -CONH- bond between Thr and Lys was modified as -NHCO-. The synthesized H-(R)Thr-ψ [NHCO](R,S)Lys-Pro-Arg-OH (H-g(R)Thr-(R,S)mLys-Pro-Arg-OH) pseudopeptide, when administered orally or i.v., was more active than tuftsin in increasing the number of specific antibody secreting cells in spleen of mice immunized with sheep erythrocytes. It was also ten times as active as the parent peptide in reducing rat adjuvant arthritis. It also enhanced the cytotoxic activity of splenocytes. The pseudopeptide was demonstrated to be completely resistant to enzymatic cleavage in vitro [334]. The analog also stimulated interferon-γ and TNF-α secretion by peripheral-blood-mononuclear cells [248]. The indicated research group has published a separate paper on the anti-inflammatory effect of tuftsin and its retro-inverso-analog in rat adjuvant arthritis [19].

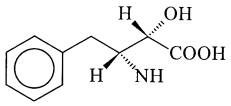
Arcoleo et al. synthesized similarly modified peptides derived from the C-reactive protein (H-gThr-(R,S)mLys-Pro-Leu-OH, H-gGly-(R,S)mLys-Pro-Arg-OH, and H-gThr-(R,S)mLys-Pro-Gln-OH) and found that they were able to induce nitric oxide synthesis by peritoneal macro-

phages. The synthesized pseudopeptides also stimulated the production of IL-1 and TNF-α. The data obtained by these authors indicate the predominant role IL-1α has in the induction of nitric oxide secretion by these tuftsin analogs [8].

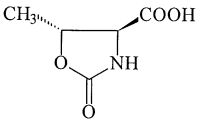
Last year, Kraus–Berthier et al., in the attempt to increase the metabolic stability of tuftsin analogs, synthesized a series of peptides in which the Pro residue was replaced by nonproteinaceous amino acid - ABO ((3S)-2-azabicyclo-[2.2.2]-octane-3-carboxylic acid), and the Thr residue by the AHPA ((2S, 3R)-3-amino-2-hydroxy-4-phenyl-carboxylic acid) or cycloThr ((4S, 5R)-5-methyl-2-oxo-oxazoline-4-carboxylic acid):



ABO



AHPA



cyT

A series of those analogs consisted of Thr-Lys-ABO-Arg, cyT-Lys-ABO-Arg, and AHPA-Lys-ABO-Arg. All those compounds were active as immunomodulators in vitro but none of them showed greater efficiency than tuftsin itself. However, in the opinion of Kraus-Berthier et al., the ABO-containing analog with a pseudoamide bond, Phe-ψ[CH_2NH]Lys-ABO-Arg, might be a promising compound in immunotherapy of infectious and neoplastic diseases [153,154].

Aiming to limit the formation of tripeptide tuftsin inhibitor, Lys-Pro-Arg, by the enzymatic splitting of Thr-Lys peptide bond, Konopińska et al. synthesized octapeptide tuftsinyltuftsin $(Thr\text{-}Lys\text{-}Pro\text{-}Arg)_2$ and found that this peptide has a stronger tumoricidal activity against leukemia cells L-1216 than tuftsin itself [145]. A canine tuftsinyltuftsin $(Thr\text{-}Lys\text{-}Pro\text{-}Lys)_2$ and a chimeric octapeptide Thr-Lys-Pro-Lys-Thr-Lys-Pro-Arg were subsequently synthesized and examined for any evidence of inhibition of murine sarcoma viruses growth. The canine tuftsinyltuftsin showed a marked inhibitory potency in the test [143]. Tuftsinyltuftsin, as well as its partial sequences successively shortened on the N-terminus, were examined for the restoration of phagocytosis of defected granulocytes from blood of children with acute lymphoblastic leukemia. Their stimulatory activity diminished rapidly with the increase of the chain length and disappeared completely in the case of octapeptide [142].

Peptides of the same series were tested for their influence on the mortality of mice infected with encephalomyocarditis virus. Tuftsin and some of its elongated analogs exerted a protective effect in this experiment. Canine tuftsinyltuftsin demonstrated an even higher activity in this test than tuftsin. Similar results were also obtained for Rauscher murine leukemia virus and Friend leukemia virus [352].

