Expression of p53 protein and the histomorphological view of epidermis in experimental animals after cladribine application

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(Received 2 Jul 2013; revised version 9 Nov 2013; accepted 24 Nov 2013)

Summary

Cladribine acts as an antineoplastic, immunosuppressive and anti-inflammatory agent. The aim of this study was to examine the possible impact of this drug on the epidermis of Wistar rats. The control group received standard feed and water. The experimental group I was treated with cladribine in a schema corresponding to the treatment of multiple sclerosis in humans. The material for microscopic and immunohistochemical evaluation was obtained 24 h after administration of the last dose of the drug. The drug in experimental group II was administered in the same way as experimental group I, but test material was downloaded following a 4 week break after the administration of the last dose of the drug. After the completion of the experiment, skin samples were taken from all rats for histomorphological and immunohistochemical studies. An analysis of experimental group I H&E samples showed visible changes in epidermis patterns, and the.tissue layer was found to be much thinner compared to the analogous structures in the control group. The difference in p53 expressions between the control and both experimental groups was found to be statistically significant ($P<0.0001$). Cladribine administration induces changes in the images of the epidermis of experimental animals. Based on morphological analysis and p53 protein expression, it can be concluded that the 4-week break in drug administration contributed to the regeneration of the rats' skin epithelial tissue.

Key words: Cladribine, Epidermis, Apoptosis, p53 protein, Caspase 3

Introduction

Cladribine (2-CdA, 2-chloro-2'deoxyadenosine) is a purine nucleoside analog (Robak *et al*., 2006, 2009; Spurgeon *et al*., 2009; Wawryk-Gawda *et al*., 2011), acting as an antineoplastic, immunosuppressive and anti-inflammatory agent (Filippi *et al*., 2000; Rice *et al*., 2000; Pérez-Gálan *et al*., 2002; Leist and Vermersch, 2007; Leist and Weissert, 2011; Tadmor, 2011).

2-CdA is equally toxic to dividing and non-dividing cells. In dividing cells, cladribine is incorporated into the DNA strand, thus inhibiting the activity of the enzymes which take part in DNA synthesis. These changes lead to disturbances in cell functions and cause cell death. In non-dividing cells, 2-CdA interferes with the balance of deoxinucleotids and causes endonuclease activation. Finally, it leads to the death of the cell by way of the apoptosis process. So far, two apoptotic pathways induced by 2-CdA have been reported (Nomura *et al*., 2000; Ceruti *et al*., 2003; Takahashi *et al*., 2006; Laugel *et al*., 2011). The apoptosis is controlled by the protein p53 system, in connection with the Bcl-2 protein family (Tyliński *et al*., 2003). Cladribine plays a major role in the activation of p53, leading to apoptosis (Bielak-Żmijewska, 2003; Budanov and Karin, 2008). Since accessible bibliographies contain little information regarding the activity of cladribine on the skin, the aim of our study was to analyze the morphology of the epidermis of experimental animals given 2-CdA in doses used for treating sclerosis multiplex.

Numerous experimental works demonstrate the drug activity mechanism to be based on apoptosis induction, thus an experimental analysis of caspase 3 (the main executive enzyme) was conducted using immuno-histochemical methods. This was done by analyzing the p53 protein.

Materials and Methods

The experiment was carried out on 15 female Wistar rats with an average body mass of 250-300 g. The animals were placed into three groups of 5 animals each: one control group and two experimental groups. They were handled according to ethical standards set out by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the European Community Council Directive of 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the local ethics committee. To carry out the research, consent No. 126/2001 was obtained from the Local Medical University of Lublin Bioethical Committee.

In addition to standard feed and water, over the course of the experiment, the animals in the control group were injected with physiological salt in quantities corresponding to the drug dosage.

The animals of experimental group I were treated with a dose of 0.07 mg/kg/24 h cladribine (Biodribin, produced by the Institute of Biotechnology and Antibiotics) for six consecutive days in the morning, in 3 cycles with a 5 week gap in the drug's administration (a schema corresponding to the treatment of multiple sclerosis in humans). The drug was administered subcutaneously. The material for microscopic and immunohistochemical evaluation was obtained 24 h after administration of the last dose of the drug.

The drug in experimental group II was administered in the same dose and according to the same scheme as experimental group I, but in order to assess the potential capacity of the recovery of the epidermis, test material was downloaded following a 4 week break after the administration of the last dose of the drug. For a period of 4 weeks, the animals received a granular form of feed and water without restrictions.

