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PRACA ORYGINALNA
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Changes in expression of genes related to caspases and BCL-2 family in RPTEC treated with amphotericin B and its modified forms

Zmiany w ekspresji genów kodujących białka związane z aktywnością kaspaz oraz białka z rodziny BCL-2 w komórkach RPTEC traktowanych amfoterycyną B i jej modyfikowanymi formami

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ABSTRACT

INTRODUCTION: The main limitation of the use of amphotericin B (AmB) – effective in the treatment of systemic fungal infections – is its high toxicity to human cells. The mechanism of AmB toxicity is not clear. Caspase-related and BCL-2 proteins participate in the regulation of apoptosis. Thus, they may be involved in drug toxicity. In this study we evaluated the influence of AmB on the transcriptional activity of genes related to caspases and the BCL-2 family. We also tested the influence of modified forms of AmB: AmB-Cu^{2+} (the complex with copper(II) ions) and the AmB-ox (oxidized form).

MATERIAL AND METHODS: Human RPTECs (Renal Proximal Tubule Epithelial Cells) were treated with AmB, AmB-Cu^{2+} and AmB-ox. Total RNA was extracted using the phenol-chloroform method. The expression profiles of genes related to caspase activity and BCL-2 were determined using oligonucleotide microarrays (HG-U133A 2.0, Affymetrix). Analysis included 67 ID related to caspases and 32 ID associated with BCL-2, according to the Affymetrix database.

RESULTS: The analysis revealed upregulation of the BCL-2 and BCL2L1genes in the cells treated with AmB-Cu^{2+}, in comparison to the control. In both the AmB and AmB-Cu^{2+} -treated cells, differentiating genes were associated with inflammation and mitophagy activated by intrinsic signals. In the cells treated with AmB-ox, the BCL-2 genes were downregulated.

CONCLUSIONS: The results suggest that AmB and AmB-Cu^{2+} activate genes involved in the regulation of inflammation and autophagy induced by intrinsic signals, but overexpression of BCL-2 and BCL2L1 may protect AmB-Cu^{2+}--treated cells from death. In the cells treated with AmB-ox extrinsic signals prevail, indicating the distinct molecular mechanism of its cytotoxicity.

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KEY WORDS
caspases, BCL-2, amphotericin B, copper complexes, oligonucleotide microarrays

STRESZCZENIE

WSTĘP: Głównym ograniczeniem stosowania amfoterycyny B (AmB) – skutecznej w leczeniu grzybic układowych – jest jej wysoka toksyczność wobec komórek ludzkich. Mechanizm cytotoksyczności nie został wyjaśniony. Białka związane z aktywnością kaspaz oraz białka należące do rodziny BCL-2 uczestniczą w regulacji apoptozy, mogą być zatem zaangażowane w procesy odpowiedzialne za toksyczność leku. W pracy oceniono wpływ AmB na aktywność transkrypcyjną genów kodujących białka związane z aktywnością kaspaz oraz białka z rodziny BCL-2. Zbadano również wpływ modyfikowanych form AmB: AmB-Cu^{2+} (kompleks z jonami miedzi (II)) i AmB-ox (formy utlenione).
MATERIAŁ I METODY: Ludzkie komórki RPTECs (Human Renal Proximal Tubule Epithelial Cells) inkubowano z AmB, AmB-Cu^{2+} i AmB-ox. Całkowity RNA wyekstrahowano metodą fenolowo-chloroformową. Profil ekspresji genów wyznaczono techniką mikromacierzy oligonukleotydowych (HG-U133A 2.0, Affymetrix). Analiza obejmowała 67 ID genów związanych z aktywnością kaspaz i 32 ID geny kodujące białka z rodziny BCL-2, zaproponowane przez bazę Affymetrix.
WYNIKI: Analiza wykazała nadekspresję genów *BCL-2* i *BCL2L1* w komórkach traktowanych AmB-Cu^{2+}, w porównaniu z kontrolą. Zarówno w komórkach traktowanych AmB, jak i AmB-Cu^{2+} geny różnicujące związane były z zapaleniem i mitofagią aktywowanymi w odpowiedzi na sygnały wewnątrzkomórkowe. W komórkach traktowanych AmB-ox geny z rodziny BCL-2 były wyciszone.
WNIOSKI: Wyniki sugerują, że AmB i AmB-Cu^{2+} aktywują geny zaangażowane w regulację zapalenia i mitofagii aktywowanych sygnałami wewnątrzkomórkowymi, jednak nadekspresja genów *BCL-2* i *BCL2L1* może chronić komórki traktowane AmB-Cu^{2+} przed śmiercią. W komórkach traktowanych AmB-ox przeważa sygnał zewnątrzkomórkowy, co wskazuje na odrębny mechanizm cytotoksyczności tej formy antybiotyku.

