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Doxycycline increases prednisolone sensitivity in childhood acute lymphoblastic leukaemia

Doksycyklina zwiększa wrażliwość na prednizolon w ostrej białaczce limfoblastycznej u dzieci

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Glucocorticoids are the most important group of drugs in the therapy of childhood acute lymphoblastic leukaemia (ALL), and resistance of lymphoblasts to prednisolone both *in vitro* and *in vivo* is one of the most important adverse prognostic factors. We tested the hypothesis that the tetracycline antibiotic doxycycline (DCC), which inhibits protein synthesis and is known as having properties to upregulate expression of glucocorticoid receptor and having no anti-leukaemic activity, can modulate the response to prednisolone in childhood ALL.

Samples of 60 childhood ALL and 12 children with normal marrow cells (NMC) were tested for sensitivity to prednisolone and doxycycline by the MTT assay and for cell cycle analysis by flow cytometry.

DCC itself caused cell death in 15% of lymphoblasts, but not in NMC. When combined with prednisolone, DCC caused increase of its cytotoxicity in ALL median 2.17-fold, but not in NMC. In 13 cases of ALL samples, sensitization exceeded the value 1000, and in 14 cases, antagonism between these two compounds was observed. Sensitization was higher in relapsed patients, but no correlation was observed between *ex vivo* resistance of lymphoblasts to prednisolone and modulatory effect of DCC.

In summary, we conclude that DCC might modulate prednisolone cytotoxicity in childhood ALL. This is possibly due to positive modulation of expression and function of glucocorticoid receptor; however, other mechanisms of cellular drug resistance might also contribute to this phenomenon.

Key words: prednisolone, drug resistance, sensitivity, glucocorticoids.

Introduction

The tetracycline antibiotic doxycycline (DCC), which inhibits protein synthesis, can upregulate expression of glucocorticoid receptor [1, 2]. DCC also increases expression of inhibitor of cyclin-dependent kinase $p16^{INK4A}$ protein, which hypophosphorylates pRb, causes cell cycle arrest in G0/G1 phase, inhibits proliferation and upregulates expression of GR [3, 4]. Therapy with DCC leads to eradication of T-cell leukaemia in the rat, due to the fact that the cellular membrane of T lymphocytes, but neither B lymphocytes nor erythroid cells, is permeable for tetracyclines [3]. DCC causes apoptosis in the Jurkat cell line, mediated by FAS/FAS-L (i.e. via activation of receptor CD95) [5]. DCC in concentration 50 μg/ml is cytotoxic and leads to apoptosis in monocytic cell lines (human histiocytic lymphoma U937 and mouse macrophage cell line RAW264) [6].

DCC induces expression of IRF-2 oncogene (interferon regulatory factor 2) and as a result, selectively upregulates expression of FAS-L, B1 cyclins and p27 protein, which is a CDK inhibitor, leading to cell cycle arrest in S and G2 phases [7]. DCC in therapeutic concentrations of 1-5 μg/ml inhibits function of activated B lymphocytes, which suppresses immunoglobulin secretion and inhibits matrix metalloproteinase in extracellular matrix [8]. In cell line K562 (pTet-on/p53) and in neuroepithelial cells, DCC increases expression of p53 protein, p21 protein and $p27^{Kip1}$ protein, decreases expression of dihydrofolate reductase and causes cell cycle arrest in G0/G1 phase, lowering the percentage of cells in S phase [9-11]. DCC induces CDK inhibitor gene $p15^{INK4B}$ in human melanoma cell line, causing cell cycle arrest in G0/G1 phase [12], induces $p16^{INK4A}$ in CCRF-CEM cell line [4], and, like dexamethasone, induces $p57^{Kip2}$ protein in HeLa cell line [13] and regulates expression of c-myc [14]. In CCRF-CEM cell line, DCC by induction of $p16^{INK4A}$ sensitizes leukaemic cells to apoptosis in the presence of glucocorticoids in physiological concentrations [4]. By regulating expression of c-myc, tetracyclines increase apoptosis induced by glucocorticoids [14].

Since doxycycline might upregulate expression of glucocorticoid receptor [1, 2], the aim of this study was to analyse the modulating effect of doxycycline on prednisolone cytotoxicity in childhood acute lymphoblastic leukaemia.

