Tuftsin – Properties and Analogs

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Abstract: Immunomodulation is one of the significant therapeutic strategies. It includes both stimulation and suppression of the immune system by a variety of substances called immunomodulators, designed to regulate the immune response of the organism to infections of varying etiology. An example of such a substance is tuftsin (TKPA) **3** (Fig. (**1**)). Tuftsin is an endogenous immunomodulator of a wide spectrum of biological activity. Tetrapeptide **3** provides also antitumor, antimicrobial, anticoagulant and analgesic properties. In this paper, we present tuftsin derivatives described over the years, its biological activity and potential clinical applications.

Keywords: Tuftsin, immunomodulator, biological activity, Selank, tuftsin analogs, regulatory peptides.

1. INTRODUCTION

The immune system is intended to protect the body against attack by microorganisms. In the case of the reduced activity, it can lead to pathogenic agents, therefore, immunomodulators play a key role in regulating the immune response to infections of varying etiology. Many low molecular weight peptides were characterized by properties regulating the immune response. The source of these compounds is the fragments of antibodies or peptidoglycans of bacterial cell wall. Until now well recognized peptides characterized by immunomodulatory properties include cyclosporin, muramyl dipeptide (MDP) **2** and tuftsin **3** (Fig. **1**). Cyclosporin **1** (Fig. (**1**)) has been isolated from the fungus *Tolypocladium inflatum*. It is a cyclic peptide consisting of 11 amino acids (undecapeptide). This compound inhibits cellular and humoral immune responses and modifies the inflammatory reactions. Cyclosporin affects the process of activation of T_h lymphocytes, which indirectly inhibits the production of antibodies, activation of macrophages and inhibits B cells. MDP **2** induces the release of endogenous mediators, such as interkulin and cytokines, has antitumor properties and inhibits the replication of human immunodeficiency virus. Tuftsin **3** shows not only the immunostimulatory activity, but also antibacterial, antiviral, antimycotic or antitumor properties making it a promising subject of research [1].

Despite numerous advances in the use of immunosuppressive drugs in organ transplantation, it has failed so far to find the perfect drug which is characterized by a high selective inhibitory effect on the reaction of transplant rejection, which would not cause adverse effects and would be inexpensive to produce. Price of immunosuppressive drugs limits their widespread use. Current trends suggest that efforts should be made to produce a drug that would provide not only immunosuppressive activity, but also improve tolerance.

In 1970, at Tufts University in Boston, Nishioka and Najjar isolated and synthesized tuftsin **3**, which is an endogenous tetrapeptide having the sequence Thr-Lys-Pro-Arg, naturally occurring in human blood. In the body, tuftsin is active only as a free peptide [2,3]. This peptide **3** is a fragment of the heavy chain Fc (289-292) immunoglobulin G (IIg), and is released by the action of specific enzymes such as leucokinase and endocarboxypeptidase tuftsin spleen [4,5]. In the case of endocarboxypeptidase this process is followed by cleavage

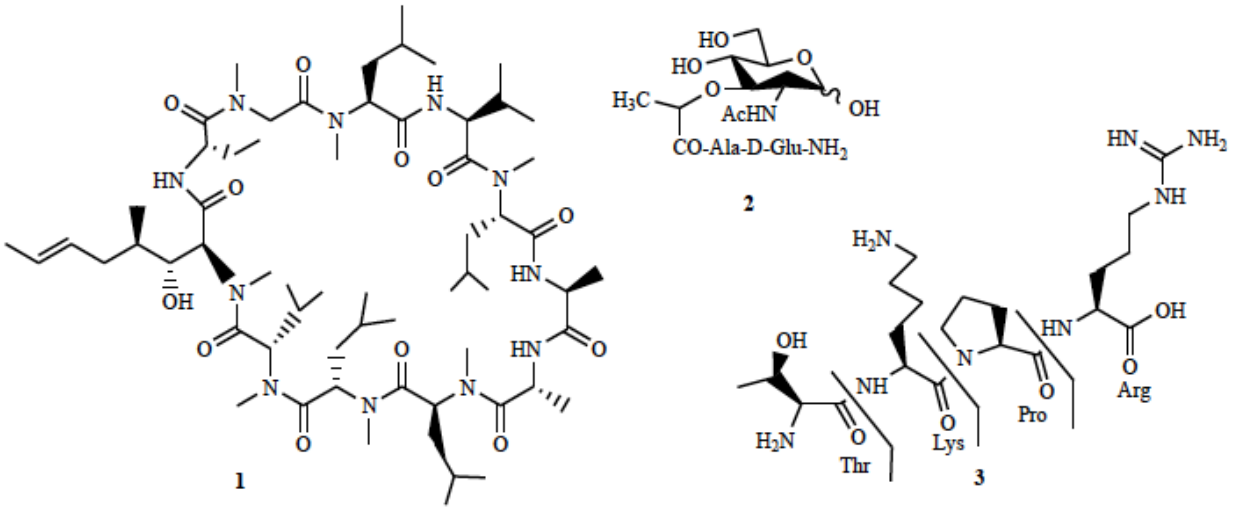

Fig. (1). Structure of cyclosporine **1**, muramyl dipeptide (MDP) **2** and tuftsin (TKPR) **3**.

of the peptide bond between Arg292 and Glu293. On the other hand, leucokinase acts on the bond between Lys288 and Thr289. The mechanism of action tuftsin **3** is not fully understood. There is a proposition that at the beginning, the peptide binds to the phagocytosis cell using electrostatic forces, moves sequentially to the selected receptors and internalizes peptide-receptor complex. Released tuftsin **3** increases motility, phagocytosis, chemotaxis and pinocytosis [5-7], activates macrophages to treat bacterial infections, and improves communication between the immune system (macrophages, T cells and antibody-producing B lymphocytes), as a result, antibody production is increased. The peptide enhances the immune response against the tumor and retards tumor growth. It stimulates the production of blood cells by the bone marrow, and is also useful for combating yeast infections [8]. Tuftsin **3** also regulates the levels of norepinephrine, serotonin and dopamine. It can cause changes in the synthesis and degradation of monoamines, protein, in the foothills of the brain and the structures of the cerebral cortex [4]. In patients without spleen observed adverse to the body deficiency tuftsin **3**. Thus, patients who have a damaged spleen are significantly more susceptible to severe infection due to the lack of tuftsin. The deficit of the immunomodulator also demonstrated in the case of diseases such as sickle cell anemia, leukemia and AIDS Hidgkina [9]. In this work we review the medicinal applications proposed for tuftsin analogs with emphasis on the more recent publications.

2. PROPERTIES OF THE TUFTSIN

Tuftsin **3** has a wide spectrum of biological activity. It interacts with human *C*-reactive proteins (CRP) and can be involved in immunological processes during acute-phase reaction [10-13]. Peptide **3** shows not only the effect of immunoactive but also antineoplastic, antiviral, antifungal and antibacterial properties. It has also been shown that tuftsin acts analgesic [14], which confirms its direct impact on the nervous system. This ability of tuftsin **3** corresponds to dipeptide Pro-Arg moiety [15]. The peptide **3** can inhibit axonal degeneration, delays neuronal death as well as converts microglial cells in less branched and oval shapes. It also improves the functioning of the neurological system [16]. In addition, it relieves withdrawal of symptoms [17], increases blood pressure [18] and inhibits contraction of the lymphatic vessels [19].

It was observed that the inhibition of fragment 1-3 tuftsin according to the vitreous body of the eye, results in increased axonal regeneration of retinal ganglion layer and reduces the amount of phagocytes in the retina [20].

The tripeptide (TKP) acts as an inhibitor of macrophage/microglia that plays a protective role in preventing intracerebral haemorrhage in animal model studies [21]. Fragment of 1-2 tuftsin (TKP) also reduces the production of free radicals and the number of neurons degradable, often the result is to reduce the damaged area and improves the nervous system [21].

Signaling pathway of tuftsin **3** is not yet fully understood, despite the fact that it is known for forty years. Recently it was identified that the tuftsin **3** binds to neuropilin-1 [22,23]. Neuropilna-1 receptor plays a major role in angiogenesis, vascular permeability, and in the development of the nervous system. Tuftsin containing a sequence similar to the end of the VEGF-A165, binds to neuropilin-1 by competing with VEGF. Peptide **3** is combined with NRP-1 interaction *via* a

network, in which the most important is the *C*-terminal Arg residue coming from tuftsin binding of Asp-320 and NRP-1. It has been found [24], that the presence of receptors for tuftsin **3** on endothelial cells reflects the ability of these cells to participate in the inflammatory response. Therefore, the use of appropriate probes would be effective for visualizing inflammation *in vivo*.