SŁOWA KLUCZOWE
kaspazy, BCL-2, amfoterycyna B, kompleksy miedzi, mikromacierze oligonukleotydowe

INTRODUCTION

Caspases are intracellular enzymes belonging to the group of cysteine proteases. Up to now, 17 different mammalian proteins belonging to this family have been identified. [1]. Depending on the function, pro--apoptotic (caspase-2, -3, -6, -7, -8, -9, -10) and pro-inflammatory caspases (caspase -1, -4, -5, -11, -12) are recognized [2, 3]. The division of pro-apoptotic caspases is based on their role in apoptosis: initiator (-2,-8,-9,-10) and effector caspases (-3,-6,-7) [2,3,4]. The role of initiator caspases rely primarily on the initiation of this process and the activation of effector caspases. They hydrolyze structural and functional proteins necessary for proper functioning of the cell, resulting in morphological changes leading to its death. Thus, caspases are key enzymes in the process of apoptosis [5]. However, the initialization of this process depends on many factors, including the activation of genes encoding proteins of the BCL-2 family (B-cell CLL/lymphoma 2), both pro- and anti-apoptotic [6]. These membrane proteins regulate mitochondrial membrane permeability. In response to the induction of apoptosis, pro-apoptotic proteins facilitate the formation of pores in the mitochondrial membrane, and then release cytochrome c and other pro-apoptotic factors to the cytosol. Anti-apoptotic proteins inhibit this process by binding pro-apoptotic proteins. There are three classes of BCL-2 family proteins: 1) anti-apoptotic, having four BH (BCL-2 homology) domains (1-4): BCL-2, BCL2L1/BCL-XL (BCL2 like 1), BCL2L2/BCL-W (BCL2 like 2), MCL-1 (myeloid cell leukemia sequence 1) and BCL2A1/BFL-1 (BCL2 related protein A1); 2) pro--apoptotic, strongly interacting with anti-apoptotic proteins, with three BH domains: BAX (BCL-2-associated X protein), BAK (BCL-2-antagonist/killer-1), BOK (BCL-2 related ovarian killer); 3) BH3-only proteins, pro-apoptotic, homologous to BCL-2 only within the BH3 domain: BIM (BCL-2 interacting mediator), PUMA (p53 upregulated modulator of apoptosis), BAD (BCL-2 associated death promoter), BID (BH3 interacting domain death agonist), BIK (BCL-2 interacting killer), BMF (BCL-2 modifying factor), HRK (Hara-kiri) and NOXA (from Latin: damage) [7].

Both, caspase-related and BCL-2 proteins, due to participation in the regulation of apoptosis, may be involved in processes related to drug toxicity. It has been found, among others, that caspases are activated

during renal cell injury in response to antibiotics [8,9]. One of most important drugs used for systemic fungal infection treatment is amphotericin B (AmB) – a natural macrolide antibiotic produced mainly by actinomycetes of the genus *Streptomyces*. It is characterized by a broad spectrum of activity, including *Cladosporium cladosporioides* [10]. The main limitation of the use of amphotericin B, however, is its high toxicity to human cells. One of the proposed mechanisms of the nephrotoxicity of amphotericin B is the induction of oxidative stress [11]. However, the mechanism of AmB toxicity has not been clearly elucidated. Whether the antibiotic affects the expression of genes encoding caspase-related and BCL-2 family proteins has not been examined. The expression of BCL-2, BAX and BAK proteins is constitutive [7], while genes encoding BH3-only proteins are activated in response to pro-apoptotic signals. Therefore, their transcriptional activity may indicate initiation of the process. In this study we have examined the influence of amphotericin B on the transcriptional activity of genes encoding caspase-related and BCL-2 proteins. Additionally, we studied the influence of modified forms of AmB: the complex of AmB with copper(II) ions (AmB-Cu^{2+}) and the oxidized form of AmB (AmB-ox). The first of them is less toxic for RPTECs while maintaining its antifungal activity [11,12]. Oxidized forms of AmB may occur in the patient's circulation during therapy, causing the observed side effects [13].