Material and methods

Patient samples

Leukaemic samples were obtained from 60 children with ALL aged 0.1-17.2 years (median 7.8 years), including 46 initial and 14 relapsed patients. Fresh lymphoblasts obtained from bone marrow were isolated by Ficoll

Glikokortykoidy są najważniejszą grupą leków stosowanych w terapii ostrej białaczki limfoblastycznej (ALL) u dzieci, jednak oporność limfoblastów na prednizolon zarówno *ex vivo* oraz *in vivo* jest aktualnie jednym z najsilniejszych niekorzystnych czynników rokowniczych. Celem pracy jest weryfikacja hipotezy, czy doksycyklina, antybiotyk z grupy tetracyklin, który ma właściwości hamowania syntezy białek oraz zwiększa ekspresję receptora glikokortykoidowego, lecz nie ma właściwości leku przeciwbiałaczkowego, może modulować *ex vivo* odpowiedź na prednizolon w ALL u dzieci.
Próbki 60 dzieci z ALL oraz 12 dzieci z prawidłowym szpikiem kostnym (*normal marrow cells* – NMC) poddano badaniu na wrażliwość *ex vivo* na prednizolon i doksycyklinę, stosując test MTT oraz analizie cyklu komórkowego metodą cytometrii przepływowej.
Doksycyklina powodowała zniszczenie 15% limfoblastów, nie wpływała natomiast na żywotność NMC. W skojarzeniu z prednizolonem, doksycyklina powodowowała wzrost jego cytotoksyczności 2,17-krotnie (mediana) w komórkach ALL, lecz nie w NMC. W 13 próbkach ALL, wskaźnik uwrażliwienia (komórek ALL na prednizolon) przekroczył 1000, lecz w 14 przypadkach obserwowano antagonizm pomiędzy tymi dwoma lekami w stosunku do komórek ALL. Wskaźnik uwrażliwienia był wyższy u pacjentów we wznowie ALL, jednak nie wykazano korelacji pomiędzy opornością limfoblastów *ex vivo* na prednizolon a efektem modulacyjnym doksycykliny.
Podsumowując, doksycyklina może modulować cytotoksyczność prednizolonu w dziecięcej ALL. Zjawisko modulacji jest związane najprawdopodobniej ze zwiększeniem ekspresji i funkcji receptora glikokortykoidowego, jednak inne mechanizmy oporności komórkowej mogą odgrywać znaczącą rolę.

Słowa kluczowe: prednizolon, oporność na cytostatyki, wrażliwość, glikokortykoidy.

gradient. Only samples with at least 90% of lymphoblasts were included in the study. Also 12 children with normal bone marrow cells (NMC) were included. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients and their parents.

Chemicals

Prednisolone (Fenicort, Polfa, Jelenia Gora, Poland) was tested in concentration 0.0076-250 μg/ml. Doxycycline (Doxycycline hydrochloride, Polfa, Tarchomin, Poland) was dissolved in 0.9% NaCl, kept in stock in concentration of 1.0 mM in –20°C. Its effect on normal marrow cells was tested in concentrations 12.5-100 μM, and it was used in final concentration of 20 μM in all experiments with prednisolone.

Viability assay

In vitro drug sensitivity to prednisolone, doxycycline and their combination was done by the MTT assay, as described previously [15]. The value of *ex vivo* resistance was expressed by the IC50 value, which is the concentration of drug inhibitory to 50% of tested cells after 72 hours of incubation.

Modulation of prednisolone cytotoxicity

Leukaemic cells were treated *in vitro* with prednisolone and/or doxycycline. The effect was analyzed by the percentage of cells surviving in the highest concentration of prednisolone ± doxycycline. Control cell survival was assumed as 100%. Sensitization factor (SF) was calculated as the ratio of IC50 for prednisolone divided by IC50 for combination of prednisolone and doxycycline (after correction of doxycycline cytotoxicity).

Cell cycle analysis

For DNA content analysis, cells were stained with hypotonic propidium iodine solution (concentration 20 μg/ml, Sigma, No. P4170), dissolved in citric buffer and Triton X-100. Cells were incubated with propidium iodide for 30 minutes at room temperature in the dark, and then at least 20000 events were analyzed with an Epics XL flow cytometer (Coulter, Miami, FL, USA) after 24 hours of incubation. This flow cytometer is equipped with an argon laser with an excitation wavelength of 488 nm. Cell cycle was calculated by Multicycle software (Phoenix Flow Systems, San Diego, CA). Percentage of cells in the sub-G1, G0/G1, S and G2/M phases were expressed as mean ± standard deviation.

