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Expression profiles of genes related to melatonin and oxidative stress in human renal proximal tubule cells treated with antibiotic amphotericin B and its modified forms

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Abstract: Melatonin protects cells from oxidative stress caused by antibiotics. Through the generation of reactive oxygen species (ROS), amphotericin B (AmB) causes oxidative injuries of the kidneys and liver. Generation of oxidized forms of AmB (AmB-ox) in a patient's circulation may also generate ROS, causing side effects. Studies aimed at elimination of toxic properties of AmB are focused on forming complexes of AmB with copper(II) ions (AmB–Cu^{2+}). However, the influence of such modified forms on renal cells and their transcriptome has not been studied so far. Therefore, the aim of this study was to answer the question of whether AmB–Cu^{2+} complexes and AmB-ox influence the transcriptional activity of melatonin-related and oxidative stress-related genes in human renal proximal tubule epithelial cells (RPTECs) and whether these changes can result in less toxicity of modified forms of AmB. Gene expression profile was evaluated with the use of oligonucleotide microarrays. At high concentrations AmB–Cu^{2+} was two times less toxic than AmB and AmB-ox. AmB–Cu^{2+} caused downregulation of oxidative stress-related genes and *RORA*, and upregulation of *AANAT* and *MTNR1B*. AmB-ox caused similar molecular changes. Based on cytotoxicity tests results and changes in melatonin- and oxidative stress-related genes expression profiles, we conclude that AmB–Cu^{2+} is less toxic for RPTECs.

Key words: Amphotericin B, copper complexes, oxidative stress, melatonin, oligonucleotide microarrays

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized in the central nervous system by the pineal gland, and also in the retina, epithelium, gastrointestinal tract, skin, and immune cells (Bubenik, 2002; Tan et al., 2003; Pandi-Perumal et al., 2008; Slominski et al., 2008; Danielczyk and Dzięgiel, 2009; Lin and Chuang, 2010). It acts through membrane receptors (MT1 and MT2), nuclear receptors (ROR/RZR - retinoid orphan receptors/ retinoid Z receptors), and nonreceptor-mediated mechanisms (Slominski et al., 2012). Melatonin, through its antioxidant properties, protects against damage of cellular components including DNA, cytosolic proteins, and cell membrane lipids (Yürüker et al., 2015). Studies on the antioxidant properties of melatonin are most commonly associated with agents that induce free radicals in cells (Nazıroğlu et al., 2013). Antibiotics also induce the generation of free radicals. One of them, amphotericin B (AmB), causes lipid peroxidation through generation of reactive oxygen species (ROS). AmB belongs to the group of polyene antibiotics and is an effective antifungal drug commonly used to treat systemic mycoses. It is believed that the fungicidal activity is due to the binding of AmB with the ergosterol present in the cell membrane of fungi, resulting in membrane permeabilization and osmotic imbalance (Brajtburg et al., 1990; Paulo et al., 2013). One of the mechanisms leading to fungal cell death is leakage of potassium ions caused by formation of ion channels in the cell membrane as a consequence of AmB binding with ergosterol (Chudzik et al., 2015). This situation leads to the generation of ROS and lipid peroxidation (Mesa-Arango et al., 2012). Unfortunately, AmB, besides its affinity to ergosterol, also shows an affinity to cholesterol present in human cell membranes, causing nephrotoxic and hepatotoxic side effects (Antonowicz-Juchniewicz et al., 2006; Gagoś and Arczewska, 2010; Patel et al. 2011). Nephrotoxic mechanism is not clearly understood; hypothetically, it is caused by induction of oxidative

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stress in renal cells. The cell is provided with a number of enzymatic and nonenzymatic antioxidant systems (Stępniewska et al., 2015). However, in disease conditions increased concentration of free radicals and reduced antioxidant protection causes serious damages in the cell (Lowes et al., 2013).