Dagan et al. obtained a new class of tuftsin double sequences combining together tuftsin and retrotuftsin fragments. The group found that tuftsinyltuftsin, retrotuftsinyltuftsin, and tuftsinylretrotuftsin all compete with [^{3}H-Arg^4]-tuftsin for binding to macrophages, a fact that, in the opinion of these authors, suggests that the antitumor activity of tuftsinyltuftsin may be a direct one rather than occurring through enzymatic splitting to yield tuftsin [65].

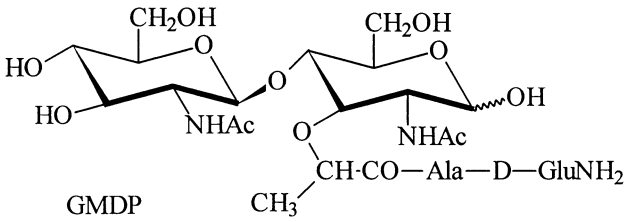
To enhance the stability of tuftsin preparations taken in physiological conditions, in 1984, Rocchi et al. started the synthesis of tuftsin analogs bearing the carbohydrate residue [267]. They elaborated the methods of introduction of monosaccharide moiety on the N-terminus (in reaction with D(+) gluco-1,5-lactone) and on the C-terminus (by utilization of 2-amino-2-deoxy-D-glucopyranose) of tuftsin, and also the method of *O*-glycosylation of the N-terminal Thr residue of this peptide (by reacting 2,3,4,6,-tetra-*O*-benzyl-D-glucopyranose with Z-Thr-*O*-nitrophenyl ester) [268]. It was found that Thr-Lys-Pro-ArgNHGlc slightly enhanced phagocytosis but could not displace [^{3}H-Arg^4]-tuftsin from macrophages. [Thr($(\alpha+\beta)$-*O*-glucosyl)^1]-tuftsin did not stimulate phagocytosis, even though it replaced [^{3}H-Arg^4]-tuftsin from its receptor sites. Thus, the glucosylation of tuftsin at those positions produced inactive compounds [266].

Solution syntheses of 4 stereochemically defined tuftsin analogs containing D-glucopyranosyl or D-galactopyranosyl unit, covalently linked by α or β *O*-glycosidic linkage with Thr residue, were elaborated thereafter [79]. Incorporation of *O*-glycosylated Thr to the nascent tuftsin peptide chain coupled with 4-hydroxymethylphenyloxyacetylnorleucyl derivatized polydimethylacrylamide Kieselguhr support resin (Pepsyn KA) was also accomplished [79].

Using the solution procedure, Biondi et al. synthesized tuftsin derivatives with a 2-acetamido-2-deoxy-D-galactopyranosyl unit linked to the side chain function of Thr by α or β *O*-glycosidic bond [26]. The authors tested six Thr(*O*-glyco) derivatives of tuftsin for their influence on the release of IL-1 and TNF from mouse peritoneal macrophages and human monocytes, and found that the substances did exert modulatory effects on these processes [269].

Biondi et al. also utilized the hydroxyl group of hydroxyproline analog of tuftsin for the glucosylation reaction and synthesized the correspondent α- or β-*O*-D-glucosylated derivatives of tuftsin. Both substances, as well as the unsubstituted [Hyp^3]-tuftsin, evoked the release of IL-1 by mouse macrophages and augmented the immunogenic capacity of antigen-fed macrophages to the level comparable with that produced by tuftsin itself [27].

The paper of Titov et al. also should be quoted in this context. These authors synthesized the conjugates of tuftsin with glucosaminyl muramyl dipeptide (GMDP):



GMDP

that the γ-carboxylic group of D-Glu was attached to tuftsin either through the α-amino group of terminal Thr residue or through the ε-amino group of Lys. The products were examined for their stimulation of antibody production against ovalbumin, as well as their stimulation of DTH reaction and phagocytosis. It was found that GMDP facilitates the macrophage-stimulating activity of tuftsin [323].

Tuftsin moiety was also conjugated with some natural peptides. Thus the conjugate of luteinizing hormone releasing hormone (LHRH) with Ala-Ala-tuftsin was used in the studies on the binding of different LHRH derivatives to monoclonal and conventional anti-LHRH antibodies produced by the immunization of animals using LHRH linked to tetanus toxoid or BSA [299,300].

Potential uses of thymosin-tuftsin conjugate as a new immunomodulator in cattle were discussed in a review paper by Khanasari and Jafari [131].