Further research [25] supports the idea of Nrp1 having a dual role; first as a mediator of long interactions between microglia and Treg and also as a ligand for tuftsin **3** that promotes the microglial inflammatory shift. Microglia preferentially interacts with Treg on an Nrp1- mediated basis, which preferentially activates these immunosuppressive cells. While normally during EAE the overall environment would become more inflammatory due to deelimination and cell death, tuftsin **3** promotes an M2 shift in microglia through Nrp1 which further supports an environment in which Treg and Th2 responses can predominate. Received by the authors data indicate that the effects of tuftsin **3** on Nrp1 signaling may constitute a potential therapeutic opportunity for EAE/MS, in particular with the development of nonpeptide, small-molecule tuftsin mimetics.

Tuftsin **3** induced pinocytosis and reduced processd phagocytosis with the superoxide ion, O^{2-}, causing additional forms of oxygen, leading to the excited electronic states and luminescence phagocytes. The effect of this is to increase the phagocytes bactericidal, as the resulting oxygen species are toxic to microorganisms. The cells and tissues surrounding phagocytes, including tumor cells are destroyed. Studies have shown that tuftsin **3** administered with the nystatin in mice *Candida albicans* enhanced antifungal drug efficacy [26]. Noteworthy, concentration of tuftsin **3** is lower in the case of cirrhosis of the liver or spleen damage functions and contributes to the weakening of the phagocytic activity of granulocytes [27]. The ability of tuftsin to reduce ulcers, administration of this tetrapeptide restricts the ulcer as well as faster healing of the diseased tissue was reported [28].

To protect against infection before hatching chicks, embryonic stage in conjunction with tuftsin was vaccinated. Vaccination is proved to be very effective against Marek's disease, Gumboro and Newcastle (NDV) and against hemorrhagic enteritis and bronchitis. Studies show that after embryonic vaccination against NDV with tuftsin, after several weeks of increased peptide levels, thus protecting against diseases of the immune system [29].

Tuftsin **3** also used to obtain the radiopharmaceutical compound 99mTcRP-128, which exposes the affected areas in rheumatoid arthritis. It comprises the antagonist tuftsin - TKPPR, which has the ability to four times stronger binding *in vivo* to receptors on the cell phagocytic surface. It allows to detect and market areas of inflammation in the central nervous system [30].

Tetrapeptide **3** was also joined to the antibody *via* a linker Gly-Ser-Gly-Gly, to give a fusion protein scFv-Tuftsin. Protein expression was carried out in the system *Pchia pastoris*. Immunization with the protein reinforced humoral immune response by hanging the production of anti-idiotypic antibodies Ab2 and anti-idiotypic Ab3 [31].

Due to the repeated examination of the properties tuftsin **3** on human and animal, it was decided to test the tetrapeptide **3** on fish. The research was carried out on carp species Labeo rohita where tuftsin **3** was injected four times at two-week intervals. After the study, there was an increased resistance to infections and increased non-specific immune response. The test results can be used to protect fish farming [32].

Despite such advantageous properties of tuftsin **3**, the peptide **3** is not stable, its half-life in blood is 16 minutes. It is hydrolyzed by leucine aminopeptidase, carboxypeptidase B, proteinase and subtilisin. The hydrolysis appears tripeptides such as Lys-Arg and Pro-Thr-Lys-Pro. The resulting compounds inhibit the activity of tuftsin. Therefore, in case of tuftsin **3** properties can be fully utilized, more stable tuftsin analogs and its connections to various biologically active compounds are desired.

3. TUFTSIN USE IN VACCINE

In 2015 [33] a novel mucosal vaccine based on HE-ORF2 and HA-VP1 (Hepatitis E virus - HEV and Hepatitis A virus - HAV) was constructed. VP1 appeared to be the dominant structural protein among the capsid proteins of HAV, and was demonstrated to be highly immunogenic [34]. HEV-ORF2 naturally occurs as a homodimer and higher-order oligomers, and it was strongly recognized by HEV reactive sera in the oligomeric state. Furthermore, it was found to be more highly immunogenic compared with other viral peptides [35]. Two proteins were constructed by adding lysine linkages at their *C*-terminal ends with or without a tetrapeptide tuftsin molecule **3** as a stem. The results indicated that the tuftsin group could generate stronger humoral immune response than the no-tuftsin group. The results also showed that the tuftsin group showed a

significantly higher level of mucosal immune response after intranasal inoculation. IgA effective dose elicited by intramuscular administration in mice were not as strong as those by the intranasal route, and no difference was observed between the tuftsin group and no-tuftsin group except the gut. The tuftsin group displayed greater activation of CD4+ and lower activation of CD8+ than did the no-tuftsin group with intranasal administration. Thus, an antigen fused to tuftsin may promote stronger immune responses in mice by stimulating production of antibodies, improving CD4+ T cell proliferation and inhibiting CD8+ T cells. By integrating HE-ORF2, HA-VP1 and tuftsin **3** in a vaccine, this study validated an important concept for further development of a combined mucosal vaccine against hepatitis A and E infection [35].

Tuftsin **3** was also used by another team of researchers [36] with matrix vaccine against influenza virus type A. Tuftsin **3** was used as an immunostimulant molecule peptide. The authors designed a new peptide vaccine against influenza A based on M2e. M2e was chosen because of the most promising targets for a universal influenza vaccine, amino acids with highest frequency in every position from 1505 influenza A virus strains isolated from humans between year 1918 and 2006 as the sequence for vaccine candidate [37]. Unfortunately, this 24-amino acid has a relatively low antigenicity and immunogenicity, which is not favorable for the vaccine. Accordingly, using the system MAP (multiple antigen peptide), a new peptide vaccine was developed. A branched peptide composed of four copies of M2e was synthesized by adding lysine linkages at their *C*-terminals with a tuftsin molecule as a stem. This solution increases the weight and increases the immunogenicity. In the second case tuftsin was replaced by four molecules of glycine. The results showed that the humoral and cellular immune response in (M2e)4-tuftsin **5** and (M2e)4-G4 **6** groups was greater than M2e **4** monomer group (Fig. (**2**)). Protective acting of groups M2e-MAP was also better than those observed M2e **4** monomer group. Subsequent studies have shown that after boosting vaccine the M2e-specific serum IgG titers in (M2e)4-tuftsin **5** group was 89.125, which is much higher than the (M2e)4-G4 **6** group (25.119). This is probably related to the immunostimulatory effect of tuftsin [26]. It was also found that (M2e)4-tuftsin **5** may induce a stronger cellular immune response than (M2e)4-G4 **6**. Probably this explains the better protective performance (M2e)4-tuftsin **5** than (M2e)4-G4 **6** group. The anti-M2e cannot neutralize the virus immediately, as in the case antibody: anti-Ha and anti-NA. Rather, it comes to playing a role in antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [38,39]. It has been found that the M2e-based vaccines may have a protective effect through antibody-dependent NK cell-mediated cytotoxicity [38].

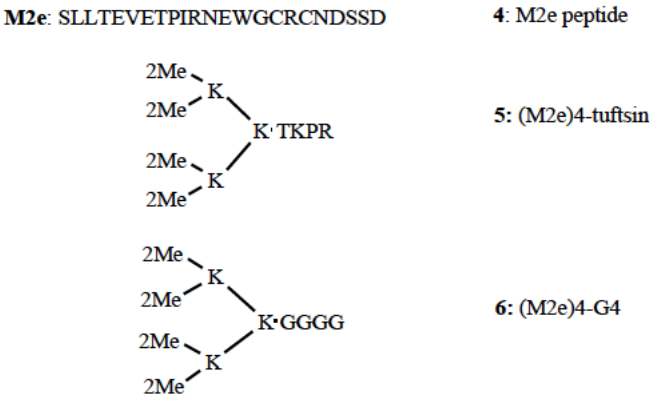

Fig. (2). Structures of the compounds **4**: M2e peptide **5**: (M2e)4-tuftsin and **6**: (M2e)4-G4.

In the literature [40], a combination of tuftsin **3** with fulleren C_{60} was reported (Fig. (**3**)), which is nanomaterial and its derivatives have been used in biomedicine, for example: in the photodynamic antitumor therapy, to treat viral activity of the human immunodeficiency virus. This fullerene penetrates the cell membrane and localizes in the mitochondria, therefore, can be used as a drug carrier, particularly during the administration of anticancer drugs. Fullerene C_{60} as xenobiotic, driven mainly by the immune system and can trigger a series of immune responses. One of studies show that a polyhydroxylated C_{60} (fulerol, $C_{60}(OH)_{24}$) can activate the innate immunity in mice with cancer, resulting in inhibition of tumor growth [41].