The aim of numerous studies is to eliminate toxic properties of AmB while maintaining the antifungal activity. One of the methods is to form a complex of AmB with copper(II) ions. Such an AmB–Cu^{2+} complex was shown to be stable at physiological pH and to retain high bioactivity (Gagoś et al., 2011; Chudzik et al., 2013). The reasons for using the copper ions are their fungicidal and bactericidal properties (Spampinato and Leonardi, 2013). These properties are related to the ability of the element to transport electrons, resulting in the formation of free radicals (Chudzik et al., 2013). A hypothesis suggests that the presence of copper ions in the molecule of AmB can increase its antifungal activity in lower concentrations (Chudzik et al., 2015). This could be crucial for lowering the nephrotoxic and hepatotoxic effects of this antibiotic. AmB toxicity may also result from the lipid membrane oxidation caused by free radicals generated during oxidation reaction of the AmB chromophore molecule. Occurrence of such oxidized forms of AmB during therapy cannot be excluded (Gagoś and Czernel, 2014). In the aqueous solution of AmB after addition of Fe^{3+}, redox reactions occur. AmB is oxidized, and the Fe^{3+} ions are reduced to Fe^{2+}. Such a reaction could also take place in a patient's circulation, causing side effects.

Some studies have shown beneficial effects of melatonin on liver condition during chemotherapy (Madhu et al., 2015). It has been shown that melatonin, due to its antioxidant properties, has the ability to reduce the hepatotoxic effects of AmB (Hossain et al., 2013). Studies have also shown that AmB affects the mitochondrial P450 systems. Due to its antioxidant properties melatonin is a potential agent involved in the protection of kidneys from inflammation and oxidative injuries (Slominski et al., 2012). A very important aspect of studies concerning nephrotoxicity of AmB is assessment of gene expression in cells treated with this antibiotic. Such analysis could reveal numerous changes at the transcriptional level, which is the first step of cells' reaction for the presence of AmB. Therefore, the aim of this study was to assess the changes in transcriptional activity of genes involved in the pathway of melatonin and oxidative processes in normal human renal proximal tubule epithelial cells (RPTECs), depending on the modified form of amphotericin B, the AmB–Cu^{2+} complex, and the oxidized form of AmB (AmB-ox, with the use of Fe^{3+}). For this purpose, oligonucleotide microarray analysis was involved.

2. Materials and methods

2.1. Cell culture conditions

Normal human RPTECs were obtained from Lonza (CC-2553, Lonza, Basel, Switzerland) and routinely maintained at 37 °C in a 5% CO_2 incubator (Direct Heat CO_2; Thermo Scientific, Waltham, MA, USA) with the use of a REGM Bullet Kit (CC-3190, Lonza), which contained renal epithelial basal medium (REBM) and supplements and growth factors (SingleQuots). In this study, gentamicin/AmB from SingleQuots was replaced with pure gentamicin 100 µg/mL (17-5182, Lonza).

To test for the effects of AmB on gene expression, different forms of this antibiotic were added to cell culture: amphotericin B (AmB), a complex of AmB with copper(II) ions (AmB–Cu^{2+}), and oxidized forms (AmB-ox). Complexes of amphotericin B with copper(II) ions were prepared as previously described (Gagoś et al., 2011). To obtain oxidized forms, dissolved AmB was diluted in acidic water (pH 2.2) and then incubated with 0.050 mg of iron(III) chloride (per 0.5 mg of AmB) for 165 min at room temperature. The reaction was stopped with the use of 1 mg of bathophenanthrolinedisulfonic acid disodium salt trihydrate (Fluka-Sigma-Aldrich). The final concentration of AmB was designated by cytotoxicity test with the use of the sulforhodamine B colorimetric assay (TOX6-1KT; Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's instruction. Cytotoxicity test was performed on cells after 24 h of incubation with modified forms of AmB at three concentrations: 50, 5, and 0.5 µg/mL. The control cells were not treated with AmB.

RPTECs were treated with modified forms of amphotericin B for 6 h. Controls were left untreated. After incubation cells were harvested and stored for RNA extraction.