Very recently, Granoth et al. have obtained 2 chimeric peptides: H-THFγ2-tuftsin-OH and H-tuftsin-THFγ2-OH, by combining tuftsin with THFγ2, an immunologically active principle in thymic humoral factor (THF). Both these peptides induce IL-6 production by mouse macrophages. In antigen presentation test the H-tuftsin-THFγ2-OH peptide was much more potent than tuftsin itself or the H-THFγ2-tuftsin-OH peptide [99]. Notably, the fragment Asp-Gly Pro-Lys of the THFγ2 sequence resembles the sequence of rigin (Gly-Gln-Pro-Arg), a 341–344 fragment of IgG H-chain, for which Veretennikova et al. established a phagocytosis stimulating activity of the same range as that found for tuftsin [335].

The conjugate of tuftsin with adenylic acid should also be noted here, namely, it was shown that tuftsin, when coupled to adenylic acid through the ε-amino group of Lys, can serve as a substrate for the lysyl-(N^{ϵ}-5′-phospho)-adenosylphosphoamidase identified in *Dictyostelium discoideum* [270]. The compound was examined for its chemotaxis stimulation of *D. discoideum*, but in this test it was found to be inactive [271].

Another approach in the search for superactive tuftsin analogs consists in the synthesis of tuftsin's rigidified analogs, that could fit the tuftsin receptor site better than conformationally labile tuftsin. The rigidification of the peptide's spatial structure could reduce a negative entropy of activation for the process of interaction of peptide with the receptor. However, for such approach to be possible, some knowledge of the biologically active conformation of tuftsin is a prerequisite. Such analogs, because of their cyclic structure, may also be more resistant to the enzymatic degradation.

From the beginning the problem of tuftsin conformation in solution was the subject of heated scientific discussions. The debate was briefly reviewed some years ago by Nikiforovich [220]. Thus, in 1976, taking into account its ^{13}C-NMR characteristics, Konopińska et al. suggested that the biologically active conformation of tuftsin may resemble the β-turn conformation [146]. The IR spectroscopic investigation of the folding tendencies, conducted on a large series of protected peptides belonging to the tuftsin group, appeared to support this proposition [312].

The examination of the biologic activity of some tuftsin analogs containing D-amino acid residues also appeared to support that conformational hypothesis [215]. However, when the folding tendencies of those protected precursors of tuftsin analogs containing D-amino acid residues were checked using the IR spectroscopic method [313], they did not clearly support it.

The results of ^{13}C-NMR and CD spectroscopic measurements, performed by Siemion et al. [290] in water solution, were interpreted by those authors in terms of the presence of type III β-turn conformation in conformational equilibria. In particular, the group interpreted changes in tuftsin's CD spectrum caused by pH increase to be a result of an increasing population of β-turn conformers in solution.

However, most recent CD measurements of the same research group, performed in connection with tuftsin and a series of its sequential isomers, showed that the tendency to form a β-turn decreases in the following order: Thr-Arg-Lys-Pro > Thr-Lys-Arg-Pro > Thr-Pro-Arg-Lys > Thr-Lys-Pro-Arg. It follows that tuftsin itself demonstrates the lowest tendency of all for turn formation [297].

The solution conformation of tuftsin was also investigated using the NMR spectroscopy by Blumenstein et al. [31]. It was found that no preferred conformation exists for tuftsin in water solution. In dimethylsulfoxide solution, however, some conformational preference was observed for the structure with the amide NH proton of Arg^4 residue shielded from solvent.

A criticism of the "β-turn proposition" was offered by Blumenstein in a separate article [30].

However, it appears worth noting that the ^{13}C-NMR measurements performed by both groups mentioned above clearly documented the absence of a cis-amide bond on Pro^3 residue of tuftsin dissolved in water. This result indicates limited conformational lability of the peptide in water solution.

The CD spectroscopic properties of tuftsin were initially investigated by Vičar et al. in 1976 [338]. The authors concluded that in the strongly acidic medium tuftsin exists in extended conformation because of the repulsive interactions of its three positive charges. According to these authors, at natural pH, the pseudocyclic structure of tuftsin may exist in solution due to the interaction between Arg^4 carboxyl group and Lys^2 ε-amino group.