Tuftsin **3** was connected with fullerene C_{60} in two ways to give NH_2-tuftsin-C_{60} **7** and C_{60}-tuftsin-COOH **8**. In both cases the compound is combined *via* a covalent bond to C_{60} amino or carboxyl terminus end of tuftsin **3**. The compounds were tested for stability against degradation leucine aminopeptidase (LAP), and the immunostimulants peritonitis in mice. During the study of macrophages *in vitro* strongly enhanced chemotaxis phagocytosis was observed. Both compounds showed total resistance to enzymatic hydrolysis and toxic effects of macrophages. In carrying out the biological assays it was observed that immunomodulatory compounds stimulate the activity tuftsin **3**, it even increases. Furthermore, both derivatives have a much greater stability than the native tuftsin **3**. The results indicate that new conjugates were received from

fullerene C_{60} and tuftsin **3** which can be used as potential immunomodulatory compounds and adjuvants in vaccines [42].

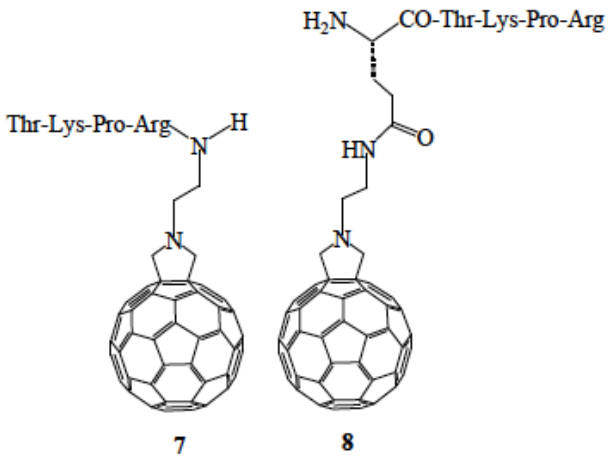

Fig. (3). Structures of conjugate NH_2-tuftsin-C_{60} 7 and C_{60}-tuftsin-COOH 8.

There were synthesized oligopeptides based on pentapeptide $(TKPKG)_n$ derivative of tuftsin with differing chain length (n = 2,4,6,8) [26]. The compounds were found to be non-toxic to mouse spleen cells were not immunogenic, and their effects immunostimulants resulted in an increase of the antibody response to the antigen (SRBC) in mice. Analyzing the results, it was found that oligopeptide derivatives may be promising carriers for synthetic vaccines 3'-azido-3'-deoxythymidine, *Zidovudinum* (AZT) conjugate (a drug used in the treatment of acquired immunodeficiency syndrome lub acquired immune deficiency syndrome (AIDS)) [43] was conntected with tuftsin **3**. The compound displayed the properties of the two components. Inhibited the reverse transcriptase activity and the expression of the human immunodeficiency virus (HIV) antigen and stimulate the release of IL-1 by murine macrophages. Tuftsin-AZT conjugate proved to be non-toxic as compared to T cells, and may be of potential use in the treatment of AIDS.

Received also polituftsin $(TKPR)_{40}$ as a carrier for synthetic peptides derived from the proteins gp41 and gp120 of HIV. Polituftsin exhibits the same biological activity as the tuftsin **3**, at the same time increases the production of IL-2 and IFN-γ and has a longer half-life in the body [44].

4. TUFTSIN ANALOGS HAVENIG ANTITUMOR ACTIVITY

Since tuftsin **3** was reported as a macrophage targeting molecule, its possibility by targeting tuftsin-grafted C7P3 (C7P3-T/DNA) has been explored to reach macrophages in comparison to unmodified C7P3/DNA complex [44]. Authors selected conjugate C7P3 because it was validated by *in vitro* delivery of siRNA, intracellular trafficking and *in vivo* transfection efficiency in Balb/c mice. In addition, for targeting specific cell type, they modified the chitosan-PEI conjugate, C7P3, to examine its efficacy for wider applications. The receptor-mediated targeted gene delivery in macrophages was achieved by tethering tuftsin, a natural macrophage activator peptide, to the C7P3 conjugate. C7P3-T/DNA complex was found to be biologically safe in both mice peritoneal macrophages and human lung cancer cells under experimental conditions. The result suggested that tuftsin **3** tagging connected significantly the target-specificity without affecting its cytotoxicity. Thus, the presented study shows that tuftsin **3** tagging can improve targeting ability of the nano-carriers and C7P3-T as a result may be a potential chemical vector for targeted gene delivery [45].

It was found that tuftsin **3** combined with an anti-CA125 can cause immune protection against ovarian cancer *in vivo* [46,47]. The activity of cancer may be associated with phagocytosis and protein CD47 it has focused the attention because of the role it could play in the process of suppression of phagocytosis, which interferes with the growth of cancer cells. Expression of CD47 on the cell surface is a mechanism that protects against immune system response [46].

Lidamycin has very potent cytotoxic properties against tumor cells, it consists of an apoprotein (LDP) and an enediyne chromophore (AE). Ec is an EGFR-targeting oligopeptide.

Liu and co-workers [46] during their research generate tuftsin-based fusion proteins, the LDP-TF (LDP-TF the fusion proteins composed of the LDP and TF) and Ec-LDP-TF (Ec-LDP-TF The fusion proteins composed of Ec, the LDP and TF) and the corresponding AE (active enediyne from clindamycinum) integrated analogues, LDM-TF (LDM-TF the enediyne-energized fusion protein composed of the LDP, TF and AE) and Ec-LDM-TF (Ec-LDM-TF the enediyne-energized fusion protein composed of Ec, LDP, TF and AE), in order to increase the effectiveness of anti-related immunomodulation. Genetically designed by the authors LDPTF fusion protein consisted of tuftsin and LDP; further Ec-LDP-TF consisted of the oligopeptide Ec (the *C*-loop of epidermal growth factor (22 amino acids of EGF COOH terminal)) and LDPTF. Genetically designed in this way EGFR-targeting fusion protein

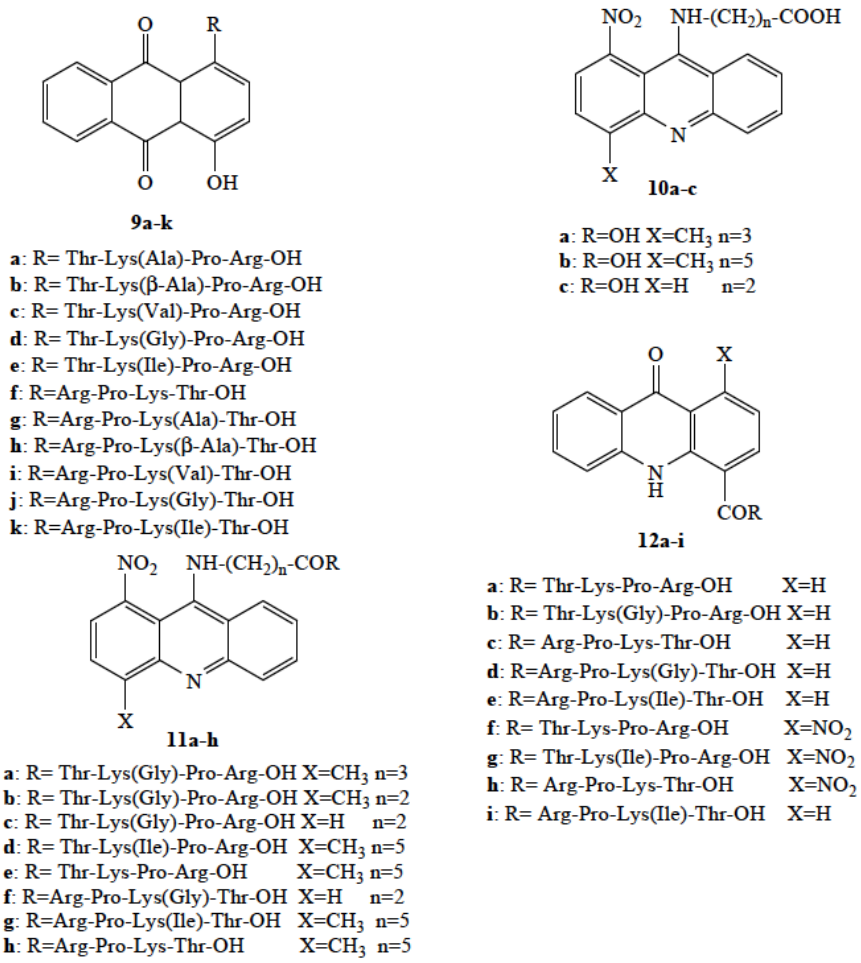

Fig. (4). Structures of new tuftsin conjugates with anthraquinone and acridine/acridone derivatives.

