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Identification of a primycin producing industrial bacterial strain and *in vitro* investigation of the active substance.

PhD thesis

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Introduction

The discovery and widespread use of antibiotics has not only been a breakthrough in medical practice in the fight against infectious diseases, but has to date decisive influence on the course of human history [1]. In the middle of the twentieth century, which we regard as the golden age of antibiotics, antibiotics appeared to be the ultimate medicine for bacterial infections, but it soon became clear that antibiotics do not prove to be almighty drugs. Although a large number of natural, semisynthetic and synthetic antibiotics are currently available in healing, the therapeutic process used against pathogens are increasingly ineffective. One of the reasons for this is that multi-resistant bacterial strains develop against antimicrobials that are constantly expanding. Extensive antibiotic-resistant *Mycobacterium tuberculosis*, methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* are now serious public health threats [2]. Due to the slowdown in the development of new antibiotics, the use of new strategies has become necessary against the increasing threat of antibiotic resistance worldwide. Based on the above phenomenon one of these strategies is aimed at reviving old antibiotics that are not or less used in current clinical practice [3–5]. Primycin the first isolated and manufactured antibiotic in Hungary [6] shows outstanding efficacy against the current populations of the most common Gram-positive pathogens including the current multi-resistant strains [7]. The bactericidal effect of non-dividing bacteria [8] and the extensive and high efficacy of multidrug resistant Gram-positive bacteria makes primycin very valuable for clinical practice. However from the point of view of drug development it is absolutely necessary to reassess with modern testing methods and expand our knowledge to meet today's industrial and scientific requirements. The basis of

the work was the joint research program of the Department of General and Environmental Microbiology of PTE TTK and Pannonpharma Ltd.

Aims

The aim of this work was to expand the avaible information of the primycin producing industrial strain and the primycin by using multidisciplinary, system-based biological testing methods.

- To investigate the antimicrobial activity of primycin on microbes that have not yet been tested, and to re-evaluate existing studies using modern, standardized microbiological methods.
- Microbiological characterization of the production strain and identification of its taxonomic status by molecular biological methods.
- Development of a human three-dimensional (3D) liver model for in vitro toxicological studies.
- In vitro toxicological testing of primycin by different methods.
- Gene expression analysis of the effect of primycin treatment with microarray and real-time qPCR techniques

Materials and Methods

Microbiological methods

Determination of minimum inhibitory concentration

Tests for susceptibility to antimicrobials were performed on the basis of International Standards (CLS-Clinical and Laboratory Standards Institute). Testing the susceptibility of microbes to primycin we used the standard for the appropriate organism; testing yeasts for M27 [10], filamentous fungi testing in M38 [11]; testing bacteria in M7 [12]; standard. As a technical control, amphotericin B was used, which, like primycin, had a membrane attack. Microplates were evaluated visually and with an ELISA reader (Bioscience, Multiskan EX). Total inhibition was considered to be the minimum inhibitory concentration.

Carbon source utilization and bacterial indentification tests

The purpose of carbon source utilization studies is to determine whether the microorganism is able to use the available carbohydrate in the nutrient solution as the single carbon source. During the identification of the species, the following tests were performed: starch hydrolysis, gelatinase test, casein hydrolysis, nitrate reduction, catalase test.

Microbiological assay of antibiotics

Microbiological assay of primycin was performed on industrial primycin producing strain and *Saccharomonospora* species starting from shake flask fermentation. For the assay a 24-hour culture of *B. subtilis* ATCC 6633 was used in a Latin square design.

Scanning electron microscopy

The primycin-producing industrial strain (NCAIM 00028) was incubated in LB medium at 37 ° C for 48 hours. Scanning electron microscopy was performed using a JSM 6300 (JEOL) microscope.

Analytical methods

High Performance Liquid Chromatography (HPLC)

The supernatant of the microbial cells was tested with an Agilent 1100 HPLC. The components were separated by a 4.6 x 150 mm, 5 μm BDS Hypersil C18 (Shandon) column. The separation time is 60 minutes, from which the 16-26 minute chromatogram section has been integrated.