Theoretical calculations of Nikiforovich, performed in 1978, suggested that such a conformation is actually the low-energy conformation of tuftsin [219]. The conformation of the Pro residue in the computed model structure was found to be the same as that proposed by Konopińska et al. on the basis of ^{13}C-NMR measurements [146].

However, the ^{1}H and ^{13}C-NMR measurements performed by Sekacis et al. did not confirm the existence of salt bridges in the molecule of tuftsin in polar solvents such as water, or water-methanol. However, some tendency for β-turn formation was observed by these authors for DMSO solution [276].

To resolve the question of tuftsin's biologically active conformation by the way of experiment, Chipens et al. synthesized a cyclic analog of tuftsin,

with the amide bond located between Arg carboxyl group and Lys ϵ-amino group. This analog manifested a strong phagocytosis-stimulating activity. On the other hand, the cyclic peptide

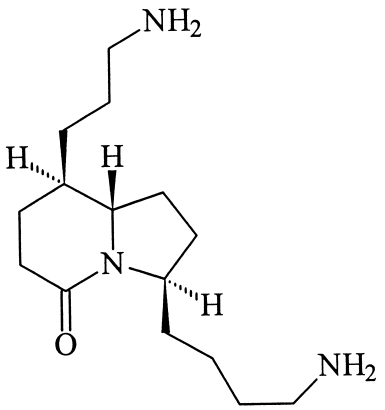
was devoid of such activity. On this basis, Chipens et al. concluded that quasi-cyclization of tuftsin (with the salt bridge between ϵ-amino group of Lys and Arg carboxyl group) in the apolar biophase of receptor may play a role in the generation of tuftsin biologic effects, i.e. that such a structure may be considered as the biologically active conformation of tuftsin [51]. The same point of view was also expressed by Nikiforovich et al. [221]. However, the hypothesis has found no support in the data concerning the binding activity of defective tuftsin mutant, a tetrapeptide Thr-Glu-Pro-Arg [201], to the tuftsin receptors. This mutated peptide is an effective tuftsin inhibitor and has four times as great affinity to the receptors as tuftsin itself (the formation of quasi-cyclic structure of the type proposed for tuftsin is impossible in this case).

Such formation is also hardly possible for rigin (Gly-Gln-Pro-Arg), a tetrapeptide that connects the domains C_H2 and C_H3 of human Ig G immunoglobin. This peptide, however, is, according to Chipens et al. [51], as effective phagocytosis stimulant as tuftsin itself.

In parallel, conformational energy studies of tuftsin were performed by Scheraga's group. These studies produced the "hairpin" type conformation with 2 "split ends" and defined it as the low energy conformation of this peptide [80]. According to those calculations, a β-bend conformation is also possible but only for the peptide with a cis Lys-Pro amide bond.

Parallel computations of the low energy conformations of a sequence variant of tuftsin, a contraceptive tetrapeptide Kentsin, Thr-Pro-Arg-Lys, found in the oviducal lumen of 2-day pregravid hamsters [7], showed that, in that case, the bend conformation of γ-turn type, situated on the Pro residue, would be the most privileged one.

Assuming that tuftsin exists in a turn (or hairpin) conformation, Kahn and Devens [120] synthesized indolizidine, the compound possessing the following structure:



The structure in question may mimic Lys-Pro-Arg fragment of the tuftsin molecule, but in its folded conformation only. This compound inhibited tuftsin's stimulatory effect on macrophages in a dose-dependent manner. The results suggested that the biologically active conformation of tuftsin could be of that folded type.

Some years ago, new calculations of tuftsin's low energy conformation were performed by O'Connor et al. using high temperature quenched molecular dynamics (QMD) [237]. For the linear tuftsin sequence, a type IV β-turn conformation (with the Lys and Arg side chains positioned on the same face of the molecule) was suggested on the basis of these calculations. (The term "type IV β-turn" denotes a bend with non-typical conformational angles ϕ and ψ for (i + 1) and (i + 2) amino acid residues, see [164].) Direct cyclization of tuftsin through N- and C-termini resulted in a complete loss of the type IV β-turn conformational characteristics. However, calculations performed for two cyclic analogs of tuftsin: c-(Thr-Lys-Pro-Arg-Gly) and c-(Thr-Lys-Pro-Arg-Asp), showed that, for these compounds, the type IV β-turn at Lys-Pro moiety is possible. In the case of c-(Thr-Lys-Pro-Arg-Lys) analog, on the other hand, a type III β-turn conformation at the Lys-Pro fragment would prevail, according to these calculations.