Ec-LDP-TF, effectively reduced cell carcinoma cells A431 and mouse macrophage J774A.1. It was found that EC-LDP-TF possesses a higher anti-tumor efficacy of LP-TF compared to epidermoid carcinoma A431 model. The level of inhibition by Ec-LDP and LDP-TF-TF at equimolar/kg dosage level on A431 tumor growth was respectively 84.2% and 76.3%. It was observed that Ec-LDM-TF augmented the therapeutic efficacy, inhibiting the growth of A431 xenograft by 90.9%, especially the Ec-LDM-TF caused marked down-regulation of CD47 in A431 cells [46]. Parameters of TNF-α, IFN-γ were used to examine the impact of anti-tuftsin [23,48]. Studies revealed a decrease of the level of TNF-α in the A431-bearing animals compared with healthy, untreated animals. Raised levels of TNF-α expression were observed in animals treated with tuftsin-based fusion proteins. The expression levels of TNF-α compounds Ec-LDP-TF, LDP-TF and LDP in tumor-bearing mice treated were increased 250-fold, 7-fold and 5-fold compared to those treated with PBF. It was found also that the levels of IFN-γ are significantly higher in mice treated with Ec-LDP-TF than LDP. In addition, it was noted that the weight loss effects of the treatment Ec-LDP-TF in different doses, did not exceed 10% of pre-treatment weight. The results suggested that tuftsin-based fusion proteins might affect the level of cytokines and could modulate the immune response. Therefore, it could be potentially used in cancer immunotherapy. The best therapeutic effect was observed in the group of animals treated with the combination of Ec-LDP-TF with Ec-LDM-TF. The authors found that the tuftsin-based, enediyne-energized, and EGFR-targeting fusion proteins gave very high efficacy with CD47 modulation [48].

Tuftsin was modified at the ε-amino group of lysine to increase blood plasma stability [49]. In 2011 new tuftsin conjugates with anthraquinone and acridine/acridone derivatives (**9a-k**, **10a-c**, **11a-h**, **12a-i**) were designed (Fig. **4**) [50].

The received compounds were evaluated for cytotoxic activity against human cancer cell lines, such as lung adenocarcinoma A549 cells and myeloid leukemia HL-60. Compounds **9f**, **9g**, **9i**, **12b**, **12d**, **12e** showed

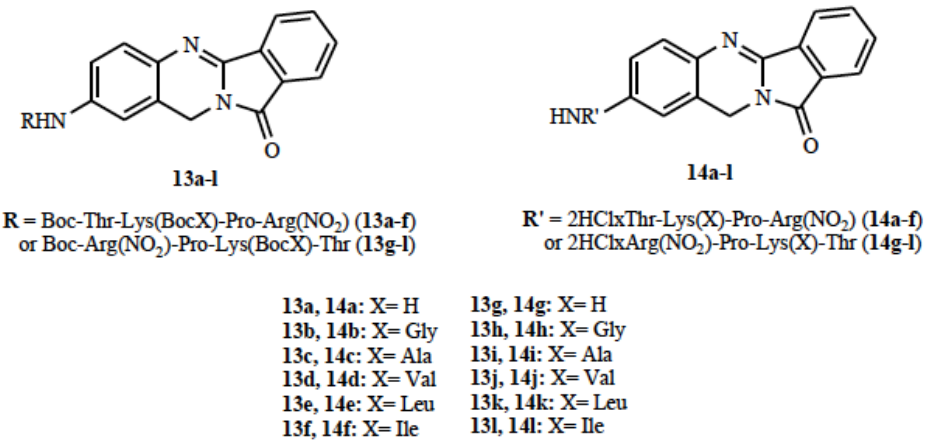

Fig. (5). Structures of conjugates BAT with analogues tuftsin and retro-tuftsin.

no cytotoxicity even at a concentration greater than 100 mM. Other tuftsin analogs were characterized by a low cytotoxicity at high micromolar concentrations. In the case of compound **11d** was obtained 2-fold greater cytotoxicity to both cell lines than its precursor **10b**. A compound **11a** exhibited a 6-fold greater cytotoxicity to A549 lines than its precursor **10a**. Accordingly, tuftsin conjugates caused an increase in reduction in cytotoxicity in comparison with their precursors [50].

Structure activity relationships analysis revealed that the element having the greatest influence on the cytotoxicity of the tuftsin conjugates was the type of the peptide and the manner of engagement with a corresponding precursor. The most active cytotoxic compound conjugated with precursor *via* a linker COR occured to be compound **11d** (IC_{50} = 8.7 ± 1.8 mM for A549 cell line), the partial modifications of structure with the molecule of isoleucine. In contrast, analysis of derivatives tuftsin connected with the precursor by linker CONHR, resulted that the most active compound was **11a** (IC_{50} = 3.0 ± 0.68 mM for A549 cell line), which was modified at the ε-amino group of lysine throughout glycine. Notheworthy, the cytotoxic activity depended on the length of linker between the chromophore moiety and tuftsin analogue. Compound **11b** having a longer linker (n = 5) showed a significantly lower cytotoxicity than the compound **11a** (n = 3) [50]. The authors also conducted a study of inhibition of the catalytic activity of human types I and II of DNA topoisomerases. The results showed that none of the tested compounds affected significantly DNA relaxation mediated by both type I and type II DNA topoisomerases.

Subsequently, the effects of tested compounds on cell cycle progression and induction of cell death of tumor cells were described, both on lung adenocarcinoma cells and myeloid leukemia at concentrations at equitoxic corresponding to the IC_{90} concentrations. In the cell cycle in the early S phase and in G2 and M biologically active conjugates were arrested. Exposure of tested precursors to both human tumor cell lines led to the emptying phase S [50]. Analysis of the nuclear morphology of tumor cells exposed to the conjugate **11a** and precursor **10a** revealed that cell death was induced in different ways depending on the type of tumor cells. The cell line HL-60 exposed to the precursor **10a** induced rapid apoptosis, in the case of conjugate **11a** there was no apoptotic figures. In the case of the A549 cell line did not noticed any change in nuclear morphology [50].

Recently new series of BAT analogues with tuftsin/retro-tuftsin derivatives containing isopeptide bond between ε-amino group of lysine and carboxyl group of aliphatic amino acids such as Gly, Ala, Val, Leu, Ile (Fig. (**5**)) was received [51].

The authors conluded that the conjugates BAT with tuftsin/retro-tuftsin exhibited greater cytotoxicity. In the case of derivatives BAT with a retro-tuftsin modified with leucine **14k** or isoleucine **14l** cytotoxicity was increased more than 10-fold. BAT and tuftsin/retro-tuftsin conjugates inhibited the catalytic activity of the type II topoisomerase DNA and at cytotoxic concentrations rapidly caused DNA damage (double stranded DNA breaks) in drug-treated tumor cells. The authors argued that the increased cytotoxicity was probably related to the differences in the mechanism of action at the cellular level. Therefore it should be associated with increased bioavailability of obtained conjugates. This led to their much higher accumulation in tumor cells and much effective tumor cell killing [51].

In 2016 a derivative of tuftsin with branched-structure by connecting four tuftsin peptides through a

linker lysine (Thr-Lys-Pro-Arg)$_4$-Lys$_2$-Lys-Gly-OH (TP) as potential antitumor agent was designed [52]. The study was performed in beagles. The pharmacokinetics and bioavailability of TP were conducted by using ELISA method. Dose-dependency and non-linear dynamics of TP after single-dose pharmacokinetic studies were performed at the doses ranged 2, 6 and 18 mg/kg, respectively and the half-time of TP was 1.3-2.8 h. Moreover, the results of *in vitro* metabolic stability of TP and tuftsin in both the beagle's plasma and liver homogenate system showed that TP was much more stable than tuftsin *in vitro*. In turn, the absolute bioavailability of TP with subcutaneous administration was 31.1 ± 6.2% [52].

Furthermore, it was examined if tuftsin **3** could serve as a biomarker. This peptide in combination with fluorescein isothiocyanate (FITC tuftsin), Tβ-DOTA and Gδ-DOTA ($CH_2CH_2NCH_2CO_2H$), and pentapeptide (Thr-Lys-Pro-Arg-Pro) were tested *in vitro* for the fluorescent properties. The results confirmed that the FITC-conjugated tuftsin could be suitable for the detection of macrophage cells in the body. It turned out that the Tβ-DOTA pentapeptide had a greater affinity for the macrophage than Tβ-DOTA with the same tuftsin. Macrophages labeled Gδ-DOTA were detected by magnetic resonance imaging (MRI), for example stroke [53].

