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 (H₂O₂). 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 (O₂⁻) is dismutated into H₂O₂, which is then used in the process of myeloperoxidase-mediated halogenation (3). Halides converted into reactive hypohalogen 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 leukokinase. 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 (O₂⁻) and then to hydrogen peroxide, which is involved in phagocytosis (7).

It has already been proved that combining those two immunomodulators: 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 (Thr-Lys-Pro-Arg) and then to hydrogen peroxide, which is
(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 (H2DCFDA) to the parent fluorescent dye 2',7'-dichlorofluorescein (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. The skyrocketing amount of ROS stabilized in the first hour of stimulation and remained at the same level till the fourth hour. 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.
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.

REFERENCES

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 b5 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
some cytochromes P450 are also present in other tissues.

Being the major elimination route for many drugs, P450 enzymes play a very important role in the detoxification of xenobiotics, but may also lead to toxic or tumorigenic metabolites.

All P450 enzymes are heme-containing proteins. The heme iron is usually ferric (Fe$^{3+}$) that once reduced to the ferrous (Fe$^{2+}$) state, allows cytochromes P450 to bind O$_2$ or carbon monoxide (CO). The complex between ferrous cytochrome and CO absorbs light maximally at 450 nm, from which CYP derives their name.

The human liver microsomal CYPs belong mainly to four gene families (CYP1, CYP2, CYP3 and CYP4). These gene families generally correspond to a single or two subfamily(ies) i.e. CYP 1A, CYP 3A or CYP 4A, with the exception of the CYP2 gene family which contains six subfamilies (CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP2J). The number of CYP enzymes in each subfamily differs from one species to another.

Praziquantel (PZQ), 2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinoline-4-one (Figure 1), is an anthelmintic drug with a broad activity against trematodes and schistosomes. It is included in the WHO Model List of Essential Drugs and it is the drug of first choice in the treatment of schistosomiasis. Currently it was used in malaria treatment. The usefulness of PZQ as antimalarial drug is very important, because malaria is one of the most dangerous endemic diseases; nowadays rapid development of resistance to usually applied drugs is observed.

PZQ is used in the therapy as its racemate although the anthelmintic activity is mainly associated with the $R$-(-)-enantiomer (2). The drug undergoes extensive metabolism in human body, mainly in liver, by two cytochrome P-450 isoenzymes: 2B1 and 3A (1), yielding numerous monohydroxylated and dihydroxylated metabolites. Also, polyhydroxylated metabolites have been described. The hydroxylated metabolites are also excreted in the urine as conjugates with glucuronic and sulphuric acids. Up to now in human body, only the monohydroxylated metabolites trans-4'-hydroxypraziquantel (main metabolite in humans) and cis-4'-hydroxypraziquantel have been fully identified (by means of chemical proof and spectral data analysis) and described (2, 3). Recently next new metabolite 8'-hydroxypraziquantel was identified and described by means of electrospray ionisation-mass spectrometry (ESI-MS), $^1$H and $^{13}$C NMR spectroscopy (4). However, the exact chemical structure of the most metabolites is still unknown.

Up to now to create different in vitro and in vivo models of PZQ biotransformations: laboratory animals (rats), microsomal preparations and microorganisms were used. Till now, successfully obtained metabolites of PZQ were the result of biotransformations by Cunninghamella echinulata and Beauveria bassiana (5). There were derivatives in position C7, and in D ring (the position of substitution is not known).

In the following study biotransformation of PZQ was examined. In our research on in vitro model of PZQ biotransformation was created by using modified human cytochrome P-450 3A4 expressed in *Escherichia coli* DH5$\alpha$ (6) and microsomes from engineered yeast strain *Saccharomyces cerevisiae* with coexpressed human cytochrome CYP 3A4, human NADPH-P450 reductase and human cytochrome b$_5$ (7).

In the first experiment isolated protein of CYP 3A4 was used. This cytochrome was expressed in *Escherichia coli* DH5$\alpha$. The expression plasmid pCW/NF14 was obtained from Professor F. Peter Guenguerich (Department of Biochemistry and Centre in Molecular Toxicology, Vanderbilt University School of Medicine). This plasmid contains modified human cytochrome P-450 with a (His)$_3$ tag at the C-terminus and with N-terminus modified (the residues 3-12 were deleted). In the second experiment we have applied microsomes isolated from engineered *Saccharomyces cerevisiae* as a source of human cytochrome CYP 3A4, NADPH CYP-reductase and cytochrome b$_5$. The reactions were monitored by HPLC.

**EXPERIMENTAL**

**Chemicals and reagents**

Racemic PZQ was obtained as a gift from prof. G. Blaschke from University of Münster. The expression plasmid pCW/NF14 was obtained from Professor F. Peter Guenguerich (Department of Biochemistry and

![Figure 1. Structure of praziquantel (PZQ).](image1)

![Figure 2. pCW/NF14 vector with modified cytochrome P-450.](image2)
Expression of plasmid in E. coli

E. coli DH5α was transformed with plasmid pCW/NF14 (Figure 2) and selected on LBamp plate. Single isolated colony was grown at 37°C overnight in LBamp media and then diluted 1:100 in TBamp media containing 100 mg/L ampicillin for induction of P450 3A4 enzyme. Induction was carried out at 30°C for 24 h before harvest. Harvested culture was chilled on ice and centrifuged at 5000 rpm (4°C, 5 min). The cell pellet was resuspended in 100 mM potassium phosphate buffer (pH = 7.6) containing 20% glycerol and 0.1 mM DTT, and frozen at −80°C. The cells were thawed at room temperature and resuspended in 10 mL of PBS buffer. Suspension was sonicated in ice-salt bath with the Bandelin instrument, and centrifuged at 10000 rpm (4°C, 20 min).