2.2. Extraction of total RNA

Total RNA was extracted from harvested cells with the use of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Extracts of total RNA were purified with the use of the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and treated with DNAse I (Fermentas International Inc., Burlington, ON, Canada) according to the manufacturer's protocol. The RNA concentration was determined with the use of a Gene Quant II spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, UK). Agarose gel electrophoresis was performed to check the quality of RNA extracts.

2.3. Oligonucleotide microarray analysis.

Total RNA was reversely transcribed and used for the synthesis of biotinylated aRNA. Obtained aRNA was fragmented and subsequently hybridized with the HG-U133A 2.0 (Affymetrix, Santa Clara, CA, USA). The whole procedure was performed with the use of a GeneChip 3' IVT

Express Kit (Affymetrix) according to the manufacturer's instructions. Staining with streptavidin-FITC was carried out with the use of a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) according to the manufacturer's instructions. Gene Chip Scanner 3000 7G and GeneChip Command Console Software (Affymetrix) were used to measure fluorescence intensity.

2.4. Statistical analysis

Microarray data analysis was performed with the use of the GeneSpring 12.6.1 platform (Agilent Technologies, Inc., Santa Clara, CA, USA) and PL-Grid Infrastructure. For finding significant genes, comparative analysis was performed with the use of one-way ANOVA testing with Benjamini–Hochberg multiple testing correction and the Tukey HSD post hoc test. Genes were considered as differentiating when $P < 0.05$ and $FC \geq 1.0$ (fold change).

3. Results

3.1. Cytotoxicity measurement

At a concentration of 50 µg/mL, significant growth inhibition of cells treated with AmB and oxidized AmB was observed, while % of growth inhibition of cells treated with the AmB–Cu^{2+} complex was two times lower (Figure 1). At a concentration of 0.5 µg/mL there was no growth inhibition of cells treated with AmB and its modified forms. This concentration was chosen for transcriptome analysis.

3.2. Transcriptome analysis

Sixty-six ID mRNAs of melatonin-related genes and 67 ID mRNAs of oxidative stress-related genes were found in the Affymetrix database (http://www.affymetrix.com). One-way ANOVA testing with Benjamini–Hochberg multiple testing correction showed 13 differentiating transcripts of melatonin-related genes and 10 transcripts of oxidative stress-related genes ($P < 0.05$; Table 1).

In order to identify differentially expressed genes in cells treated with AmB, AmB–Cu^{2+} complexes and AmB-ox transcriptomes of those cells were compared to controls. Analysis was performed with the use of the Tukey HSD post hoc test ($P < 0.05$ and $FC \geq 1.0$). In the transcriptome of AmB-treated cells 2 ID mRNAs of melatonin-related genes and 2 ID mRNAs of oxidative stress-related genes were differentiated (Table 2). Those ID mRNAs revealed downregulation of the *RGS4* gene and overexpression of *STC2* and *SOD2* genes. The AmB–Cu^{2+} complexes caused overexpression of *AANAT*, *MTNR1B*, and *GPLD1* genes, while *RGS4*, *AKAP17A*, *PER2*, and *RORA* genes were downregulated (Table 3). All differentiated oxidative stress-related genes had low expression levels (Table 3). In the transcriptome of cells treated with AmB-ox only the *GPLD1* gene was overexpressed, while *RGS4* and *RORA* were downregulated (Table 4). Like in the cells treated with AmB–Cu^{2+} complexes, all differentiated oxidative stress-related genes had low expression levels.