5. DERIVATIVES OF TUFTSIN HAVING ANTI-INFLAMMATORY PROPERTIES

Bhasin *et al.* [54] showed that using the tripeptide macrophage/microglia inhibitory factor MIF (the TKP, tuftsin fragment) and the tetrapeptide macrophage/ microglial stimulator tuftsin **3** attenuated experimental autoimmune encephalomyelitis (EAE) symptoms. Moreover, it has been proved that the disease progression could be potentially manipulated favorably at early stages by altering the timing of macrophage/microglial activation, which altered the systemic immune response to favor upregulation of T helper cell 2 genes and was critical for the clinical outcome of EAE. Tuftsin **3** or MIF was supplied by mini-osmotic pumps at a concentration of 500 μM, with an infusion rate of 0.5 μl/hour, where four different modalities were used for modifying macrophage/microglial activation in MOG-injected wild-type mice. Three of them, MIF treatment as EAE symptoms started, tuftsin **3** treatment either before or at the time of symptom resulted in improved EAE clinical scores, in turn pretreatment with MIF was of limited benefit. It has been noticed that a balanced T cell response with a predominance of a Th2 fate for the activated T cells is one correlation that connected the three successful modalities. Another factor was the requirement for macrophages/microglia to migrate to local sites of injury [54].

In another experiment it was proved that tuftsin-mediated microglial activation resulted in shifting microglia was an anti-inflammatory phenotype and attenuated symptoms in experimental autoimmune encephalomyelitis [15]. To determine the outcome of MS / EAE was considered a balance between pro-inflammatory response of Th1 and Th2, in conjunction with the effects of regulatory T cells. The authors found, that after treatment with tuftsin **3**, markers of Th2 and Treg cells were stimulated, suggesting activation of Th2 and expansion of immunosuppressive regulatory T cells (Treg). Studies confirmed the idea of early activation of microglia by tuftsin **3** in the coordination of the immune response, it was conducive to the protection of autoimmunity as opposed to autoimmune disease [16].

The direct and indirect impact of tuftsin **3** on T cells during the test was examined, the direct impact of tuftsin **3** on T cells, found a 6-fold increase in the levels of TNF-α, but there was no apparent effect on the production of IL-10. To investigate potentially indirect impact, culture conditions were designed to mimic the effects of *in vivo* tuftsin **3** infusion in the EAE model. The culture was stimulated by treating primary neurons to generate neuronal-conditioned medium (NCM). It was found that the combination of NCM and tuftsin **3** stimulation of microglia yielded a microglial-conditioned medium, had a huge impact on the effect of the TNF-α and IL-10 levels. The level of release of TNF-α was reduced four-fold as the level of the release of IL-10 increased 10-fold. This led to > 35-fold shift towards immunoprotection. It gave immunosuppressive effect in accordance with the Th2 phenotype. The authors suggested that the combination of tuftsin **3** and NCM microglia could cause a shift to an anti-inflammatory M2 phenotype, which could explain the prevalence of Th2 cytokines. Further studies showed that the combination of NCM and tuftsin **3** promoted an anti-inflammatory M2 phenotype in microglia, which might cause the polarization of T cells towards a Th2 phenotype [16]. The study was extended by performing RT-PCR on mRNA from T cells cultured in differing types of conditioned medium, using an array of primers to analyze a panel of cytokines and their receptors. The PCR array results revealed that modulating microglia activity with NCM and tuftsin **3** effi-

ciently down-regulated Th1-type responses, upregulates Th2-type responses, and released factors that promote immunosuppressive regulatory T cell formation. Sequentially rated regulators pathways were potentially involved in the transcriptional changes. Administration of tuftsin **3** strongly blunted the increase in phospho-STAT1 while increased synthesis of IL-10, IL-4, FoxP3 and CD25. It was found that the tuftsin **3** administration shifted the balance of the immune response from pro-inflammation (phospho-STAT1) to an anti-inflammation (IL-4 and IL-10) and immunosuppression (FOXP3, CD25). The results indicated that tuftsin **3** promoted *in vivo* inhibiting the signaling promotes Th1, Th2 cell activity and increased the expansion of Treg cells [16].

During the last decades many studies reported that infection with parasitic helminths, or systemic treatment with helminths extracts and secretory molecules, could reduce inflammation associated with autoimmune diseases [55-61]. Therefore commenced examinations were being aimed [62] at investigating and evaluating a novel therapeutic approach which was based on a worm derivative. Their studies consisted of deciphering the tolerogenic potential of tuftsin-PC (tuftsin extended at its *C*-terminal i.e THR-LYS-PRO-ARG-Gly-Tyr coupled to diazotized 4-aminophenyphosphoryl chloride (PC) to obtain TPC) compound in mice genetically prone to develop lupus. Authors' data indicated that TPC had the ability to attenuate lupus nephritis. Received results showed that TPC attenuated the development of glomerulonephritis in lupus prone mice, in particular, it ameliorated proteinuria, and reduced immunoglobulin deposition in the kidney mesangium. TPC also enhanced the expression of TGF-β and IL-10 and inhibited the production of IFN-γ and IL-17. TPC treated mice had a significant elevated phenotype expression of CD4þCD25þFOXP3þ Treg cells and in concert with their ability to accelerate anti-inflammatory cytokines expression, it contributes to the delay in the onset of lupus nephritis and prolong the lupus mice survival time. Summarizing this study showed that the treatment of lupus mice with TPC composed of phosphorylcholine conjugated to tuftsin, could mitigate evolution glomerulonephritis in NZBxW/F1 lupus prone mice [62]. Bashi *et al.* [63] continued their research, their study addressed TPC therapeutic efficacy in a mouse model of collagen-induced arthritis (CIA). TPC immunomodulatory effect was associated with a significant reduction in arthritic score, prevention of joint damage accompanied by immunomodulation of the cytokines profile and enhanced expansion of T and B cells regulators. These studies demonstrated the ability of TPC to attenuate joint inflammation in CIA mice. Analyzing the results found that, prophylactic treatment with TPC induced the expansion of $CD4^{+}CD25^{+}FoxP3^{+}$ T_{reg} and $CD19^{+}IL10^{+}CD5^{high}CD1d^{high}TIM\text{-}1^{+}$ B_{reg} cells in isolated splenocytes, thus modulating the cytokine profiles and decreasing the level of inflammation and synovial hyperplasia. Prominent B_{reg} and T_{reg} cells enabled the reduction in proinflammatory, increased cytokines and the anti-inflammatory cytokines in stimulated isolated splenocytes taken from TPC-treated mice [63].

In another research [57], the therapeutic potential of TPC to attenuate murine colitis was assessed. TPC proved to be effective in the treatment of colitis development in a murine model of colitis induced by 2% dextransulfate-sodium-salt (DSS) in drinking water. Treatment of colitis with TPC (daily oral administration of 50 mg/0.1 ml PBS per mouse) resulted in a substantial beneficial effect, including weight loss, colon length, intestinal bleeding, and disease activity score as well as histological analysis. These studies showed that activity of TPC was associated with a significant stimulation of anti-inflammatory cytokine IL-10 and down-regulation of colon pro-inflammatory IL-1β, TNF-α and IL-17 cytokines expression. Thus, TPC treatment could prevent significantly experimental colitis induction in native mice. It is noteworthy that inflammatory bowel disease (IBD) in humans in which multiple immune pathologies may intertwine is not completely imitated by chemically induced colitis model used in this experiment and future studies must focus on models with existing chronic disease [57].

Tuftsin, has also been used to modify the cross-linked alginate nanoparticles with encapsulated reporter (green fluorescent protein, GFP) and murine interleukin-10 (mIL-10) expressing plasmid DNA, using obtained nanoparticles of the size of 280-300 nm. For plasmid DNA containing alginate nanoparticle, negatively charged residues attached 6 arginine (positively charged arginine), next 4 G residues as a walk and tuftsin [64]. Transfection efficiency was determined in J774A.1 macrophages. It was noted that tuftsin alginate-modified nanoparticles were rapidly internalized within the first 15 minutes of exposure, in contrast to the unmodified and scrambled peptide modified nanoparticles, which internalized after 1 h and beyond. Due to a long cellular internalization, authors found that alginate tuftsin-modified nanoparticles were internalized by receptor-mediated phagocytosis and the peptide was indeed attached to the particles at the time of the particle uptake. Violent internalized in J774A.1 murine macrophages, led to superior transfection po-