After the experiment, the rats were decapitated, and full thickness slices of skin (of a 1 cm^2 area) were taken from all for histomorphological and immunohistochemical study.

Methods

H&E study

Skin samples were fixed in a 4% buffered formalin solution. After dehydration in ethyl alcohol, paraffin blocks were formed, cut on the microtome in 5 µm sections put onto slides and stained with haematoxylin and eosin (H&E).

Immunohistochemical study

An immunohistochemistry reaction was carried out using properly diluted primary antibodies, Sigma®, directed against the tested antigens. For this purpose, p53 protein (MONOCLONAL ANTI-TP53, Clone BP53-12 Antibody Produced in Mouse, dilution: 1:400) and caspase 3 (ANTI-HPA002643-100UL CASP3, Antibody Produced in Rabbit, dilution: 1:300) were used.

All immunohistochemistry reactions were carried out according to the indirect immunoperoxidase method. Paraffin sections were incubated overnight at a temperature of 58°C. In order to block the activity of endogenous peroxidase, the samples were rinsed in a 3% hydrogen peroxide in methanol. Afterwards, antigen channels were unmasked using thermal processes. To block all points which could bind the used antibody, the samples were incubated for 30 min with inactive serum (normal diluted serum). Following this, the material was incubated for 24-h at 8°C with the primary antibodies. The samples were then rinsed in a solution of TBS and mottled by chromogen-3-3´diaminobenzidine (Novolink DAB), which induced a bronze color in the reaction product. At the end of the process, the cell nuclei of the sample were stained by Meyer hematoxylin.

Evaluation of the material was undertaken using DP25 Olympus BX41 digital camera images. Calculations were made in Cell^D.

In order to determine the intensity of the immunohistochemistry reaction, three ×400 vision areas were selected, and three 100 cell-count trials were analyzed. Areas with positive responses were assessed in terms of the intensity of immunohistochemistry expression, 1 (+) being a weak reaction, 2 (++) being the average response and 3 (+++) being a strong reaction. The results of the statistical data were analyzed by Statistica 10.0.

The differences in p53 protein intensity and caspase 3 expressions between the groups were investigated using a Chi-quadrat test. Statistical significance was considered at $P<0.05$.

Results

H&E study

As Fig. 1 shows, the image of the test tissue from the control group appeared to be normal (Fig. 1).

Analyses of the H&E samples from experimental group I showed visible changes in the test tissue pattern; the epidermis layer was much thinner compared to the analogous structures in animals from the control group. One to two layers of keratinocytes were also evident in the epidermis. Cells from the basal layer were loosely arranged and less numerous, while the spinous layer was reduced, consisting of a single layer of cells. There were also areas where the spinous layer was not visible at all (Fig. 2).

In experimental group II, the number of cell layers (2-3) was smaller than that of the control group, but larger than experimental group I (Fig. 3).

Immunohistochemical study

Caspase 3

A significant correlation was found between the control group and experimental group I in their caspase 3 expression ($P<0.0001$). A significant correlation also appeared between both experimental groups ($P<0.0012$). However, the difference between the expression of caspase 3 between the control group and experimental

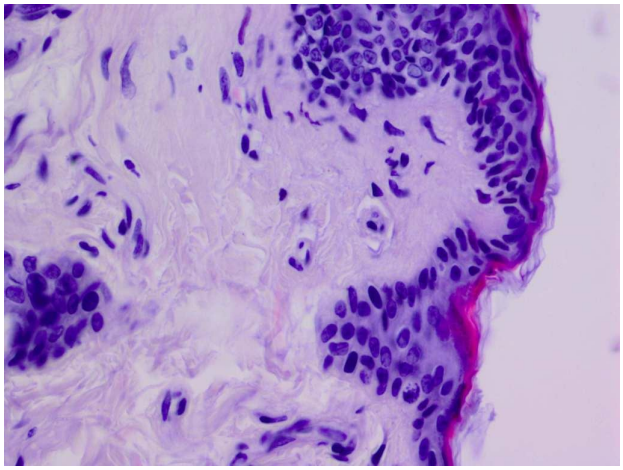

Fig. 1: Epidermal layer in the control group (H&E, ×400)