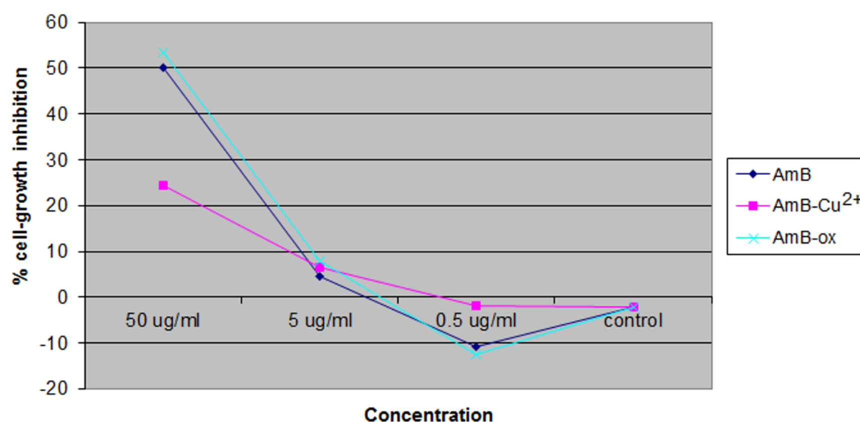

Figure 1. Percentage of cell-growth inhibition for cells treated with amphotericin B (AmB), AmB–Cu^{2+} complex (AmB-Cu^{2+}), and oxidized form of AmB (AmB-ox).

Table 1. The results of one-way ANOVA tests with Benjamini–Hochberg multiple testing correction showing 13 differentiating melatonin-related genes and 10 differentiating oxidative stress-related genes at $P < 0.05$.

Corrected P-value	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.005	P < 0.001
Melatonin-related genes	66	13	8	5	3	1
Oxidative stress-related genes	67	10	5	4	2	0

Table 2. Tukey HSD post hoc test results showing differentiating transcripts for cells treated with AmB in comparison to control cells.

ID	Gene symbol	Change (up/down)	Gene name	P	FC
Melatonin-related genes					
204337_at	*RGS4*	↓	Regulator of G-protein signaling 4	0.0034546426	−1.3174056
204339_s_at	*RGS4*	↓	Regulator of G-protein signaling 4	0.0011204381	−1.2426155
Oxidative stress-related genes					
203438_at	*STC2*	↑	Stanniocalcin 2	0.0059375763	1.2551361
221477_s_at	*SOD2*	↑	Superoxide dismutase 2, mitochondrial	0.0000287455	1.1200539

Table 3. Tukey HSD post hoc test results showing differentiating transcripts for cells treated with AmB–Cu^{2+} complexes in comparison to control cells.

ID	Gene symbol	Change (up/down)	Gene name	P	FC
Melatonin-related genes					
207225_at	*AANAT*	↑	Aralkylamine N-acetyltransferase	0.023507113	1.2842025
208516_at	*MTNR1B*	↑	Melatonin receptor 1B	0.037811667	1.3687539
206264_at	*GPLD1*	↑	Glycosylphosphatidylinositol specific phospholipase D1	0.017869767	1.266195
203624_at	*AKAP17A*	↓	A kinase (PRKA) anchor protein 17A	0.013379096	−1.1427889
205251_at	*PER2*	↓	Period circadian clock 2	0.04186887	−1.4827319
210426_x_at	*RORA*	↓	RAR-related orphan receptor A	0.005400353	−1.4796749
210479_s_at	*RORA*	↓	RAR-related orphan receptor A	0.009972014	−1.3813231
204337_at	*RGS4*	↓	Regulator of G-protein signaling 4	0.003454642	−1.3461816
Oxidative stress-related genes					
200845_s_at	*PRDX6*	↓	Peroxiredoxin 6	0.004685244	−1.0349842
201427_s_at	*SEPP1*	↓	Selenoprotein P	0.001458089	−1.1224521
208791_at	*CLU*	↓	Clusterin	0.000298733	−1.2282754
208792_s_at	*CLU*	↓	Clusterin	0.000130129	−1.2043961
208998_at	*UCP2*	↓	Mitochondrial uncoupling protein 2	0.002017573	−1.4034905
221477_s_at	*SOD2*	↓	Superoxide dismutase 2, mitochondrial	0.000028745	−1.125971
222043_at	*CLU*	↓	Clusterin	0.000442488	−1.4138653
33494_at	*ETFDH*	↓	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	0.006946974	−1.2046279
39729_at	*PRDX2*	↓	Peroxiredoxin 2	0.004766464	−1.132459

Table 4. Tukey HSD post hoc test results showing differentiating transcripts for cells treated with oxidized forms of AmB in comparison to control cells.