MATERIAL AND METHODS

Cell culture conditions

Normal human Renal Proximal Tubule Epithelial Cells (RPTEC) (CC-2553, Lonza, Basel, Switzerland) were grown at 37°C in a 5% CO_2 incubator (Direct Heat CO_2; Thermo Scientific, Waltham, MA, USA), using the REGM Bullet Kit (Lonza). SingleQuots (containing gentamicin and amphotericin-B) was replaced with 100 μg/mL of pure gentamicin (Lonza). The cells were treated with: amphotericin B (AmB) (Sigma-Aldrich, St. Louis, MO, USA), amphotericin B copper (II) complex (AmB–Cu^{2+}) and the oxidized forms (AmB-ox) were prepared as previously reported [11,14]. The concentration of antibiotics and the time of incubation were as previously described [11]. After the drug incubation was completed, the cells were immediately subjected to RNA extraction.

Total RNA extraction

The extraction of total RNA was done using the TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA extracts were purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance to the producer's protocol. Quantitative assessment was carried out using a GeneQuant II spectrophotometer (Pharmacia Biotech, Cambridge, UK). The RNA integrity was evaluated with 0.8% agarose gel electrophoresis stained with ethidium bromide (Sigma-Aldrich).

Oligonucleotide microarray procedure

All the steps of the microarray procedure were performed according to the producer of the oligonucleotide microarray HGU133A 2.0 (Affymetrix, Santa Clara, CA, USA). The synthesis of biotinylated cRNA, and its fragmentation was performed using a GeneChip 3' IVT Express Kit (Affymetrix). Hybridization with the microarray and staining of hybridized cRNA with streptavidin-FITC was carried out by means of a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix), according to the manufacturer's instructions. Fluorescence intensity was measured using a Gene Chip Scanner 3000 7G and GeneChip Command Console Software (Affymetrix).

Statistical analysis

The microarray data was analyzed using the GeneSpring 13.0 platform (Agilent Technologies, Inc., Santa Clara, CA, USA) and PL-Grid Infrastructure. To find significant genes, one-way ANOVA with Benjamini–Hochberg multiple testing correction followed by the Tukey HSD post hoc test were used ($P < 0.05$ and $FC \geq 1.1$ - fold change). The set of genes related to caspases and BCL-2 was proposed by the Affymetrix database (http://www.affymetrix.com).

RESULTS

Expression profile of genes related to caspases

The analysis of 67 ID mRNA of genes encoding proteins related to caspases showed 7 mRNA differentiating examined groups in comparison to the control (Table I). In the cells treated with AmB, two genes were upregulated: *CARD14* and *CASP5*, while *MALT1* gene was downregulated. In the cells treated with AmB-Cu^{2+}, an increase in *CARD14*, *CASP5*, *NLRP1* and *CAAP1* was noted. The *CASP9* and *MALT1* genes were downregulated in these cells. The cells treated with AmB-ox showed upregulation of *CFLAR* and downregulation of *MALT1*.
ANOVA with Benjamini-Hochberg correction did not confirm differentiating genes.

Expression profile of BCL-2 family genes

The analysis of 37 ID mRNA of genes encoding BCL-2 proteins by ANOVA with Benjamini-Hochberg correction showed 7 ID differentiating mRNA (table II). In both the cells treated with pure amphotericin B and in those treated with AmB-Cu^{2+}, all the differentiating mRNAs were upregulated whereas in the AmB-ox treated cells all these genes were downregulated. They were: *TP53BP2*, *BNIP3L* in the AmB, and *TP53BP2*, *BNIP3L*, *BNIP3*, *BCL-2*, *BCL2L1*, *MCL1* in the AmB-Cu^{2+}-treated cells. Treatment with AmB-ox caused a decrease in *BNIP3L*, *BNIP3* (2 ID mRNA) and *BCL-2*.