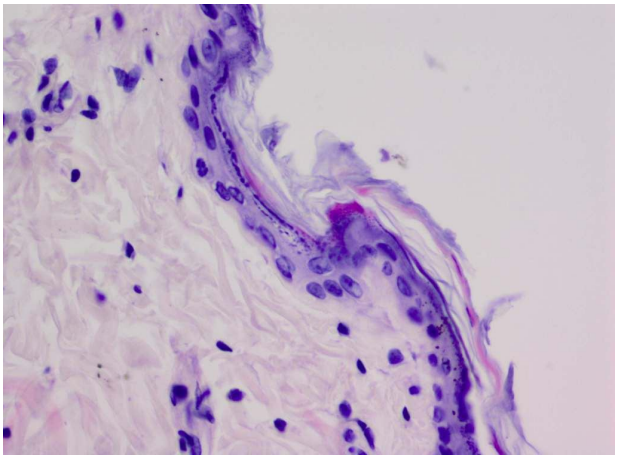

Fig. 2: Thinning of the epidermis in experimental group I. Cells in the basal layer are loosely arranged (H&E, ×400)

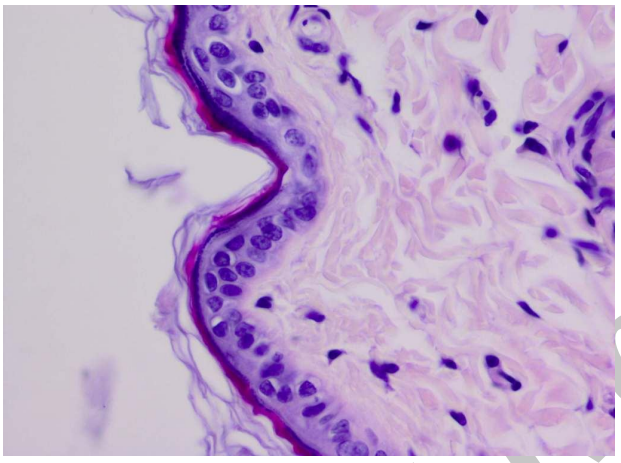

Fig. 3: Epidermis with two or three layers of keratinocytes in the experimental group II (H&E, ×400)

group II was statistically negligible (P<0.4971) (Table 1). No immuno-reactivity was found in the epidermal cells within the samples from any of the research groups: more than 37% of the cells in the control group, in experimental group II, this figure was 35% of the inactive cells, while in experimental group I, the results showed only 23%. The largest number of cells (77%) revealing positive caspase 3 reaction were observed in experimental group I (Fig. 4). In comparison to other groups, as assessed at 3 (+++), in this group, more cells (9%) showed strong expressions of caspase 3 (Table 2).

The control group was dominated by cells with negative IHC caspase 3 reactions located in the spinous and granular epidermal layers. The low expression of caspase 3, assessed at 1 (+) was seen mainly in the cytoplasm of cells in the basal layer of the epidermis (Fig. 5). In experimental group I, positive, strong expressions of caspase 3, assessed at 3 (+++) were observed in the nuclei and cytoplasm of keratinocytes located in the basal epidermal layer (Fig. 6). In experimental group II, expression of caspase 3 was visible in the cytoplasm and nuclei of keratinocytes. The reaction was less intense in comparison to experimental group I. Some cells were also seen with negative IHC reactions (Fig. 7).

P53 protein

The difference in the presence of the expression of p53 between the control group and both experimental groups was found to be statistically significant