High Performance liquid chromatography electrospray ionisation tandem mass spectrometry (HPLC/ESI-MS)

Primycin components were also investigated by HPLC / ESI-MS. For separating the components a 2.1 x 150 mm, 3.5 μm Zorbax Eclipse XDB-C18 (Agilent) column was used. Fragmentation of the samples was performed by positive electrospray ionization (70 V). Chromatographic parameters were checked and data analyzed using Chemstation (Agilent, A.08.04) software.

Genomic approach

DNA isolation

DNA isolation was carried out according to Molecular Cloning Manual (Sambrook et al., 2001, Cold Spring Harb. Lab. Press), applying 50-150 mg starting material. Sample quantitative and qualitative parameters were examined by NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalyzer DNA kit.

De novo genom sequencing

The genome sequencing of Saccharomonospora azurea SZMC 14600 was conducted by combining cycled ligation sequencing on the SOLiD 3Plus system (Life Technologies) with 454 FLX pyrosequencing (Roche) in BayGen Institute, Szeged.

The automatic annotation of the genome was assigned by using the NCBI

Prokaryotic Genomes Automatic Annotation Pipeline [13], EMBL EBI Velvet program and RAST (Rapid Annotation using Subsystem Technology) server [14].

Structural analysis of the PKS gene cluster

PKS gene clusters were identified and analyzed with CLUSTSCAN és antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) program tools [15]. Database searches for homologues genes and proteins were performed using the National Center for Biotechnology Information (NCBI) BLAST server [16]. Domain analysis and motif search were done by MAPSI (Management and Analysis for Polyketide Synthase type I) [17], SBSPKS (Structure Based Sequence Analysis of Polyketide Synthases) [18], SMART (Simple Modular Architecture Research Tool) [19] and MEME (Multiple Em for Motif Elicitation) [20] respectively. Multiple sequence alignments of the PKS gene sequences were performed with CLUSTALW [21] program tool.

Cell Biology Studies

Human tissue cultures

Human liver carcinoma cell line (HepG2, ATCC), primary human hepatocyte cells (hNHEPS, Lonza) and primary human fibroblast cells (NHLF, Lonza) were used to construct the two- and three-dimensional in vitro liver models. Cells were trypsinized with ~ 80% confluency and used for different assay models. Known hepatocyte differentiation marker genes were tested by gene expression assays for each 2D and 3D liver model involved.

RNS isolation and qPCR

RNA extraction was performed with EZ-10 total RNA (Bio Basic Inc.) isolation kit according to the manufacturer's instructions. The RNA content measured by a Qubit fluorometer (Invitrogen, version 1.0) using a Quant-iTTM RNA BR Assay Kit. The isolated RNA was treated with DNase

(DNase I, Fermentas). For cDNA transcription we used High Capacity RNA to cDNA Kit (Applied Biosystems) according to the manufacturer protocol. The cDNA content of the sample was checked with a Qubit fluorometer using a ss DNA BR Assay Kit. ddCt was used to calculate gene expression changes normalized to beta-actin internal control.

Microarray analysis

Microarray examinations were performed with the Affymetrix GeneAtlas ™ Personal Microarray (Affymetrix) using U219 array chip (Affymetrix), which is suitable for gene expression levels of 20,000 genes and 36,000 transcriptional variants. CEL files were analyzed in a statistical environment R using Affymetrix Bioconductor software (Bioconductor, version 2.10.1). Normalization between the arrays was performed using the Robust MultiArray Avarage (RMA) method.

Viability tests

In vitro toxicological study of primycin was performed using colorimetric (MTT test) and luminescent (CellTiter Glo LuminescCell Viability Assay) assays on different liver models. Cell cultures were incubated with different concentrations of primycin (0.1 µg / ml; 1.0 µg / ml; 10 µg / ml; 100 µg / ml) for 72 hours. To determine the cytotoxic activity of primycin we used a microplate reader (Multiskan Ascent, Thermo Fisher Scientific Inc.).

Lipidtox staining

Lipid staining was performed with HSC LipidTOX ™ Red Neutral Lipid Paint (Invitrogen). The sections were incubated for 30 minutes with 1: 1000 diluted dye. Images were recorded using a LSM 710 confocal microscope (Zeiss).

