THE BIOLOGICAL ACTIVITY OF NEW TUFTSIN DERIVATIVES – INDUCTION OF PHAGOCYTOSIS

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Abstract: Phagocytosis plays a crucial role in a host defense against invading microorganisms. This process can be induced by many phagocytosis stimulating factors. One of them is an endogenous tetrapeptide – tuftsin that occurs in the blood of mammals including human beings. Tuftsin is capable of potentiating granulocyte and macrophage functions such as: phagocytosis, motility, and chemotaxis as well as bactericidal and tumoricidal activity. The other particle able to induce phagocytosis is muramyl dipeptide (MDP), the smallest synthetic glycopeptide of bacterial origin that possesses an immunogenic activity. MDP is known to affect most functions of macrophages. Phagocytosis stimulating properties of a new group of tuftsin and MDP analogues (one tuftsin analogue and four conjugates of tuftsin/retro tuftsin and muramyl dipeptide or nor-muramyl dipeptide) were tested. The results of the study show that all of the examined conjugates are able to generate oxidative burst. The most promising analogues proved to be kd6 and kd7.

Keywords: tuftsin derivatives, oxidative burst, reactive oxygen species, phagocytosis

Phagocytosis is one of the essential arms of host defense against bacterial or fungal infections. The phagocytic process consists of several major stages: 1. chemotaxis – migration of phagocytes to inflammatory sites, 2. attachment of particles to the surface of phagocytes, 3. ingestion – phagocytosis and intracellular killing by oxygen–dependent (oxidative burst) and oxygen-independent mechanisms (1). If phagocytosis is effectively activated in early stages of infection, it may result in limitation of bacteria spreading and prevent ongoing infection.

Different types of cells, including: neutrophilic granulocytes and monocytes/macrophages are able to perform phagocytosis (2). Phagocytosis in neutrophils is associated with an increase in turnover of the hexose monophosphate shunt and production of large quantities of hydrogen peroxide (H_2O_2). Those changes are essential for bacteria killing (3). Activated phagocytic cells convert molecular oxygen into reactive oxygen species (ROS) that are highly reactive oxidizing agents able to destroy microorganisms (3). The enzyme playing crucial role in killing microbes by oxygen-dependent mechanisms is NADPH oxidase. It is a multisubunit enzyme assembled in activated phagocytes mainly in the phagolysosomal membrane. This enzyme reduces molecular oxygen into ROS (3). Superoxide (O_2^-) is dismutated into H_2O_2, which is then used in the process of myeloperoxidase-mediated halogenation (3). Halides converted into reactive hypohalous acids can react and kill bacteria. The process by which ROS are produced is called the respiratory burst (4).

Phagocytosis can be induced by many phagocytosis stimulating factors. One of them is an endogenous tetrapeptide – tuftsin, which occurs in the blood of mammals including humans (5). Being an integral part of a heavy chain of IgG, tuftsin is liberated by the successive action of two specific enzymes: splenic tuftsin edocarboxypeptidase and leukokininase. Tuftsin is capable of potentiating granulocyte and macrophage functions such as: phagocytosis, motility, and chemotaxis as well as bactericidal and tumoricidal activity. Due to high plasma instability of tuftsin, many derivatives of this peptide has already been synthesized and examined. Some of them are equally active as tuftsin or even display better biological properties (7, 8).

The other particle able to induce phagocytosis is muramyl dipeptide (MDP), the smallest synthetic glycopeptide of bacterial origin that possesses an immunogenic activity. MDP is known to affect most functions of macrophages. The activation of those cells results mainly in increased reduction of oxygen to the superoxide anion (O_2^-) and then to hydrogen peroxide, which is involved in phagocytosis (7).

It has already been proved that combining those two immunomodualtors: tuftsin and MDP, results in an increase of their biological activity (9,10). We have investigated two completely new groups of conjugates of MDP or nor-MDP with tuftsin (10) and MDP or nor-MDP with retro-tuftsin (11, unpublished data). The assumption, that a few of examined derivatives would exhibit positive effect on the function of phagocytic cells, was based on previously obtained result (10, unpublished data).

The aim of this study was to evaluate the impact of new tuftsin and MDP derivatives (one tuftsin analogue and four conjugates of tuftsin/retro tuftsin and muramyl dipeptide or nor-muramyl dipeptide) on the induction of the phagocytosis process through the influence on the activation of oxidative burst in phagocytic cells.