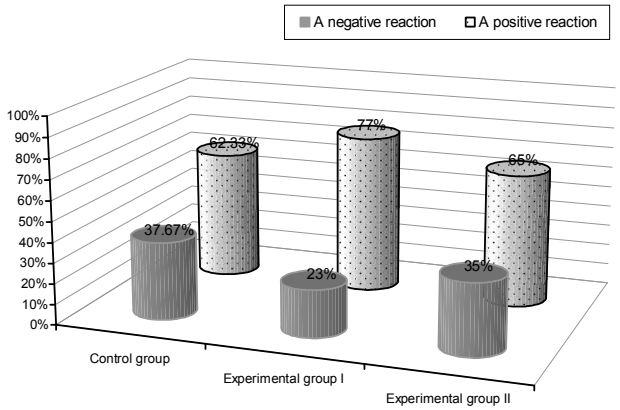

Fig. 4: Expression of caspase 3 in the experimental groups

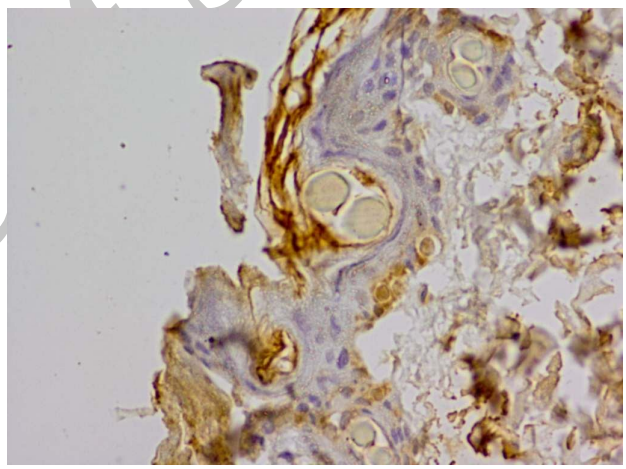

Fig. 5: Control group. Keratinocytes with negative IHC reaction located in the spinous and granular epidermal layers and single basal cells with weak expression of caspase 3 (×400)

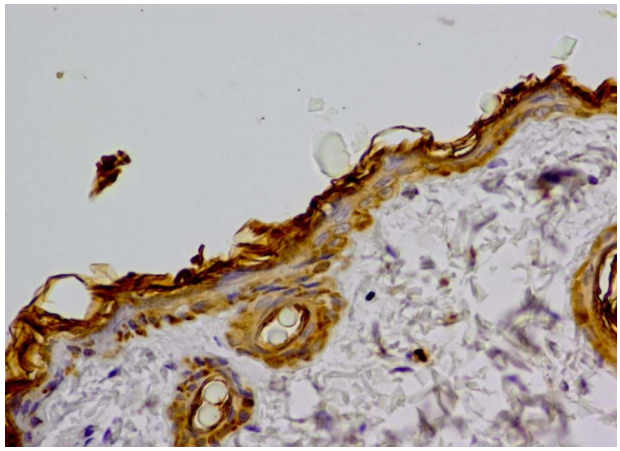

Fig. 6: Experimental group I. Positive, strong IHC expression of caspase 3 in the cytoplasm and nuclei of the epidermal cells (×400)

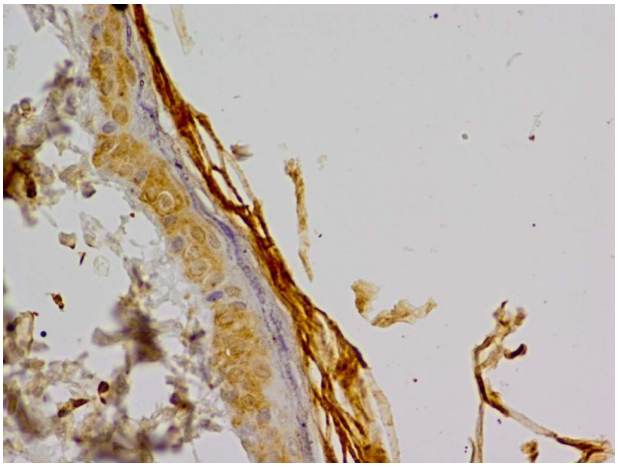

Fig. 7: Experimental group II. Positive expression of caspase 3, assessed at 2 (++), in the cytoplasm and nuclei of epidermal cells (×400)