Statistics

The Mann-Whitney U test was used for unpaired comparisons and the Wilcoxon matched pair test was used for paired comparisons. All reported *p*-values are two-sided; $p < 0.05$ was considered as statistically significant.

Results

Cell survival

Cell survival in the presence of DCC in concentration 20 μM was 97.0 ±3.8% for NMC, and 85.5 ±20.4% for ALL samples (Table 1). No proliferation of leukaemic cells was observed in the presence of prednisolone and DCC in any case of leukaemic cells. Addition of DCC significantly increased prednisolone cytotoxicity in patients with ALL ($p < 0.001$ in Wilcoxon matched pair analysis), but not NMC.

Cytotoxicity *ex vivo*

DCC itself caused cell death in 15% of lymphoblasts, but not in NMC (Table 1). When combined with prednisolone, DCC caused increase of its cytotoxicity in ALL (median SF = 2.17), but not in NMC (Table 2, Figure 1). In

13 cases of ALL samples, SF value exceeded the value 1000, indicating high synergism between prednisolone and DCC. In 14 cases, antagonism between these two compounds was observed, resulting in an SF value below 1 (Figure 2).

DCC increased prednisolone cytotoxicity at relapse 10-fold stronger than on initial diagnosis (ns) (Table 3). There was no difference in effect caused by DCC on PRN with respect to immunophenotype, bcr-abl rearrangement and response to initial therapy (initial 7-day prednisolone monotherapy and end of induction therapy).

Cell cycle

DCC and PRN caused increase of sub-G1 phase, which is a measure of late apoptosis. In combination of PRN+DCC, sub-G1 phase increased by 22.9%. DCC, like prednisolone, caused cell cycle arrest in G0/G1 phase in ALL, but not in NMC. Combination of PRN+DCC did not increase G0/G1 arrest in ALL, caused by prednisolone (Table 4).

Discussion

As glucocorticoids are the most important group of drugs in the therapy of childhood ALL, and resistance of lymphoblasts to prednisolone both in vitro and *in vivo* is one of the most important adverse prognostic factors, we tested the hypothesis that tetracycline antibiotic doxycycline (DCC), which is known as having properties to upregulate expression of glucocorticoid receptor [1, 2] and having no anti-leukaemic activity, can modulate the response to PRN in childhood ALL.

In the group of 60 children diagnosed for ALL, we have shown that DCC has a significant effect in potentiating anti-leukaemic activity of PRN by median 2.17-fold. This effect was not observed in NMC. Large interpatient variability of the modulatory effect of DCC was observed. In spite of significant overall positive modulation of PRN cytotoxicity by DCC, in 14 individual patients we could observe that DCC decreased sensitivity to PRN. On the other hand, in 13 patients, DCC increased sensitivity of leukaemic cells to prednisolone by more than 1000-fold, which suggests very strong upregulation of glucocorticoid receptor during 72 hours of *ex vivo* therapy.

In the cell cycle analysis, in most cases DCC caused increase of sub-G1 phase and cell cycle arrest. Glucocorticoids exert phase-dependent cytotoxicity [16] and in most we did not observe any modulation of DCC on cell cycle arrest caused by PRN, regardless of the impact of DCC

Table 1. Cell survival in the highest concentration of prednisolone and/or doxycycline
Tabela 1. *Przeżycie komórek w największym stężeniu prednizolonu i/lub doksycykliny*

	ALL	NMC
PRN	25.6 ±16.7*	44.2 ±22.1
DCC	85.5 ±20.4	96.0 ±4.8
PRN+DCC	23.4 ±22.6*	43.5 ±26.3

*Cell survival is presented as mean ± SD in %. # p < 0.05, * p < 0.01 for ALL samples in comparison to NMC*

Table 2. Effect of doxycycline on prednisolone cytotoxicity in patient samples
Tabela 2. *Efekt doksycykliny na cytotoksyczność prednizolonu w próbkach pacjentów*

	ALL	NMC	*p*
PRN	26.4 (0.02->250)	229.0 (139.2->250)	< 0.001
PRN+DCC	10.34 (0.01->250)	220.8 (120.7->250)	< 0.001
SF	2.17 (0.4-24949)	1.03 (0.8-1.22)	< 0.001
p(W)	0.001	0.333	