ID	Gene symbol	Change (up/down)	Gene name	P	FC
Melatonin-related genes					
206264_at	*GPLD1*	↑	Glycosylphosphatidylinositol specific phospholipase D1	0.017869767	1.2028623
210479_s_at	*RORA*	↓	RAR-related orphan receptor A	0.009972014	–1.3710381
204337_at	*RGS4*	↓	Regulator of G-protein signaling 4	0.003454642	–1.4980948
204339_s_at	*RGS4*	↓	Regulator of G-protein signaling 4	0.001120438	–1.2057906
Oxidative stress-related genes					
208791_at	*CLU*	↓	Clusterin	0.000298733	–1.1869079
208792_s_at	*CLU*	↓	Clusterin	0.000130129	–1.1686915
208998_at	*UCP2*	↓	Mitochondrial uncoupling protein 2	0.002017573	–1.2075838
221477_s_at	*SOD2*	↓	Superoxide dismutase 2, mitochondrial	0.000028745	–1.1824647
222043_at	*CLU*	↓	Clusterin	0.000442488	–1.1993396

For finding which genes were common for all analyzed transcriptomes and which were characteristic only for a particular transcriptome, Venn diagrams were used. Transcriptomes of cells treated with AmB, AmB–Cu^{2+} complexes, and AmB-ox were compared to control cells. In the group of melatonin-related genes downregulation of *RGS4* was common for all analyzed transcriptomes; however, one ID mRNA of this gene was common only for AmB and AmB-ox (Figure 2). Two genes were common for cells treated with AmB–Cu^{2+} complexes and AmB-ox: *GPLD1* was upregulated and *RORA* was downregulated. For cells treated with AmB–Cu^{2+} complexes, *AANAT*, *MTNR1B*, *AKAP17A*, *PER2*, and *RORA* genes were characteristic.

In the group of oxidative stress-related genes, *SOD2* was common for all analyzed transcriptomes, but the direction of changes (up/down) was not the same for all of them (Figure 3). For the cells treated with AmB

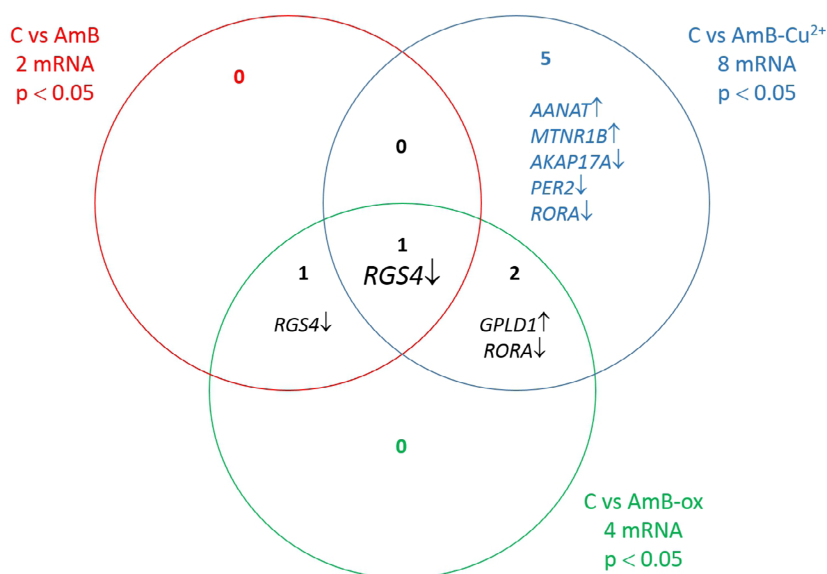

Figure 2. Venn diagram showing number of melatonin-related genes' transcripts differentiating in comparison to control cells (C) common for groups of transcriptomes: cells treated with amphotericin B (AmB), cells treated with amphotericin B–Cu^{2+} complexes (AmB-Cu^{2+}), and cells treated with an oxidized form of amphotericin B (AmB-ox).