MATERIALS AND METHODS

Chemistry

MDP (muramyl dipeptide) and nor-MDP (nor-muramyl dipeptide) analogues were modified by the incorporation of tuftsin (Thr-Lys-Pro-Arg) or retro tuftsin

Table 1. The examined compounds: A, B – conjugates of MDP and nor-MDP with retro tuftsin; T2 – tuftsin analogue; kd6, kd7 – conjugates of MDP and nor-MDP with tuftsin.

COMPOUND	CHEMICAL STRUCTURE
A	Mur(NAc)-Ala-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2
B	norMur(NAc)-Ala-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2
T2	H-Thr-Lys-Pro-Arg(NO_2)-OH
kd6	Mur(NAc)-Ala-D-Glu(Thr-Lys-Pro-Arg-OH)-NH_2
kd7	norMur(NAc)-Val-D-Glu(Thr-Lys-Pro-Arg-OH)-NH_2

(Arg-Pro-Lys-Thr-OMe) to the C-terminal of the muramyl peptides, forming a covalent bond with the isoglutamine carboxylic group (Table 1). Synthesis of these conjugates was described in details previously (10,11).

Cytometric evaluation of oxidative burst in subpopulations of PBMC (peripheral blood mononuclear cells)

Evaluation of the increase in the oxidative activity induced by examined conjugates in subpopulations of PBMC was based on oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) to the parent fluorescent dye 2',7'-dichorofluorescein (DCF). The examined substances were added to the PBMC cultures at final concentration 0,1 mg/mL. The fluorescence of the generated DCF was measured using flow cytometry just after the addition of conjugates and at 30 min, 1 h, 2 h and 4 h later.

The gates containing granulocytes and monocytes in flow cytometry analysis were established using forward angle scatter (size) and side single scatter (granularity) light characteristics of these populations. Dead cells were excluded under the same conditions. Histograms visualizing DCF fluorescence were generated from established gates. Mean and peak fluorescence signals of DCF from the examined cultures were measured and compared in arbitrary channel units.

RESULTS

Generation of oxidative burst in granulocytes (Figure 1, panel „granulocytes")

The generation of oxidative burst by the examined compounds in granulocytes from PBMC cultures started just after addition of the examined tuftsin analogues. The effect was most visible in cultures stimulated with conjugates kd6, kd7 and tuftsin derivative T2. Native tuftsin caused only a slight DCF fluorescence shift. The activity of the derivatives seemed to slightly decrease after 30 min of stimulation, because the fluorescence shift was weaker than at the start of the reaction (just after the addition of investigated compounds). Only derivative kd7 managed to sustain the same generation of oxidative burst as at the beginning of the experiment. Conjugates A and B became more active after 1 h of stimulation, but still their peaks of fluorescence signal was weaker than in cultures incubated with compound kd6, which proved to be the most active after that time. A kind of stability in obtained results was observed after 2 and 4 h of stimulation. The mean fluorescence shifts for all examined compound become steady.

Generation of oxidative burst in monocytes (Figure 1, panel „monocytes")

The examined derivatives induced an oxidation of H_2DCFDA also in monocyte cultures and the DCF fluorescence shift was similar to the one observed in granulocytes. The generation of ROS in monocyte cultures started very rapidly just after the beginning of the experiment. This effect was induced by the derivatives (especially kd6, kd7 and T2), not native tuftsin. This tetrapeptide seems to have little impact on ROS production, because after 30 minutes of stimulation the observed mean fluorescence was lower than in case of untreated cells. The level of fluorescence noted after half an hour in cultures incubated with the rest of the examined substances was a bit lower in comparison with the onset of the experiment. The longer incubation times (1, 2 and 4 h) revealed a kind of stability similar to the one observed in granulocytes cultures. But it was not so steady, while some differences were observed for conjugate A and tuftsin analogue T2. A significant difference for conjugate A became apparent after 4 hours after it addition to monocytes culture. The derivative T2 proved to be less active in comparison with other examined analogues, especially in the first and fourth hour of the experiment. The most significant and stable DCF fluorescence shift in the monocyte gate was noted in cultures stimulated with conjugates kd6 and kd7.