Values for PRN ± DCC are represented by median and range of IC50 given in μg/ml. SF (DCC) – median and range of sensitization factors IC50 (PRN)/IC50(PRN+DCC) for each tested patient sample. p = p-value tested by Mann-Whitney test, p(W) = p-value between IC50 (PRN) and IC50 (PRN + DCC) tested by Wilcoxon matched pair test

Table 3. Profile of modulation of PRN cytotoxicity by DCC in ALL *de novo* and at relapse
Tabela 3. *Profil modulacji cytotoksyczności prednizolonu przez doksycyklinę w ALL de novo i we wznowie*

	ALL *de novo*	ALL relapse	*p*
PRN	10.7 (0.02->250)	140.6 (9.0-219.8)	0.015
SF	1.32 (0.4-12287)	13.6 (0.7-24494)	0.142

Values are represented by median and range. p – value, Mann-Whitney U test

Table 4. Influence of doxycycline ± prednisolone on cell cycle
Tabela 4. *Wpływ doksycykliny ± prednizolonu na cykl komórkowy*

	Control cells after 24 hours	PRN	DCC	PRN + DCC
ALL	91.9 ±4.6	97.5 ±2.9*	96.5 ±3.9*	96.7 ±3.2*
	7.4 ±4.4	2.5 ±2.7	2.7 ±3.2	3.2 ±2.9
	0.7 ±1.0	0.1 ±0.6	0.9 ±2.5	0.3 ±1.2
	15.2 ±13.2	33.2 ±21.0	29.7 ±21.1	40.8 ±21.4
NMC	91.2 ±4.1	92.5 ±5.2	93.9 ±4.3	93.7 ±4.7
	7.6 ±3.5	5.7 ±3.7	4.8 ±3.6	5.3 ±3.9
	0.8 ±1.5	1.7 ±1.7	1.3 ±1.3	0.9 ±1.3
	7.9 ±8.0	24.1 ±8.9	24.2 ±14.5	29.3 ±7.9

Values show percentage of cells (mean ±SD) in phases: G0/G1, S, G2/M and sub-G1. For each sample, apoptotic sub-G1 phase was calculated initially; and then all other living cells in G0/G1, S and G2/M phases were counted as 100%. (*) p < 0.01 when respective value is compared to G0/G1 phase of control cells (Wilcoxon matched pair test)

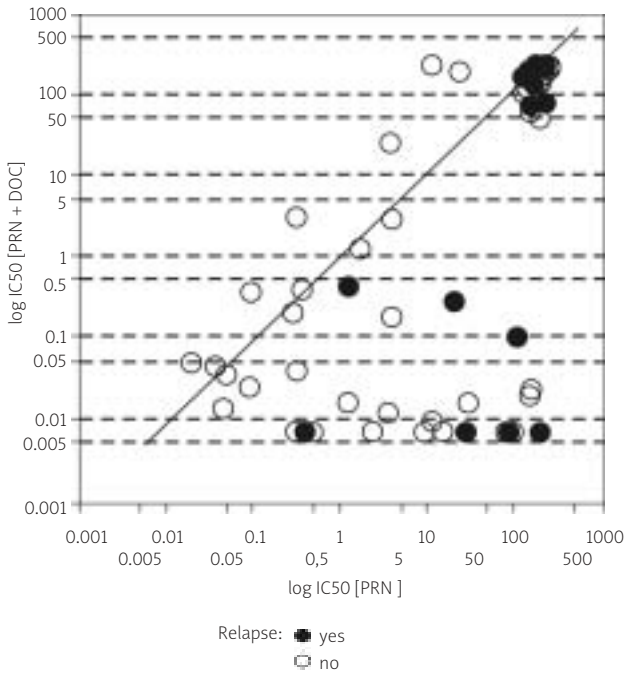

Fig. 1. Values of IC50 for PRN and PRN+DCC with respect to relapse during follow-up. Line indicates points y = x
Ryc. 1. *Wartości IC50 dla PRN + DCC z uwzględnieniem wznowy w trakcie obserwacji. Linia pokazuje wartości y = x*