Table I. Changes in expression of genes related to caspases
Tabela I. Zmiany w ekspresji genów kodujących białka związane z aktywnością kaspaz

Probe Set ID	Gene Symbol	Gene name	AmB vs C		AmB-Cu^{2+} vs C		AmB-ox vs C	
			P	change	P	change	P	change
207500_at	*CASP5*	*caspase 5*	0.0030	↑	0.0030	↑		
210017_at	*MALT1*	*MALT1 paracaspase*	0.0242	↓	0.0242	↓	0.0242	↓
220599_s_at	*CARD14*	*caspase recruitment domain family member 14*	0.0123	↑	0.0123	↑		
203984_s_at	*CASP9*	*caspase 9*			0.0210	↓		
211822_s_at	*NLRP1*	*NLR family pyrin domain containing 1*			0.0148	↑		
219276_x_at	*CAAP1*	*caspase activity and apoptosis inhibitor 1*			0.0478	↓		
210564_x_at	*CFLAR*	*CASP8 and FADD like apoptosis regulator*					0.0230	↑

Table II. Changes in expression of genes related to BCL-2
Tabela II. Zmiany w ekspresji genów kodujących białka z rodziny BCL-2

Probe Set ID	Gene Symbol	Gene name	AmB vs C		AmB-Cu^{2+} vs C		AmB-ox vs C	
			P	change	P	change	P	change
203120_at	*TP53BP2*	*tumor protein p53 binding protein 2*	0.0001	↑	0.0001	↑		
221478_at	*BNIP3L*	*BCL2/adenovirus E1B 19kDa interacting protein 3-like*	0.0001	↑	0.0001	↑	0.0001	↓
201848_s_at	*BNIP3*	*BCL2/adenovirus E1B 19kDa interacting protein 3*			0.0063	↑	0.0064	↓
201849_at	*BNIP3*	*BCL2/adenovirus E1B 19kDa interacting protein 3*					0.0053	↓
203685_at	*BCL-2*	*B-cell CLL/lymphoma 2*			0.0019	↑	0.0019	↓
212312_at	*BCL2L1*	*BCL2 like 1*			0.0030	↑		
200797_s_at	*MCL1*	*Myeloid cell leukemia sequence 1*			0.0015	↑		

DISCUSSION

Apoptosis is a process crucial for the functioning of a multicellular organism, both in physiological (development of the organism, functioning of the immune system), and pathological processes (irreversible cell damage) [15,16]. It may, therefore, be one of the mechanisms responsible for damage to kidney cells during therapy with amphotericin B. Recent reports indicate, among others, participation of the PKA pathway (protein kinase A) in the death of the cells treated with AmB, both *Candida albicans* [18] and kidney cells [18]. However, the effect of the antibiotic on the expression profile of pro- and anti-apoptotic genes in renal cells has not been studied so far, particularly those that are associated with intrinsic (mitochondrial) apoptotic pathway activation. The DNA fragmentation in the kidney cells observed by Franco et al. may indicate both apoptosis and necrosis [18]. Therefore, in this work we undertook a study to determine whether the expression profile of genes encoding caspase-related and BCL-2 proteins changes under the influence of AmB and its modified forms. A premise for the study was the literature data indicating that only part of this group of genes is constitutive, while others are activated in response to pro-apoptotic agents [7]. Recent studies showed that AmB and its modified forms at lower concentrations did not cause significant cytotoxicity. At higher doses an increase in the cytotoxic effect was observed for AmB and AmB-ox, while in cells treated with AmBCu^{2+} the

effect was much weaker [11]. In addition, transcriptome analysis showed that AmBCu^{2+} downregulates the activity of genes encoding enzymes whose expression is activated in response to oxidative stress. Furthermore, in these cells genes associated with the melatonin pathway have been activated, suggesting that additional mechanisms responsible for cell protection would be launched. Oxidative stress affects intracellular signaling, including the signal pathways related to apoptosis [19], Thus, in cells exposed to its action the expression of pro-apoptotic genes may increase.