DISCUSSION

The current study revealed that all of the examined conjugates are able to generate oxidative burst in phagocytes, but to different extend. Surprisingly tuftsin revealed little ability to induce ROS production. This result was quite surprising, because it did not confirm literature data (4). It may be assumed that the generation of oxidative burst in case of tuftsin is important but it requires additional mechanisms to obtain the maximal infection clearance. It has been suggested, that the interaction between ROS and cytoplasmic granules of phagocytes is essential for microbial killing (2). In case of tuftsin it is possible, that such interaction is needed to phagocytic activity of the tetrapeptide.

The other examined compounds seem to have more influence on bacteria elimination due to the production of large amounts of ROS. An increase in the generation of oxidative burst was observed just after the onset of the experiment. The skyrocketing amount of ROS stabilized in the first hour of stimulation and remained at the same level till the fourth hour. The same pattern of ROS production was noted in analyzed cell subpopulations, granulocytes and monocytes.

The first phase of phagocytosis, in which oxygen – dependent mechanisms are turned on, is extremely rapid

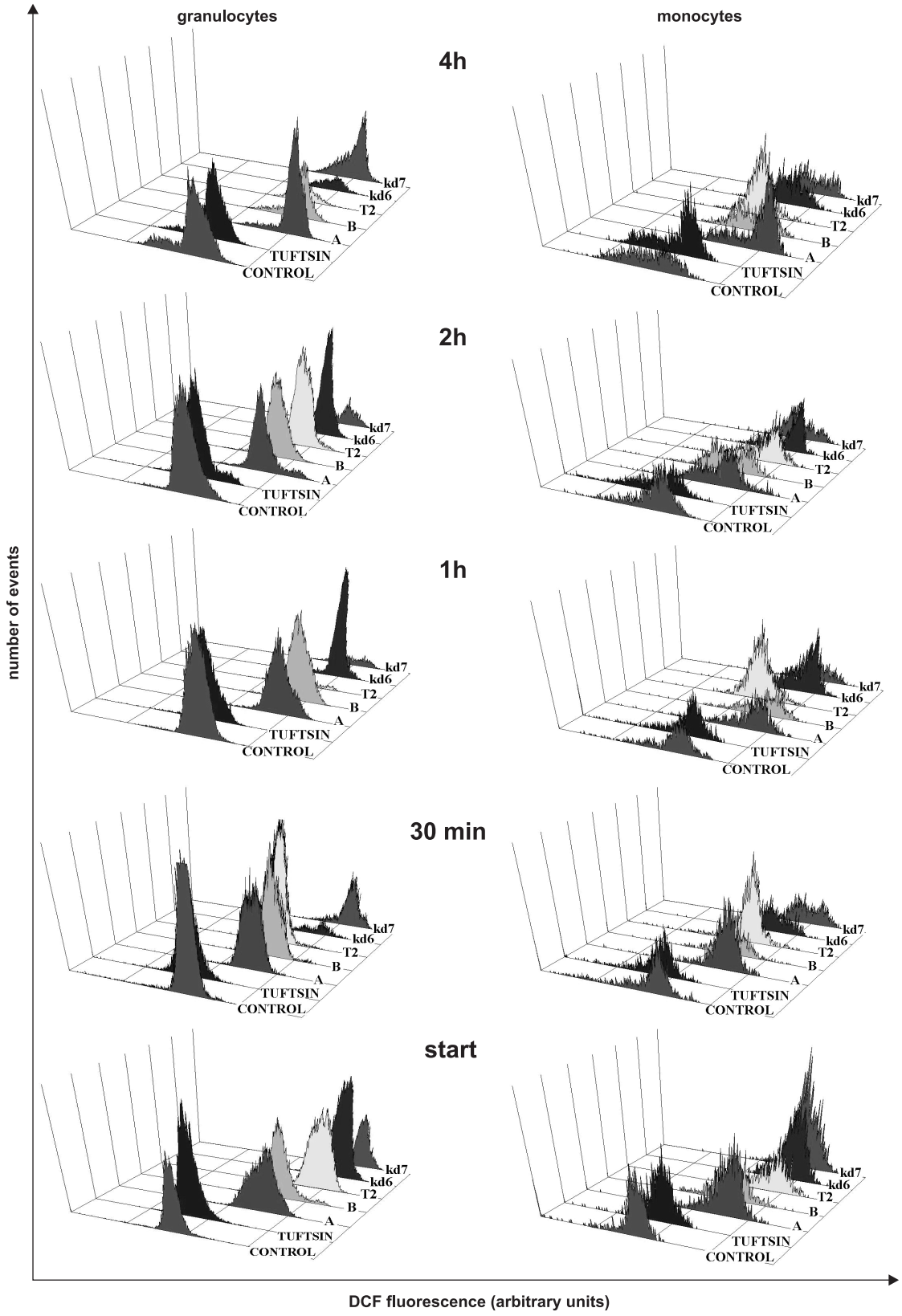

Figure 1. DCF fluorescence shift after stimulation of PBMC with the examined compounds. The presented panels "granulocytes" and "monocytes" were obtained from granulocytes and monocytes gates, respectively. The examples of overlaid histograms obtained from cultures are presented on the charts in the following order: start (just after the addition of the examined analogs), 30 min, 1 h, 2 h and 4 h of incubation. Arrows on the charts indicate the beginning of a significant difference in the peak fluorescence signal between the cultures stimulated with the particular compounds and those untreated.

(4). Our results obtained for tuftsin analogues are a confirmation of this statement, as the most significant DCF fluorescence shift was observed until the first hour of the investigation. The rapid start of ROS production and the following stabilization of mean fluorescence suggest that conjugates are able to activate NADPH oxidase to produce lethal agents in a swift but long-standing manner. So the outburst of bacterial infections can be promptly and successfully fought back by triggering the innate immune response.

Of course, as in case of tuftsin, presumably, the ROS production is still not the only way in which examined compounds help to eliminate bacteria. It is simply one of the stages of phagocytosis. Oxidative burst is crucial in stimulation of oxidative-dependent mechanisms, but also in other intravacuolar killing processes connected with the granule contents (2).