Peroxisome ploriferator activated receptor (PPAR) gamma activity measurement

To measure PPAR gamma activation, a BrightGlo luciferase assay (Promega)

was used which was detected by a microplate reader (Synergy ™ HT, BioTek Instruments).

Results

Antimicrobial activity of primycin

During the developing of antibiotics the antibiotic efficacy testing (MIC) is very important for therapeutic use, since the determination of the effective dose of the antibiotic and its therapeutic spectrum is based on this knowledge. We tested the primycin susceptibility of some *Candida* species from the strain collection and from clinical specimens. Based on our results primycin is effective in 4-64 μg / ml concentration against *Candida* species, while in literature it is 2-10 μg/ml. The intra-species variability of the minimal inhibitory concentration of primycin was assessed by clinical *Candida albicans* isolates. The examined *C. albicans* isolates show that their sensitivity to primycin varies only slightly. We found no primycin-resistant human pathogenic *Candida* species and have not been described in the literature. In the study of filamentous fungi, which play a major role in eye infections, we found that the sensitivity of *Aspergillus niger* is the highest against primycin, but the MIC is still 64-fold compared to amphotericin B. MIC data from Gram-negative and Gram-positive bacteria showed consistent data to literature. However, the tolerance of *S. azurea* to primycin excelled from the Gram-positive species included in the studies, therefore a further comparative study was performed which revealed that *S. azurea* is capable of producing antibiotics, which is not known in the literature. Following the biological value measurement, we also confirmed by analytical methods that the antibiotic produced by *S. azurea* is the same as primycin. Analytical studies identified the amino molecule described in the margolactone production, which was also identified from the fermentation broth of the

primycin-producing industrial strain and the *S. azurea* strains from the strain collection. There is no data on the biological activity of deguanidino-amino component A1 in primycin.

Strain identification

Most of the literature on primycin was published in the last century. Since then, not only the relationship between scientific testing methods and clinical relevance of microbial resistance has changed, but also regulatory expectations. Since the industrial production of primycin occurs during fermentation, the exact taxonomic identification of the production strain is indispensable for subsequent product development. In the taxonomic and diversity analysis of bacteria, the 16S rRNA is the most commonly tested gene on the basis of which the determination of the taxonomy status of the primycin-producing industrial strain was performed. Based on the homology of the 16S rRNA, we found that the industrial strains NCAIM 00028, NCAIM 000181 and ES-23 are not directly related to the reference species assumed on the basis of the literary data, but to the species described as *Saccharomonospora azurea*. After complete genom sequencing, we extended our studies beyond the 16S rRNA gene by molecular analysis of the 23S, 5S and ITS sequences and the ribonuclease P and gyrase B gene coding region. Phylogenetic analyzes for all the genes tested confirmed that primycin-producing industrial strains are identical to *S. azurea*. The primycin-producing industrial strain was deposited under the name *Saccharomonospora azurea* SZMC14600 in the Szeged Microbiological Collection.

After the identification of genes encoding the PKS I enzyme complex involved in the production of primycin, a comparative analysis of the partial PKS sequences of the industrial primycin producing strains was performed, which revealed that the two industrial strains exhibited a difference in only

one position at the 5 'end of the test sequence, resulting difference in the protein primary structure (amino acid sequence).

In vitro toxicological and pharmacological testing of the active substance

Models

Based on the differentiation markers and the functionality test, the 3D HepG2: Fb liver model is suitable for in vitro toxicological tests. Studies with the HepG2 hepatic carcinoma cell line do not significantly differ from studies with primary human hepatocytes. Since the procurement, sustainability and reproducibility of HepG2 cells are much easier to solve than primary cells, their use makes it much easier and less costly to carry out toxicological and pharmacological tests.

Viability tests

Primycin acts in a dose-dependent manner in acute toxicity tests. Primycin was toxic at 10 and 100 μg / ml. At lower concentrations (0.1 and 1.0 μg / ml), primycin was neither toxic to primary hepatocyte nor to liver carcinoma cell models. The MMT test results were also confirmed in an independent test to be non-toxic. The results predict that primycin may be useful for the development of an oral preparation for systemic infections caused by Gram-positive pathogens.