Microarray analysis of the transcriptomes of RPTEC treated with AmB and its modified forms revealed a few changes in the activity of genes encoding caspase-related and BCL-2 proteins. Moreover, in the case of genes encoding proteins related to caspases, unlike those encoding BCL-2 proteins, the Benjamini-Hochberg correction did not confirm the statistical significance of differentially expressed genes. Statistical analysis of the results obtained by the oligonucleotide microarray technique may identify distinct differentially expressed genes, depending on the statistical method [20]. The Benjamini-Hochberg correction permits the exclusion of false positive results, increasing the precision of indicating statistically significant differences in a gene expression [21]. On the other hand, the rejection of uncertain results may cause an omission of changes that may be important from the biological point of view [21]. Therefore, the results of the analysis of genes encoding caspase-related proteins should be treated with caution, as a premise to undertake further research in which the use of different experiment conditions (concentration and incubation time) will more accurately determine the importance of these genes in the molecular mechanism of cytotoxicity of the tested drugs. However, the obtained results, in combination with a group of genes encoding BCL-2 proteins, have a biological sense and enable better understanding of the observed effects of AmB and its modified forms. The cells treated with AmB showed an increase in the *CARD14/CARMA2* gene. Recent studies, in addition to the basic long form (CARMA2FL – full length), have proven the existence of two alternatively spliced variants of mRNA: the shorter variant (CARMA2*sh* – short) and a variant lacking the CARD domain (CARD2cardless – *cl*) [22]. The *sh* variant, like the *FL* variant, activates NFκB (nuclear factor kappa B). Scudiero et al. have suggested that the *sh* and *cl* variants, due to their structure, may be involved exclusively in the transduction of intracellular signalization [22]. In addition, they showed the influence of both isoforms on apoptosis induced by endoplasmic stress caused by thapsigargin and tunicamycin. In our study the increase in activity of this gene appears to confirm our previous results which indicate the initiation of oxidative stress in cells treated with AmB [11]. In addition, there was activation of the *TP53BP2* and *BNIP3L* genes, whose products are involved in the regulation of autophagy [23,24]. Autophagy is a process in which a cell gets rid of large and potentially toxic structures, and also performs "recycling" of damaged proteins, nucleic acids, fatty acids, ATP and eliminates dysfunctional mitochondria (mitophagy - autophagy of mitochondria) [24]. Therefore, this is a defense mechanism against damaging factors. Furthermore, in the cells treated with AmB an increase in *CASP5* and decrease in the *MALT1* gene was noted. The exact function of caspase-5 is not yet fully understood, but its presence together with the pro-inflammatory caspase-1 in inflammasome NLRP1/NALP1 speaks for the fact that it is involved in the inflammatory process and regulated by proinflammatory factors such as lipopolysaccharide and interferon gamma [25,26]. In turn, the downregulated *MALT1* gene encodes a scaffold protein that plays a role in signal transduction [27]. In addition, it has a catalytic activity (hence termed paracaspase) and is involved in the regulation of NFκB and JNK pathways (c-Jun N-terminal kinase) [27]. Recent studies have shown that CARD14 interacts with MALT1 activating its proteolytic activity and leading to the expression of proinflammatory cytokines in keratinocytes [28]. The activation of proinflammatory cytokines is one of the proposed mechanisms of AmB nephrotoxicity [17,29]. Therefore, the decrease in *MALT1* gene activity with a simultaneous increase in *CASP5* and *CARD14* expression may indicate the activation of stress and proinflammatory pathways by intrinsic signals.