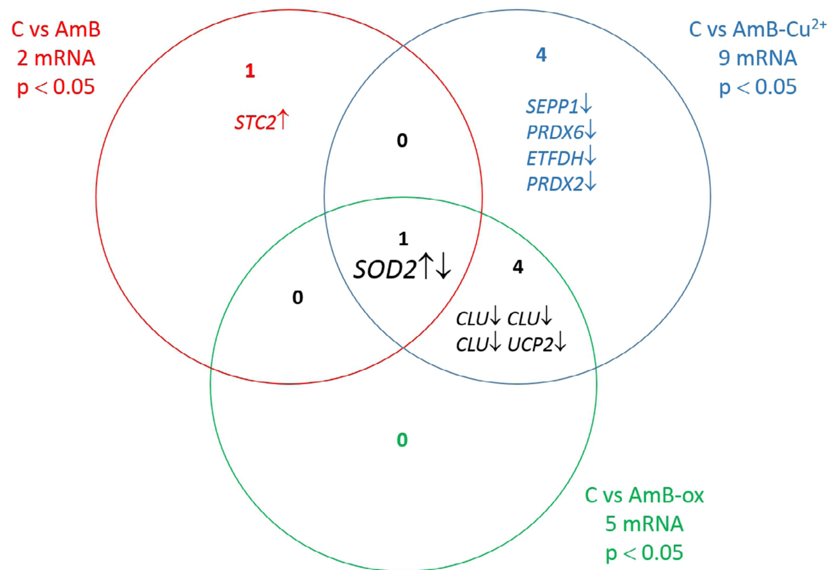

Figure 3. Venn diagram showing number of oxidative stress-related genes' transcripts differentiating in comparison to control cells (C) common for groups of transcriptomes: cells treated with amphotericin B (AmB), cells treated with amphotericin B–Cu^{2+} complexes (AmB-Cu^{2+}), and cells treated with an oxidized form of amphotericin B (AmB-ox).

only the *STC2* gene was characteristic. There were no differentiating genes common for AmB-treated cells and other transcriptomes. For cells treated with AmB–Cu^{2+} complexes and AmB-ox, *CLU* and *UCP* were mutual. For cells treated with AmB–Cu^{2+} complexes, *SEPP1*, *PRDX2*, *PRDX6*, and *ETFDH* genes were characteristic.

4. Discussion

The use of antibiotics is one of the greatest achievements of the twentieth century. Despite their all-around use, a large group of antibiotics have side effects. AmB belongs to the group of antibiotics having serious side effects, acting directly on the kidneys and liver. Therefore, it is essential to understand the mechanisms responsible for the formation of side effects and to create a form of the drug safe for the patient while maintaining its strong antifungal properties. Already in the mid-1980s, the relationship between AmB and oxidative processes was documented, based on its influence on increase of malondialdehyde concentration, a product of peroxidation (Brajtburg et al., 1985). Simultaneous treatment of erythrocytes with AmB and antioxidant substances inhibited their lysis (Brajtburg et al., 1985). However, the influence of modified forms of AmB such as AmB–Cu^{2+} and AmB-ox on oxidative processes in human renal cells has not been studied until now.

Elimination of toxic properties of AmB while maintaining its antifungal activity can be achieved by forming complexes of AmB with copper(II) ions (Gagoś et al., 2011). Previous studies showed that such modifications improved fungicidal activity of AmB (Chudzik et al., 2013). The question was how such complexes can influence human cells. Thus, in our research we assessed cytotoxicity of AmB and complexes of AmB with copper ions. Moreover, concerning the possibility that toxicity may be caused by oxidized forms of AmB, we also assessed influence on human cells of oxidized forms of AmB. The research model constituted human RPTECs. Cytotoxicity tests showed that at a high concentration (50 µg/mL) AmB–Cu^{2+} complexes were two times less toxic compared to AmB and oxidized AmB, which significantly inhibited cell growth (about 50%). This result gives optimism for retaining fungicidal activity with lowered nephrotoxicity of AmB by addition of copper ions.