tential without any toxicity [64]. To provide a preliminary assessment of DNA and transfection with control and surface-modified nanoparticles were alginate Performed with EGFP-N1 plasmid. Both quantitative GFP expression by ELISA and qualitative analysis by fluorescence microscopy showed that the tuftsin alginate-modified nanoparticles was the most effective deliver vectors in J774A.1. After confirmed transfection of GFP, [64], the delivery and transfection of a therapeutically relevant mIL-10 expressing plasmid DNA with the control and peptide-modified alginate nanoparticles in macrophages J774A.1 were evaluated. PCR analysis after 12, 24 and 72 h administration showed an increase in mIL-10 transcript with the tuftsin-modified nanoparticles relative to Lipofectin or scrambled peptide-modified nanoparticles. The results of measuring the protein expression level by ELISA showed that the highest expression of the protein with tuftsin-modified alginate nanoparticles occurred from 12 to 96 hours postadministration. In the treatment of Lipofectin, protein expression levels were much lower [64]. To show that the expressed mlL-10 J774A.1 macrophages possess anti-inflammatory effect, the authors investigated the down-regulation of pro-inflammatory cytokines TNF-α upon stimulation of the cells with 100 ng/mL LPS per 200,000 cells. LPS is a part of the cell wall of gram-negative bacteria and is associated with the toll-like receptors (TLR-4) on the surface of macrophages, which leads to the production of pro-inflammatory cytokine response, including TNF-α. First, the expression of TNF-α transcript was examined after 6, 12 and 24 h LPS stimulation of cells. After 6 h the maximum TNF-α mRNA levels were observed. Next the cells were first transfected with mIL-10 expressing plasmid DNA using the control and tuftsin modified alginate nanoparticles and the levels of TNF-α signal were measured at different time points from 12 to 72 h following administration. The results showed that the protein expression profile of TNF-α gave a significant decrease in the levels of pro-inflammatory cytokines at all time points following transfection of the cells with mIL-10 expressing plasmid using tuftsin alginate-modified nanoparticles. Results provided evidence for the development of a systemic macrophage-targeted anti-inflammatory gene delivery system potentially affecting the treatment of many diseases [64].

In another experiment the same research team [65] showed the effectiveness of a non-viral natural occurring polymer based gene delivery system encapsulating IL-10 plasmid DNA to repolarize macrophages from M1 to M2 functional sub-type for the treatment of experimental arthritis. IL-10 was encapsulated into non-condensing alginate based nanoparticles and the surface of the nano-carriers, was modified with tuftsin **3** to achieve active macrophage targeting. Enhanced localization of tuftsin-modified alginate nanoparticles was observed on the limbs of arthritic rats after intraperitoneal injection. Additionally, treatment was successful in reprogramming macrophage phenotype balance as ~66% of total synovial macrophages from arthritic animals treated with targeted tuftsin/alginate nanoparticles containing IL-10 plasmid DNA were in the M2 state compared to ~9% of macrophages in the M2 state from untreated arthritic animals. Targeted nanoparticle treatment successfully reduced systemic and joint tissue pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) expression and stave off the progression of inflammation and joint damage as shown by magnetic resonance imaging and histology. Importantly, rats which were treated retained their mobility, in turn untreated animals suffered from impaired mobility. Moreover, preliminary safety assessments showed that nanoparticles were highly tolerated in pre-clinical animal model of rheumatoid arthritis and symptoms of overt particle mediated toxicity were not observed in rats [65].

Another interesting analogue of tuftsin is Selank, which was extended at the *C*-terminus (Thr-Lys-Pro-Arg-Pro-Gly-Pro). Additional sequence of tripeptide Pro-Gly-Pro was designed to improve metabolic stability and prolonged half-life in the body. It has nootropic and anxiolytic properties, and also exhibits antiviral activity. Heptapeptide compared to tufstin has less effects on the immune system, but has a number of other interesting properties. Studies in rats confirm the positive effect of this peptide on learning and memory. It was found that Selank affects the immune system. The antiviral activity of this heptapeptide was examined against influenza virus type A (H_3N_2) *in vitro* and *in vivo*. Studies revealed that the insertion of Selank *in vivo* induced the expression of IFN-α, without affecting the gene expression of IL-4, IL-10 and TNF-α. The mechanism of antiviral acting is likely related to the ability of the peptide to modulate the balance of cytokin Th1/Th2. Another interesting feature of Selank was participation in the regulation of inflammatory processes in the body. Authors of this paper analyzed the expression of 84 genes involved in processes of inflammation (including chemokines, cytokines and their receptors) in the spleen of mice. Further studies showed that BCL6 gene that plays a key role in the development of the immune system, which was characterized by a significant change in expression level in re-

sponse to the injection of the heptapeptide. Selank and its fragments caused a number of changes in the expression of genes involved in inflammation. The results confirmed the role of the peptide in regulating processes of inflammation in the body [66].

Additionally, the effect of Selank and its short fragment Gly-Pro on the temporary dynamics of C3, Casp1, Il2rg, and Xcr1 genes expression in mouse spleen after single intraperitoneal injection (100 μg/kg) of peptidesusing real-time PCR method [67] was presented. Only 30 min after Selank injection a significant 3-fold decrease in the C3 mRNA level occurred was observed. An almost equal alteration of this gene mRNA level was observed after the injection of the dipeptide Gly-Pro. A meaningful increase in the expression of the Casp1 mRNA (2.4-fold) was received 90 min and 6 h after Selank administration. In turn, after the administration of Gly-Pro a decrease in the level of the Casp1 mRNA 30 min (5-fold) and 6 h (2.6-fold) after injection was observed. Additionally, a significant alteration in the mRNA level of the Il2rg gene at early time points after Selank and its fragment injection was found. A similar reduction in the Xcr1 mRNA level was noted 90 min after the administration of Selank and Gly-Pro. Furthermore, the mRNA level of this gene increased 1.8-fold 3 h after Selank administration and 2.1-fold 6 h after dipeptyde injection [67, 68].

Tuftsin **3** and its addictions were also administered intraperitoneally to rats and mice for anti-stress [30]. The radiopharmaceutical compound of Tc-99m RP128 [69] built with Tc and tripeptide *N*,*N*-dimethyl-GSC (Acm) connected by a glycine residue with pentapeptide TKPPR which binds *in vivo* to a specific membrane receptor of tuftsin [70,71] was used for the detection and identification of immflamatory in the central nervous system [72]. This compound has been selected for Phase I clinical trials. It has been found that it is safe and effective applicable to the visualizing pathological sites in patients with chronic rheumatoid arthritis [73].

T-peptide is an analogue of tuftsin. Its structure is as follows $(Thr\text{-}Lys\text{-}Pro\text{-}Arg\text{-}AAN)_4\text{-}(Lys\text{-}AAN)_2\text{-}Lys\text{-}AAN$, which was granted a patent in China. T-peptide was characterized by its stable structure and long half life compared with tuftsin **3**. Recently, it has been demonstrated that tuftsin-derived T-peptide could be a potential postoperative adjuvant in cancer therapy, and it showed an inhibitory effect on growth of residual tumor cells after surgical resection [74]. In 2015 Gao *et al.* [75] examined the effect of T-peptide on cell-mediated immunity in the presence of lipopolysaccharide (LPS) and the survival rate in septic mice. They noticed that administration of T-peptide enhanced the secretion of Th1 associated cytokine (IFN-γ), while the secretion of Th2 associated cytokine (IL-4) level was significantly lowered, and the response of cell-mediated immunity shifted to Th1. Subsequently authors showed that T-peptide had the ability to regulate immune dysfunction of CD4+ T lymphocytes *in vitro*. Then they investigated the apoptosis of CD4+CD25− T cells and IL-2 formation, and noticed that the proliferation of CD4+CD25− T cells was correlated with its apoptotic rate or IL-2 release. After administration of T-peptide, Th1 response was increased, while Th2 response was diminished. The balance of Th1/Th2 was gradually reversed to normal range, contributing to the improvement of host immune response as a result of septic episode. In this study, it was noted that T-peptide could down-regulate the suppressive activity of CD4+CD25+ Tregs, and the expression of Foxp-3 and CTLA-4 as well as the secretion of TGF-β were significantly lowered by treatment with immune stimulatory agent. In addition, treatment with T-peptide diminished the suppressive ability of CD4+CD25+ Tregs on CD4+CD25− T cells, and the proliferative activity as well as IL-2 formation of CD4+CD25− T cells were obviously increased together with reversed balance of Th1/Th2 response [75,76].