Purification of modified cytochrome P450 3A4

The supernatant was loaded on Ni-NTA-column, and the column was washed with loading buffer (buffer A: 0.1 M phosphate buffer, 0.5 M NaCl, 5mM imidazole, pH = 8.0). Next, the column was washed with two types of washing buffer (buffer B: 0.1 M phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH = 8.0; buffer C: 0.1 M phosphate buffer, 0.5 M NaCl, 20 mM imidazole, pH = 8.0). The P450 3A4/NF14 was eluted by 3 x 5 mL elution buffer (buffer D: 0.1 M phosphate buffer, 0.5 M NaCl, 500 mM imidazole, pH = 8.0).

Preparation of microsomes from Saccharomyces cerevisiae with overexpression of NADPH cytochrome P450 reductase

W(R) strain of Saccharomyces cerevisiae was used, which is derived from W(n) (leu2, his3, ttp1, uaa3, ade2-L, can4, cyr1) in which the P-450 reductase gene was placed under the control of the hybrid promoter GAL10-CYC1. This strain was grown in YPLA medium to late log phase (2-4 x 10⁷/mL). The reaction was performed in PBS buffer in total volume of 250 µL. The reaction mixture contained: 25 µg NADPH, 2.5 µL DMF, 50 µL suspension of microsomes with yeast NADPH cytochrome P450 reductase, 50 µL solution of modified cytochrome P450 3A4 and 100 µg of PZQ. The incubation was performed at 37°C for 30 min and was stopped by 5 µL TFA. The extraction procedure was as follows: adding 500 µL of dichloromethane, vortexing at maximum speed for 1 min., centrifuging for 5 min at 10000 rpm, removing the upper aqueous phase and evaporating the organic phase under vacuum. The dry residue was dissolved in 50 µL of isopropanol and HPLC analysis was carried out using column Chiralpak AD, mobile phase: hexane/isopropanol (75:25, v/v), flow: 1 mL/min, injection: 20 µL, UV detection (210 nm).

RESULTS

Two types of biotransformations were carried out: 1) with the modified isolated cytochrome P-450 3A4 from Escherichia coli mixed with microsomes isolated from yeast Saccharomyces cerevisiae with overexpressed yeast NADPH reductase CYP and 2) with microsomes isolated from Saccharomyces cerevisiae with coexpressed human cytochrome P-450 3A4 and human NADPH reductase CYP mixed with human cytochrome b₅.
In the first experiment, to create in vitro metabolism of PZQ, isolated human modified cytochrome P-450 3A4 from Escherichia coli and microsomes from Saccharomyces cerevisiae with NADPH-cytochrome P-450 reductase and NADPH were used. The analysis of cytochrome P-450 3A4 was performed by SDS-PAGE electrophoresis to confirm the presence and purity of cytochrome (Figure 3).

In these experiments one metabolite of PZQ-M1 (Figure 4) was obtained. The retention time of the metabolite M1 was almost identical to that of 1,11b-dehydropraziquantel (Figure 5).

In the second experiment microsomes isolated from engineered Saccharomyces cerevisiae were used as a source of human cytochrome CYP 3A4, NADPH CYP-reductase and human cytochrome b5.

As a result of this experiment only one metabolite-M2 (Figure 6) was obtained. The spectral data of this metabolite were identical with the metabolite M1 from the first experiment.

CONCLUSIONS

Bacteria offer many potential advantages as an expression system. High levels of production may be obtained rapidly and at low costs, the cells are relatively easy to break, and, in principle, purification should be easier than in the other systems. Created system for the expression of human cytochrome P-450 3A4 is relatively simple and reproducible. A slightly shorten protein version can be easily produced and purified. The enzyme shows catalytic activity toward PZQ. The efficiency of this reaction was moderate. It may be caused by used in the experiment yeast NADPH-CYP-reductase which cooperate inadequately with the human cytochrome P-450.

The yeast expression system appears to be very useful in evaluation and simulation of the metabolism of drugs and of other xenobiotic molecules by human cytochrome P-450. Since the yeasts are eucariotic microorganisms, this in vitro system is easier to handle, and it is more efficient than bacterial system.

The created in vitro biotransformation systems are similar, but the yeast system is more efficient and easier to use comparing with bacterial system.

In both experiments only one metabolite of PZQ was obtained: M1 in the first and M2 in the second experiment. Taking into consideration the spectral data, the identity of both products was confirmed. This sug-

Figure 3. SDS-PAGE gel electrophoresis of cytochrome P-450 3A4. A – protein ladder, B – before purification of protein, C – protein after purification on Ni-NTA column.

Figure 4. Results of HPLC analysis on Chiralpak AD column.

Figure 5. Results of HPLC analysis of 1,11b-dehydropraziquantel on Chiralpak AD column.
gests that cytochrome P-450 3A4 is responsible for PZQ biotransformation.

The obtained metabolite is more lipophilic than PZQ, because the retention time on Chiralpak AD column is shorter for the metabolite and on Hypersil ODS column its retention time is longer than for PZQ. The retention time for $M_1 = M_2$ is similar to 1,11b-dehydropraziquantel.

These systems may find applications in the multi-step bioconversion systems, detoxication systems or pollution-clearing systems.

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