The most promising analogues proved to be conjugates kd6 and kd7. The reaction induced by the two compounds was the most noticeable. Other substances also surpassed tuftsin in the generation of reactive oxygen species, but their ability to induce that process was a bit weaker and less steady. Nevertheless, all examined tuftsin conjugates can be considered as potential therapeutic agents enhancing drugs impact on immune system.

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BIOTRANSFORMATION OF PRAZIQUANTEL BY HUMAN CYTOCHROME P450 3A4 (CYP 3A4)

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Abstract: Praziquantel (PZQ) is the drug of choice for the treatment of human schistosomiasis. It is estimated that about 200 million people in the world are currently affected by this tropical disease. Now PZQ is also used in malaria treatment. The usefulness of PZQ as antimalarial drug is important because of rapid development of resistance to usually applied drugs. PZQ undergoes extensive metabolism in human body, mainly in liver by two cytochrome P-450 isoenzymes 2B1 and 3A. As the result of these biotransformations numerous mono- and dihydroxylated derivatives in B, C and D ring are formed. Two metabolites have been fully identified and described, as *cis*- and *trans*-4-hydroxypraziquantel. Up to now there were created many different *in vitro* and *in vivo* models of PZQ biotransformations. *In vitro* model of PZQ biotransformation was created by using human cytochrome P-450 3A4 expressed in *Escherichia coli* and *Saccharomyces cerevisiae*. In the first experiment we have used human cytochrome P-450 3A4 from *Escherichia coli* (isolated on NTA-column). In the second experiment microsomes isolated from *Saccharomyces cerevisiae* containing coexpressed human CYP 3A4, human CYP-reductase and human cytochrome b_5 were used. The reactions were monitored by HPLC and MS.

Keywords: Praziquantel, *Escherichia coli*, *Saccharomyces cerevisiae*, cytochrome 3A4, metabolism

Xenobiotic biotransformation is the principal mechanism for maintaining homeostasis during exposure of organism to different molecules such as drugs. It is accomplished by a limited number of enzymes with broad substrate specificities. Reactions catalyzed by xenobiotic-biotransforming enzymes are divided into two groups, called phase I and phase II, leading usually to increase of hydrophilicity of xenobiotics, enhancing greatly their elimination.

Among the phase I biotransforming enzymes, cytochromes P450 (CYPs) rank first in terms of oxidation catalytic versatility and the broad number of xenobiotics they detoxify or activate to reactive intermediates, that may or may not been taken over by phase II enzymes.

The highest concentration of P450 enzymes involved in xenobiotic biotransformations are found in endoplasmic reticulum of liver (microsomes) but