There were also synthesized conjugates of tuftsin containing muramyl dipeptides or nor-muramyl dipeptides (**17a-f)** (Fig. (**6**)), which were tested by using *in vitro* cultures of human monocytes and lymphocytes [8]. Muramyl dipeptide **2** is a part of peptidoglycan, which is a component of the bacterial wall. It is responsible for stimulation of the various functions of macrophages and enhances the non-specific response of the organism against a large number of infectious agents. Most of the compounds showed a suppressive effect on the viability of cells from PBMC cultures and their biological activity were similar to tuftsin **3** and MDP **2**. The results were dependent of time and dose. Moreover, studies confirmed the inhibitory effect of the examined analogs on the viability of both lymphocytes and monocytes. Furthermore, studies proved the inhibitory effect of the examined analogs on the viability of both lymphocytes and monocytes and the most active molecules were **15**, **16**, **17a**, **17c** and **17d**. The decrease in the viability of cells treated with the molecules was related to the generation of free radicals by monocytes and the stimulation of redox enzymes in lymphocytes. The fundamental difference between tuftsin **3** and its derivatives was associated to an increased viability of

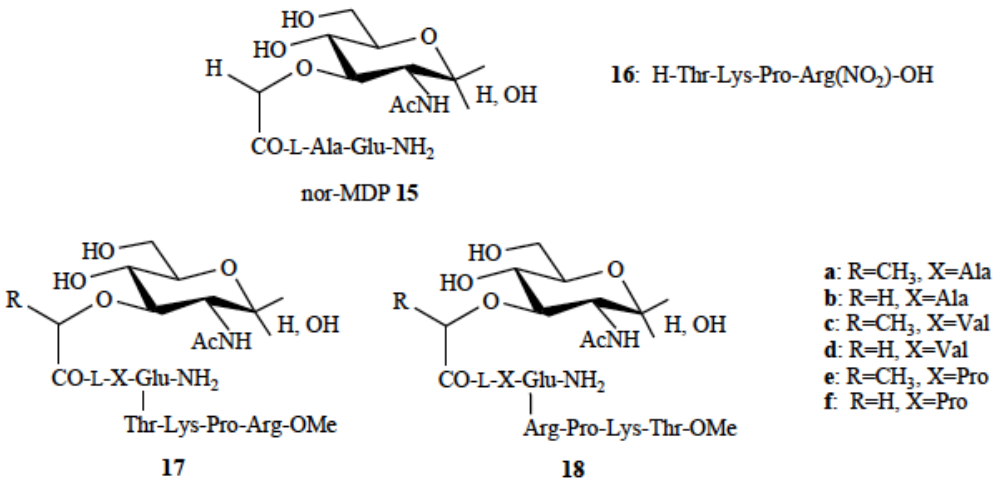

Fig. (6). Structure of nor-MDP **15**, tuftsin derivative **16**, conjugates muramyl dipeptide and nor-muramyl dipeptide of tuftsin **17a-**f and of retro-tuftsin **18a-f**.

leukocytes from short-lasting cultures treated with tuftsin **3**, which was not revealed in the presence of the most of the examined compounds. Tetrapeptide **3** undoubtedly stimulates mitochondria in the production of enzymes and metabolites that belong to the most important targets used in the viability tests and flow cytometry analysis. In turn, derivatives of tuftsin with shorter time from stimulation to reaction, visible in the activity of some compounds compared with tuftsin, might mask the activating effect of the tuftsin part of the derivatives. Additionally, the derivatives proved to be efficient stimulators of TNF-α and IL-6 secretion by both monocytes and lymphocytes. However, the secretion of cytokines did not affect the viability of the leukocyte population and seemed to play only an accessory role in their model. Moreover, most of the examined analogues did not increase the cytotoxic activity of Natural Killer (NK) cell. Only in case of some examined compounds (**17d**: 0.1 mg/ml, **17f**: 0.1–1.0 mg/ml) a trend was observed towards an increase in the cytotoxic activity of NK cells [8].

Successively, the same team studied the immunomodulatory properties of muramyl dipeptide and nor-muramyl dipeptide with retro-tuftsin (**18a-f**) (Fig. (**6**)) by using *in vitro* cultures of human subpopulations of white blood cells (peripheral blood mononuclear cells, peripheral blood lymphocytes, monocytes) [7]. Retro-tuftsin showed biological activity similar to tuftsin **3**. However, retro-tuftsin was more stable and resistant to degradation, as evidenced by the viability tests performed on subpopulations of white blood cells. The protective effect of tuftsin lasted only until the fourth hour of incubation, while retro-tuftsin demonstrated considerable activity after 24 h of stimulation. This means, that retro analogue had a higher resistance to the peptidase attack. Studies of retro-tuftsin derivatives showed suppressive effect of some compounds (**18a**, **18b**), which were dependent of time and dose. The viability inhibition was stronger when time was longer and concentration higher during stimulation. Because of low durability of tested derivatives (**18a**, **18b**), the increase in viability of cells was observed only after 4 h of stimulation. Primarily, these derivatives were rather not suppressive when incubation time was longer than 12 h and 24 h. Additionally, viability tests were performed by using *in vitro* cultures of human monocytes and lymphocytes. Experiment confirmed the positive effect of derivatives **18a-b** on the viability of PBL and monocyte cell cultures [7]. The secretion of TNF-α was visible in all analysed types of cultures, but only some compounds were able to induce it. Only the stimulation with higher doses of derivative **18a** showed a significant increase in the amount of TNF-α in the cultures of PBMC. In the PBL cultures, the secretion was induced mainly by the conjugates. Retro-tuftsin expressed a slight, similar with the tuftsin, impact on TNF-α production. Isolated monocytes were the most efficiently stimulated by MDP and higher concentrations of conjugates **18a** and **18b**. Native immunomodulators **3** and retro-tuftsine did not influence the production of TNF-α by monocytes. Derivative **18a** had ability to increase the secretion of IL-6 in the cultures of PBMC comparable to MDP, tuftsin and its retro analogue. Furthermore, only derivative **18a** had a minimal effect on secretion of the cytokine. In the case of PBL cultures, the secretion was most significantly induced by native peptide **3**. Studies proved that there was a trend towards an increase in the cytotoxic activity of NK cells treated with tuftsin and lower concentrations of its retro analogues. Also conjugates **18a** and **18b** showed some ef-

fect, but not as significant as tuftsin **3** and retro-tuftsin. MDP did not have ability to induce this cytotoxic activity [7].

Additionally, [77] conjugates muramyl dipeptide and nor-muramyl dipeptide of tuftsin as well as a retro-tuftsin containing isopeptide bond between the ε-amino group of lysine, and the carboxyl group of amino acids (Ala, Gly, Val). This modification should improve the resistance to proteolytic degradation and activity of conjugates compared to tuftsin **3**, provide immunomodulatory MDP conjugates with tuftsin as new potential drugs. The good *in vivo* activity in a murine model of sepsis, showed three compounds: *N*-acetyl-muramyl-Ala-D-isoGln-Thr-Lys-Pro-Arg, *N*-acetyl-muramyl-Val-D-isoGln-Thr-Lys-Pro-Arg and Thr-Lys-Pro-Arg. First compound was characterized by the highest efficiency, caused a reduction in the number of bacteria in the body, which is associated with increased activity of macrophages in all tissues examined and the increase of IL-10 (other compounds stimulated mainly IL-6). Increased levels of inflammatory factors, *i.e.* IL-10 in sepsis and septic shock may be particularly advantageous since they are able to stop or at least delay the development of septic shock [78,79]. MDP **2**, in addition to macrophages and monocytes, also stimulated dendritic cells (DC), which play an important role in the induction of specific immune response. As a result of the disease development was followed as decrease activity of DC, which is a result of inhibition of cell maturation, preventing antigen presentation immunocompetent cells thereby blocking the immune response. Studies carried out by the method of flow cytometry demonstrated an effect of conjugates tuftsin with MDP to stimulate the maturation and activity of DC derived from human monocytes [78].

In 2014 the effects of different repetitive peptides expressed by *Lactobacillus casei* (*L. casei*) were studied, specifically the MDP and tuftsin fusion protein (MT) repeated 20 and 40 times (20MT and 40MT), in mice also expressing the D antigenic site of the transmissible gastroenteritis virus (TGEV) spike (S) protein on intestinal and systemic immune responses [80]. Treatment of mice with *L. casei* expressing MDP, 20MT and 40MT stimulated humoral and cellular immune responses. The enzyme-linked immunosorbent assay showed that 20MT as well as 40MT induced an increase in IgG and IgA levels against TGEV. Furthermore, both 20MT and 40MT stimulated the differentiation of innate immune cells, such as T helper cell subclasses and regulatory T (Treg) cells, which induced robust T helper type 1 and T helper type 17 (Th17) responses and reduced Treg T cell immune responses in the 20MT and 40MT groups, respectively. Additionally, *L. casei* expressing 20MT and 40MT enhanced the anti-TGEV antibody immune responses of the humoral and mucosal immune systems. Thus, treatment of mice with *L. casei* expressing MDP and tuftsin possesses essential immunopotentiating properties, due to induced humoral and T cell-mediated immune responses upon oral administration, and it may be useful in oral vaccines against TGEV challenge [80].

6. TUFTSIN ANALOGS HAVING ANTIBACTERIAL PROPERTIES

In another study [81], tetramer tuftsin derivative $[TKPKG]_4$ (OT20) was used as a carrier for the peptide molecule TB5 (Fig. (**7**)). As the carrier/targeting moiety was used a granulysin derived peptide (GranF2). The main aim of the study was to enhance the cellular uptake and intracellular antimycobacterial efficacy of the TB5 *in silico* identified drug candidate.