Affymetrix microarray analysis

Primycin causes differences in the expression of inflammatory cytokines and chemokines as well as genes regulating lipid metabolism at non-toxic concentrations. The expression of the IL11 and IL24 genes is much stronger than the expression of the inflammatory IL6 and IL8 genes. Since both IL11 and IL24 have described the role of these genes in carcinogenesis and are mentioned as a potential target for anti-cancer therapies, it is worthwhile justifying this effect of primycin in further studies. The expression change of genes belonging to the CXC subfamily of chemokines was also observed.

Chemokines (CXL1, CXCL2, CXCL3, CXCL4, CXCL5) showing the increase of primycin gene expression belong to the same subfamily. The effect of primycin on lipid metabolism was confirmed by microarray data. Primycin increased the expression of PDK4 gene in the regulation of glucose and fatty acid metabolism and the SGK1 gene involved in response to cellular stress. In parallel, primycin gene expression causes a decrease in APOB and FABP1 genes. Another evidence of the effect of primycin on lipid metabolism is that the amount of both cellular and membrane lipids has drastically decreased in the lipid staining studies of the primycin-treated 3D liver model.

PPAR gene family examination

Increased gene expression of inflammatory cytokines (IL6, IL8) is associated with lipid metabolism by the PPAR gene family. In contrast to prednisolone with anti-inflammatory effect, primycin does not increase the expression of PPAR genes (PPAR α, PPAR γ) and its target ADRP gene, and also causes a decrease in PPAR γ activity in the activity assay. Although the decrease in gene expression by primycin compared to the untreated control is not significant, the level of PPAR α gene expression in both concentrations (0.1 and 1.0 µg / ml) has decreased and the PPAR α target gene, CYP3A4 expression showed significant decreasing. Although there are many active substances that affect the activity of CYP3A4, these drugs inhibit the activity of CYP3A4 and not the transcription of the CYP3A4 gene.

Summary

- We have shown that primycin has no effect on Gram-negative strains, and we have tested its antifungal effect on yeasts and filamentous fungi using modern, standardized microbiological methods
- Antibacterial effects studies have shown that *Saccharomonospora azurea* from strain collections, which showed high tolerance to primycin, is capable of producing antibiotics. The antibiotic was identified as primycin components by HPLC and HPLC / ESI-MS methods.
- We have identified large quantities of the non-investigated deguanidin-amino primycin A1 component in the industrial production strain. The amino primycin component A1 was also detected in strains of *S. azurea* from the strain collections.
- Due to the taxonomic uncertainties of the industrial strain, systematic identification of the strain by molecular biological methods was performed, which proved that the production strains are representatives of the *Saccharomonospora azurea* species described by Runmao et al.
- By analyzing genomic sequencing data, we identified the PKS domains responsible for antibiotic production in the primycin-producing industrial strain and compared these gene sections.
- An in vitro toxicological study of primycin was performed on human tissue model, we developed a model suitable for toxicological studies.
- In the in vitro toxicology study primycin was found to be non-toxic at concentrations of 0.1 and 1.0 μg / ml. In this concentration range, primycin is effective against most Gram-positive bacteria including multidrug-resistant strains.
- A comprehensive study was performed using microarray and qPCR assays. Primycin causes differences in the expression of inflammatory cytokines and chemokines as well as genes regulating lipid metabolism at non-toxic

concentrations. Lipid staining studies have shown that the amount of both cellular and membrane lipids is reduced in the primycin-treated 3D liver model.

- In the context of lipid metabolism we investigate the expression and activity of the primycin PPAR gene family, not only PPAR α and PPAR γ but also their target genes. Studies have shown that primycin significantly reduces expression of the CYP3A4 gene.

The above results are preliminary information on the effect of primycin. The lipid metabolism modulating effect of primycin is clear, but further studies are needed to gain a comprehensive understanding of the complex effect of primycin on human tissue.

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