The next phase of our research was to assess how AmB and its modified forms influence gene expression at the transcriptional level. The transcriptome is a dynamic system and changes quickly (within hours) in response to variations in cells' environment. These alternations cause changes in cells' phenotype, and thus analysis of the transcriptome can help in determination of the direction for further research. We assessed changes in the transcriptome of cells treated with AmB and its modified forms with the use of oligonucleotide microarrays. This technology allows us to assess changes of thousands of genes at the transcription level in one analysis. To achieve a sufficient number of living cells and to obtain high RNA amounts for transcriptome analysis with the use of

oligonucleotide microarrays, we lowered the dosage of the antibiotic (0.5 µg/mL) for this step.

Previous research showed that use of melatonin significantly reduces drug-induced nephrotoxicity caused by oxidative stress. Such a mechanism was described for amikacin (Parlakpinar et al., 2003), vancomycin (Celik et al., 2005), gentamicin (Ozbek et al., 2005), methotrexate (Abraham et al., 2010), AmB (Lin and Chuang, 2010), and doxorubicin (Hrenák et al., 2013). At physiological concentrations (1 to 100 mM) melatonin affected the expression of genes involved in oxidative stress in cells treated with AmB (Lin and Chuang, 2010). To answer the question of whether AmB and its modified forms influence expression of genes responsible for oxidative imbalance regulation, we assessed changes of melatonin-related and oxidative stress-related genes at the transcriptional level. The sets of genes were proposed by the Affymetrix database. Analysis of transcriptomes of cells treated with AmB revealed overexpression of two genes, *STC2* and *SOD2*. The product of the *STC2* gene is involved in calcium homeostasis and also in the regulation of oxidative stress (Fazio et al., 2011). Ito et al. (2004) showed that induction of hypoxic or endoplasmic reticulum stress causes upregulation of *STC2* expression in N2a neuroblastoma cells. The *SOD2* gene encodes the antioxidant enzyme superoxide dismutase 2. This enzyme converts superoxide into hydrogen peroxide and diatomic oxygen. It was also showed that AmB induces increase of *SOD2* gene expression (Lin and Chuang, 2010). Increased expression of *SOD2* may indicate a defensive reaction of RPTECs to AmB-induced oxidative stress. From the set of genes related to melatonin, only the *RGS4* (regulator of G-protein signaling 4) gene was differentiating. This gene encodes a negative modulator of expression of G protein-coupled receptors (Ji et al., 2011). RGS4 protein also modulates melatonin receptor MT1 function (Witt-Enderby et al., 2004). Dupré et al. showed that *RGS4* is a melatonin-responsive gene and its expression increases after melatonin treatment (Dupré et al., 2011). However, this research concerned photoperiodic response mechanism in the pars tuberalis. Little is known about the influence of melatonin on *RGS4* expression in oxidative stress conditions caused by antibiotics.