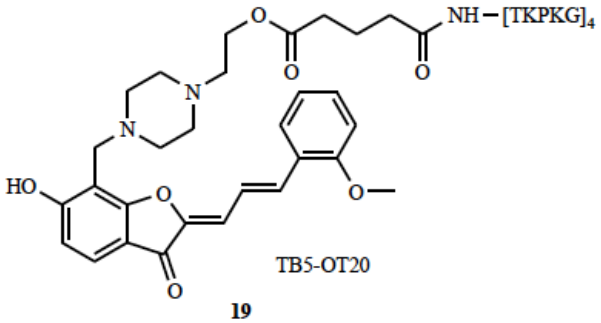

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Fig. (7). Structure derivative of tuftsin connected with the peptide molecule TB5.

The efficacy against intracellular bacteria was determined. MonoMac6 human monocytes were infected with *M. tuberculosis* H37Rv and treated with the new compounds. MonoMac6 established as a cell line, which seems to have phenotypic and functional characteristics of mature blood monocytes. Therefore MonoMac6 was often used as a host cell model to measure activity against phagocytosed intracellular bacteria. During the study it was observed that the treatment of MonoMac6 cells with the TB5 compound evoked almost the same intracellular fluorescent intensity as that of the untreated control. This means that the uptake rate of the free drug candidates is limited. After incubation conjugates of tuftsin and granulysin with TB5 showed a significantly higher the mean fluorescent intensity. Compound TB5 coupled with tuftsin caused less cytotoxicity of conjugate and the intracellular fluorescent intensity of TB5-OT20 **19** (Fig. (**7**)) treated cells was five-times higher than that of TB5 treated cells. Conju-

gate TB5 of tuftsin was extremely effective against phagocytised intracellular *M. tuberculosis* bacteria (a significant decrease in CFU was enumerated at 50 μM concentration). At the same concentration, Grand F2 and TB5-Gran2 conjugate caused 35-fold higher CFU even in the presence of a pore forming perforin. Analyzing the test results, it can be concluded that the coupling factors of antituberculosis with peptide carrier is a promising approach to enhance cellular uptake and *in vitro* selectivity. According to Horvati *et al.* [81], it can be stated that the conjugate OT20 tuftsin effectively inhibits the intracellular *M. tuberculosis* bacteria.

In 2014 a palmitoylated tuftsin derivative (a sequence of TKPKG) of isoniazid (INH) (Fig. (**8**)) [82] was presented as potential antitubercular agent. Conjugate **20** was assayed for the inhibition of *Mycobacterium tuberculosis* H37Rv (Mtb). Analog **20** was effective against intracellular Mtb and significantly reduced the viability of persisting bacteria. Interestingly, compound **20** showed more effective the treatment of Mtb infected monocytes, because resulting only a few colonies at 100 mg/L, in turn the treatment with INH resulted in confluent colonies which represent the inefficacy of the free drug against intracellular bacteria. Thus, the lipopeptide carrier used was efficient to deliver INH to its intracellular target. To assess the *in vitro* selectivity Horvati group also tested the cytotoxicity and hemolytic activity of the compounds on human peripheral blood mononuclear cells (PBMC) and erythrocytes. Studies indicated that the INH and derivative **20** were not cytotoxic to human PBMC and expressed no hemolytic activity to human erythrocytes even at the highest concentration (for both compounds: $IC_{50} > 1000$ μM; $HC_{50} > 1000$ μM) [82].

Additionally, to improve bioavailability, the compound **20** was encapsulated into poly(lactide-co-glycolide) (PLGA) nanoparticles and was tested for the determination of *in vivo* chemotherapeutic effect in a guinea pig infection model. The encapsulation efficacy of conjugate **20** was above 90%, while the encapsulation efficacy of INH was only 10%. 40 mg/kg bw dose of INH was administered orally twice a week to the infected guinea pigs, in turn the dose of PLGA-conjugate **20** was 3.8 mg/kg bw, which corresponds to approximately 10% of free INH. After necropsy procedure, the histopathological examination of the tissues revealed that treatment with PLGA-conjugate **20** resulted in considerably decreased inflammation and minimal granulomatous involvement compared to untreated control. In case of INH therapy a similar result was found, while the INH content was only 1/10 that in the conjugate loaded nanocapsules [82].

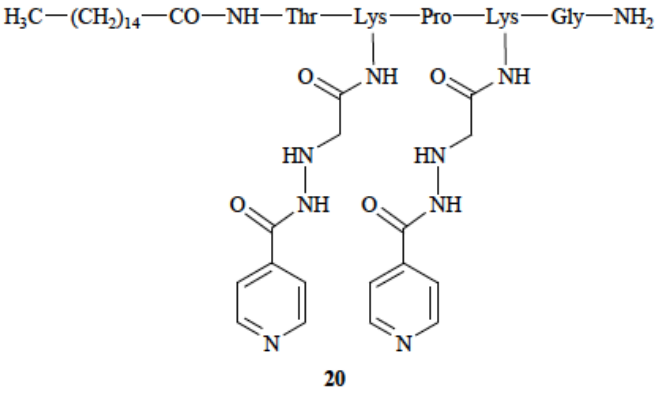

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Fig. (8). Structure of compound **20**.

During the treatment of cryptococcal meningitis in mice with leukopenia liposomes were attached to the surface of tuftsin, with modified *C*-terminal (Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-$COC_{15}H_{31}$) [83]. This modification facilitated connection to a double layer of liposomes. Analyzing the results, we can observe an increase in the activity of murine peritoneal macrophages. It has been proven that liposomal nystatin with tuftsin was more effective, safer and more stable than liposomal nystatin without tuftsin. Giving tuftsin with liposomal nystatin increased its activity against *Cryptococcus*. Due to the presence of receptors on macrophages, liposomal tuftsin served as a drug transporter, specifically by providing it to the appropriate locations, *i.e.* infections malaria [84], tuberculosis and leishmaniasis [85].

The antileishmanial efficacy of a subcurative dose of miltefosine (MF) in combination with free or liposomal palmitoyl tuftsin (p-tuftsin, **21**) (Fig. (**9**)) was investigated [86]. For the study a *Leishmania donovani*/BALB/c mouse model was used. Results were presented for two different doses of MF (2.5 and 5.0 mg/kg). Parasitic inhibition of MF at 2.5 and 5.0 mg/kg was 49.6% and 72.1%, respectively. Free p-tuftsin **21** showed an efficacy of 34%, in turn liposomal p-tuftsin revealed an efficacy of 48% ($P<0.05$). While, 2.5 mg/kg dose of MF was a combination with free p-tuftsin **21**, parasite inhibition increased from 49.6% to 66% ($P<0.01$), and in the case of addition liposomal p-tuftsin the efficacy increased further up to 81% ($P<0.001$). Significant enhancement in parasitic inhibition (93%, $P<0.01$) was observed when liposomal p-tuftsin was administered with 5 mg/kg of MF. Moreover, therapy involving free and liposomal p-tuftsin with MF increased reactive oxygen, nitrogen metabolites, and Th1 (TNF-α, IFN-γ, and IL-12) cytokine levels and reduced Th2 (IL-10) cytokine. A remarkable increase in phagocytosis index was also observed indicating

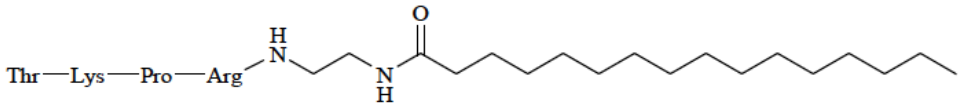

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Fig. (9). Structure of palmitoyl tuftsin.

overall immunological enhancement to antileishmanial activity of MF by free p-tuftsin **21** (20.60±1.5 (P<0.001)) and liposomal p-tuftsin (26.06±1.9 (P<0.001)) [86].

CONCLUSION

This article concerns analogues and properties of tuftsin (TKPR). Tuftsin is known as a natural immunomodulator of a wide range of biological properties**,** occuring in the blood of humans and other mammals, capable of stimulating certain white blood cells (monocytes, macrophages, and neutrophils). Tuftsin has not been applied as a drug so far because of quick biodegradation in organism (half-time is about 16 minutes). The products of its biodegradation inhibit tuftsin. Therefore, there are still efforts made to obtain more stable and active analogs and conjugates. Over the years, new molecules were prepared containing in their structure tuftsin and its derivatives. Most of the derivatives showed anti-tumor activity, anti-inflammatory or antibacterial. A large amount of the compounds may find use in vaccines. Tuftsin can also be used to prepare fusion proteins in the treatment of cancer and as carriers of many biologically active substances.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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