In the cells treated with AmB-Cu^{2+}, as in the cells treated with AmB, the activity of the *CARD14*, *CASP5*, *TP53BP2* and *BNIP3L* genes increased, while the activity of the *MALT1* gene decreased. However, other changes in the gene expression occurred – a decrease in *CASP9* and *CAAP1*, and an increase in *NLRP1*, *BNIP3*, *BCL-2*, *BCL2L1*, *MCL1*. Caspase-9 in conjunction with cytochrome c released from the mitochondria is part of the apoptosome and is a key enzyme activating the intrinsic (mitochondrial) apoptotic pathway [30]. CAAP1 blocks apoptosis by inhibiting caspases -3, -8, -9 and -10 [31]. The *NLRP1* gene is activated (at transcript and protein levels) in response to endoplasmic reticulum stress [32]. The autoproteolytic cleavage of NLRP1 leads to the activation of inflammasome and proinflammatory caspase-1, resulting in the activation of proinflammatory cytokines [33]. Thus, our results suggest that there is no activation of genes related to pro-apoptotic caspases in cells treated with AmB-Cu^{2+}. On the one hand, the increase in the activity of *NLRP1*, *CASP5* and *CARD14* genes can indicate the activation of processes damaging kidney cells through the induction of proinflammatory genes. On the other hand, anti-apoptotic

BCL-2 and BCL2L1, interacting via the loop regions with leucine-rich repeats - LRR of the NLRP1 protein, can block its activation and oligomerization, thereby actuating the additional protection mechanism of cells during stress [34]. In our research we have found overexpression of *BCL-2* and *BCL2L1*, which may indicate that at the protein level blocking of the inflammasome formation may occur. Genes *BNIP3* and *BNIP3L* were also upregulated. The proteins encoded by these genes are mediators of hypoxia-induced mitophagy and their transcriptional activity increases rapidly in response to the oxygen decline and is regulated by NFκB [24]. The expression of BNIP3 and BNIP3L proteins is associated with non-apoptotic cell death in response to stress [24]. According to Deegan et al., cellular stress leads to the activation of autophagy rather than apoptosis, and this process depends on the presence of activated caspase-9 [35]. Recent studies have demonstrated high levels of BNIP3 and BNIP3L in many normal tissues in which there was no activation of cell death [24]. It was also demonstrated that these proteins interact with BCL-2 and BCL2L1 [36]. Moreover, BCL2L1 and MCL1 block the ubiquitination of mitochondrial proteins, which may result in blocking mitophagy [24]. In our studies we have found increased activity of the *TP53BP2* gene, whose product can induce or block autophagy, depending on the cell type [23]. Thus, the results suggest that AmB-Cu^{2+} activates genes involved in the regulation of inflammation and autophagy. This may confirm the increase in oxidative stress in the examined cells, but in contrast to cells treated with AmB, molecular mechanisms that prevent cell damage there are activated. Therefore, high concentrations of AmB-Cu^{2+} were not as toxic as AmB.

In cells treated with AmB-ox there was a decrease in the expression of the *MALT1*, *BNIP3*, *BNIP3L* and *BCL-2* genes. Simultaneously, there was increased activity of the *CFLAR* gene. This gene encodes a homologue of caspase-8 and blocks its pro-apoptotic activity in response to receptor signalization [37]. These results suggest that inducing the extrinsic pro-apoptotic pathway and an increase in *CFLAR* expression may be a defense mechanism against cell death. This demonstrates that in cells treated with AmB-ox, extrinsic signals may prevail so that the molecular mechanism of the observed cytotoxicity of AmB-ox at higher concentrations [11] is different from that observed in cells treated with AmB and AmB-Cu^{2+}.

CONCLUSIONS

Cells treated with AmB and AmB-Cu^{2+} show changes associated with the regulation of intrinsic pathways related to inflammation and autophagy in response to stress. There was no increase in the activity of genes encoding pro-apoptotic caspases, however, the experiment did not include studies of caspases at the protein level. Thus, on the basis of these results, the induction of apoptosis cannot be ruled out. Changes in the gene expression profile of cells treated with AmB-ox may indicate the activation of apoptosis via receptor pathways. These results are a premise for further studies that will be aimed at a detailed explanation of the mechanisms responsible for the observed differences in the response of renal cells to amphotericin B and its modified forms.

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No conflict of interest has been declared by the authors.

Author's contribution

Study design – J. Gola
Data collection – B. Strzałka-Mrozik, C. Kruszniewska-Rajs, M. Gagoś
Data interpretation – J. Gola, K. Simka, U. Mazurek
Statistical analysis – J. Gola
Manuscript preparation – J. Gola, K. Simka
Literature research – K. Simka, J. Gola
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