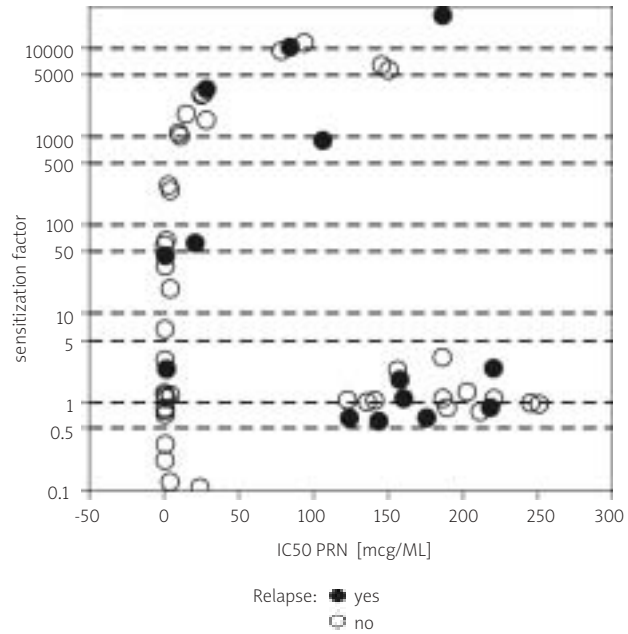

Fig. 2. Modulation of prednisolone cytotoxicity by doxycycline expressed by sensitization factor (SF), with respect to relapse during follow-up. SF > 1 indicates increase in cytotoxicity, while SF < 1 indicates antagonism between prednisolone and doxycycline
Ryc. 2. *Modulacja cytotoksyczności przez doksycyklinę wyrażoną jako wskaźnik uwrażliwienia (SF) z uwzględnieniem wznowy w czasie dalszej obserwacji pacjentów. Wartość SF > 1 oznacza wzrost cytotoksyczności, natomiast SF < 1 wskazuje na antagonizm pomiędzy prednizolonem i doksycykliną*

on PRN cytotoxicity and changes caused by DCC in the sub-G1 phase and cell cycle. In no single case of human samples did DCC increase the percentage of cells in G0/G1 phase after PRN *ex vivo* therapy, which indicates antagonism between DCC and PRN in their influence on the cell cycle. This phenomenon is related to the mechanism of prednisolone activity on the cell cycle, which inhibits cells in G1 phase and causes relative insensitivity of cells to other phase-specific drugs [16].

DCC caused significant sensitization of leukaemic cells to prednisolone cytotoxicity, and itself was cytotoxic against cell lines. It seems that addition of doxycycline significantly increases cytotoxicity of prednisolone in childhood leukaemias. Doxycycline is an antibiotic currently not often used in clinical practice in oncology. However, as more and more resistant bacterial strains occur with sensitivity to this compound, its use during anti-leukaemic therapy is possible. If this is the case, one should be aware of the possibility of increase of the anti-leukaemic effect of prednisolone. This might be mainly due to augmentation of function of the glucocorticoid receptor, but other mechanisms may possibly play a role. As a stronger lympholytic effect of glucocorticoids was observed during extended inhibition of protein synthesis in mitochondria [17], doxycycline can positively modulate glucocorticoid activity by this mechanism. Doxycycline is an agent with multidirectional activity, which in mammalian cells causes changes related to proliferation, migration and apoptosis. Doxycycline is an inhibitor of metalloproteinases MMP-2 and MMP-9 in vivo in the arterial wall, and an inhibitor of proliferation of smooth muscle cells after arterial wall injury (e.g. after balloon angioplasty) [6, 18]. A moderate toxic effect of doxycycline, as well as colistin, ciprofloxacin and tobramycin, on epithelial cells in patients with chronic lung infections was observed [19]. This effect was not observed after use of amphotericin, ceftazidime, chloramphenicol, imipenem, meropenem, piperacillin, sulfamethoxazole/trimethoprim or vancomycin [19]. Doxycycline inhibits production of mitochondrial cytochrome C oxidase [20], which can contribute to the function of these cellular organelles and activation of the mitochondrial apoptotic pathway.

In summary, in spite of the fact that a group of patients was identified for whom antagonism between prednisolone and doxycycline was observed, we might conclude that doxycycline significantly modulates prednisolone cytotoxicity in childhood ALL. This is possibly due to positive modulation of expression and function of the glucocorticoid receptor. However, other mechanisms might also contribute to this phenomenon.

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