Cells treated with AmB–Cu^{2+} complexes revealed many more changes in their transcriptome. All differentiating oxidative stress-related genes were downregulated. One of them, the *CLU* gene, encodes clusterin. This protein was shown to be involved in induction of apoptosis in RPTECs (Sansanwal et al., 2015). Silencing of this gene causes inhibition of apoptosis, indicating that clusterin plays a crucial role in the renal cell injury mechanism (Sansanwal et al., 2015). Among downregulated genes in cells treated with AmB–Cu^{2+} complexes were also two genes that were upregulated in cells treated with AmB: *STC2* and *SOD2*. This result may indicate the lack of necessity of activation of antioxidant enzymes in cells treated with AmB– Cu^{2+} complexes. Perhaps this is related to their lower toxicity and generation of free radicals in small amounts compared to AmB. As shown by Lin and Chuang (2010), melatonin reduces the effects of AmB-induced increase in *SOD2* gene expression. In our study we did not treat cells with melatonin. From the set of melatonin-related genes *AANAT*, *MTNR1B*, and *GPLD1* were upregulated. *AANAT* encodes N-acetyltransferase, which converts serotonin to *N*-acetylserotonin, the precursor of melatonin. Increase of *AANAT* expression may indicate an increase of melatonin synthesis in the human RPTECs after treatment with AmB–Cu^{2+} complexes. Upregulation of *MTNR1B* gene expression (encoding MT2) may also point out melatonin synthesis in these cells. Increase of expression was also observed in the case of the *GPLD1* gene which encodes glycosylphosphatidylinositol-specific phospholipase D1. This protein is capable of phosphoinositol hydrolysis of proteins anchored in the cell membrane, causing their release. This is associated with the transmission and generation of cellular signals. Other differentiating melatonin-related genes were downregulated, including *RGS4* (like in the cells treated with AmB), *AKAP17A*, *PER2*, and *RORA*. The *AKAP17A* gene encodes the XE7 protein, an alternative splicing regulator (Mangs et al., 2006). *PER2* belongs to the Period family of genes and its expression was shown to be downregulated in rat's anterior pituitary after treatment with cadmium (Jimenez-Ortega et al., 2012). It is probable that the presence of copper ions in our experiment induced such an effect. *RORA* expression was also downregulated. This gene encodes nuclear receptor RORα. This is a transcriptional factor responsible for regulation of genes involved in circadian rhythm. Ram et al. (2002) showed that melatonin suppresses *RORA* expression at the transcriptional level. Observed activation of *AANAT* and *MTNR1B* genes along with downregulation of *RORA* suggests that RPTECs after stimulation with AmB–Cu^{2+} complexes may synthesize melatonin. It is possible that some copper ions not sequestered by AmB were present during our experiment. Melatonin forms a complex with copper, preventing production of free radicals and their prooxidant activity (Romero et al., 2014). Thus, the presence of copper ions could cause stimulation of antioxidative stress mechanisms, including synthesis of melatonin and its receptor. However, these mechanisms should also include activation of oxidative stress-related genes. In our experiment, these genes were downregulated.

Oxidation reaction of the AmB chromophore molecule may cause generation of free radicals and hence side effects during therapy (Gagoś and Czernel, 2014). We obtained oxidized forms of AmB by reaction with Fe^{3+}

ions. We expected that these forms should reveal stronger toxic effects, but results of toxicity tests revealed toxic effects comparable with AmB. Surprisingly, analysis of transcriptomes at lower concentrations revealed changes similar to those observed after treatment with AmB–Cu^{2+} complexes. Oxidative stress-related genes were downregulated; however, the set of genes was poorer than that observed in AmB–Cu^{2+}-treated cells. The most characteristic downregulated gene for both modified forms of AmB was *SOD2*. Other common changes for both modified AmB forms were *GPLD1* (overexpression) and *RORA* (downregulated). These results indicate that at lower concentrations both modified forms have weaker toxic effects than AmB. Probably AmB–Cu^{2+} complexes additionally cause stimulation of melatonin synthesis, which at higher concentrations of the drug form protects cells from oxidative damage, while AmB and AmB-ox cause oxidative stress leading to cytotoxic effects. The molecular mechanism underlying this phenomenon needs further research at both RNA and protein levels. Additionally, oxidant and antioxidant analyses should be performed to clarify the molecular influence of AmB–Cu^{2+} and AmB-ox on renal cells.

Influence of modified forms of AmB (AmB–Cu^{2+} and AmB-ox) on renal cells and their transcriptome has not been studied so far. We conclude that observed downregulation of oxidative stress-related genes and *RORA* along with activation of *AANAT* and *MTNR1B* genes may indicate that AmB–Cu^{2+} complexes are less toxic for RPTECs. Results of cytotoxicity tests confirm this hypothesis.

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