Tuftsin's cyclic analogs c-(Thr-Lys-Pro-Arg) and c-(Thr-Lys-Pro-Arg-Gly), and also two isomers with the c-(Thr-Lys-Pro-Arg-Asp) sequence, were synthesized in

1995 by Nishioka et al. [232]. It was found that in phagocytosis assay, the c-(Thr-Lys-Pro-Arg-Gly) peptide was 50 times as potent as tuftsin. The enhanced phagocytosis stimulation potency was also exhibited by one of two isomers with c-(Thr-Lys-Pro-Arg-Asp) sequence; whereas the other isomer was almost inactive. Those results strongly suggest that type IV β-turn conformation is the biologically active conformation of tuftsin.

New 2D NMR measurements performed in 1992 by D'Ursi et al. for DMSO-d_6 tuftsin solution revealed the presence of 2 families of conformations, with trans and cis Lys-Pro bond, respectively. According to these results, the cis family is a mixture of extended structures. In the trans family, a rigid, folded conformer prevails, characterized by an inverse γ-turn located on Lys-Pro moiety [63].

However, the most recent molecular dynamics simulations performed for tuftsin in explicit water and in a 1.0 M NaCl solution by Valdeavella et al. showed that, for tuftsin with all-trans peptide bonds, the preferred conformation of the molecule is neither the β-turn nor the γ-turn [330]. When the Lys-Pro amide bond has a cis conformation, however, the average conformation of the peptide is, according to those calculations, of the type VI β-turn. The presence of salt does not influence the peptide's backbone conformation. Interestingly, the results obtained by Valdeavella et al. for cis-tuftsin correspond well to the much earlier predictions of the Scheraga's group [80].

The calculations of Valdeavella's research group also showed that independently of salt concentration, trans-tuftsin should have a higher dielectric constant in water than the cis-tuftsin. The presence of salt reduces the dielectric constant of both these structures, but neither the peptide's conformation nor the concentration of salt influences the dielectric relaxation time of water molecules [354].

Theoretical conformational study of a larger fragment of human Ig G, containing tuftsin moiety, His-Asn-Ala-Lys-Thr-Lys-Pro-Arg (res. 285–292) and of its analogs (in which the N-terminal His was substituted by Arg, Trp, Gly, Lys, and Glu, respectively) was recently performed by Kolobov et al. [141]. The study was conducted in relation to the results of Lukas et al., who found that the 281–292 fragment of Ig G is responsible for the inhibition of the interaction of the Ig G protein with C1 component of complement [172]. An attempt was made to determine the biologically active conformation of that fragment of Ig G by comparing the conformational data for the above-mentioned series of peptides and the results of the examination of their biologic activity as well as their CD spectra.

The data presented are not sufficient to resolve the question what is, in fact, the biologically active conformation of tuftsin. However, the data suggest that, in all probability, a fragment of tuftsin peptide chain shorter than tetrapeptide may be involved in the interaction with the receptor. If so, different rigidified tuftsin analogs, based on very different conformational hypotheses, may all manifest biologic activity. In this connection, the idea expressed by Segal et al., namely that Pro-Xxx or Xxx-Pro moieties (in which Xxx is Arg or Lys) are recognized by tuftsin receptor, is especially pertinent [275].

As we mention above, the N-terminal tetrapeptide fragment of substance P, Arg-Pro-Lys-Pro (a retro-sequence of a tuftsin analog Pro-Lys-Pro-Arg) displaces [^{3}H]-tuftsin from its specific binding sites on thioglycollate-elicited macrophages with the same inhibition constant as unlabelled tuftsin [16].

In this connection, a paper by Moore et al. is also worth noting. Namely, it was shown that substance P, as well as its N-terminal tetrapeptide, co-stimulate clonal proliferation of murine marrow-derived 2 signal-dependent mononuclear phagocyte progenitors in the same manner as is the case when tuftsin is involved [188]. (The relation between tuftsin and substance P receptors was reviewed in 1984 by Watson [344]).

The modification of substance P N-terminal tetrapeptide could constitute another independent, and as yet unexplored, way of searching for active effectors of tuftsin receptor.

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