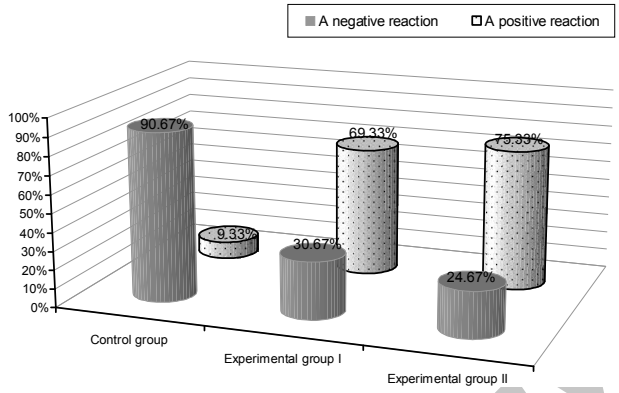

Fig. 8: p53 protein expression in the experimental groups

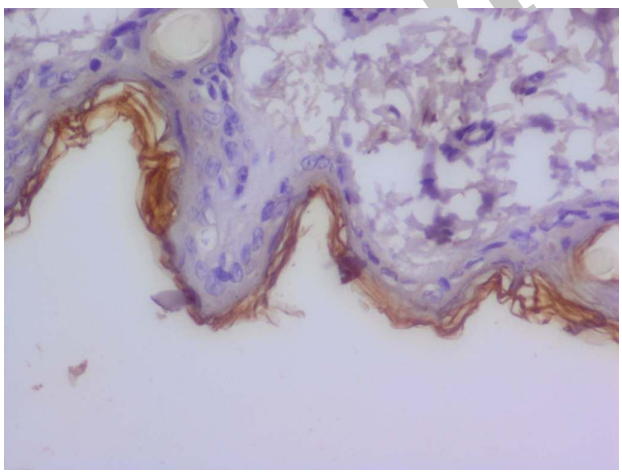

Fig. 9: Control group. Weak expression of p53 protein in the cytoplasm of keratinocytes (×400)

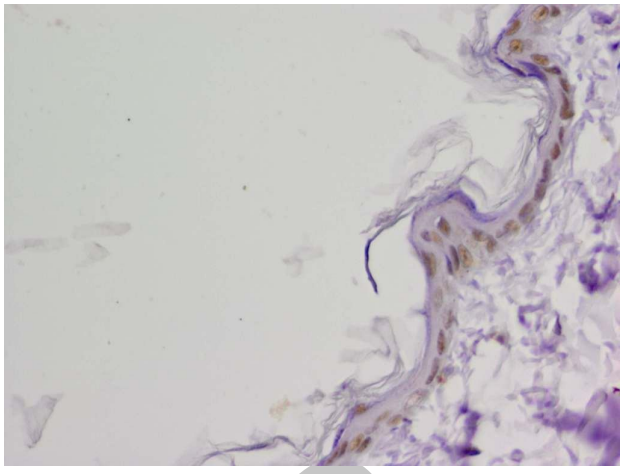

Fig. 10: Experimental group I. A strong, nuclear expression of p53 protein in the cells of the basal layer of the epidermis (×400)

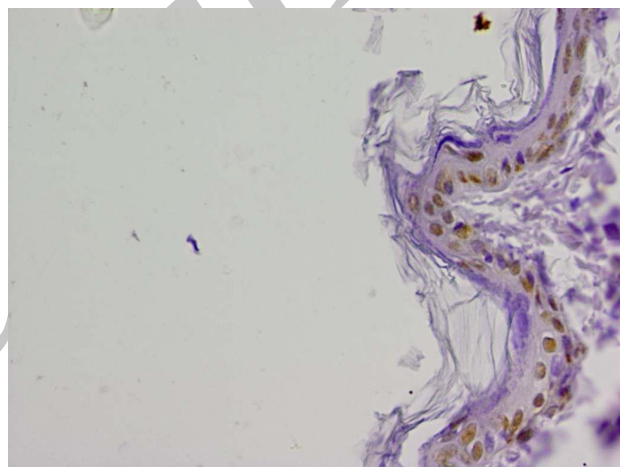

Fig. 11: Experimental group II. A strong, nuclear and cytoplasm expression of p53 protein in the cells of the basal layer of the epidermis (×400)

(P<0.0001), but no correlation was found between experimental groups I and II (P=0.1005) (Table 3). In this respect, in the control group, the majority of the cells showed a negative expression of the p53 protein (over 90%), whereas, in experimental group I, evidence of this trait was found in a little over 30% of all cells examined. In experimental group II, this was over 24% (Fig. 8). In the control group, weak immunohistochemistry responses assessed at 1 (+) appeared only in the cytoplasm of keratinocytes located mainly in the basal layer of the epidermis (Fig. 9). In experimental group I a strong expression of the p53 protein, assessed at 3 (++) appeared in the keratinocytes' nuclei in the basal

Table 1: Correlation between groups for presence of caspase 3 expression

Investigated groups	Control group and experimental group I	Control group and experimental group II	Experimental group I and experimental group II
Chi-quadrat test	P<0.0001	P<0.4971	P<0.0012

Table 2: Expression and intensity of caspase 3 immunohistochemistry reactions

Investigated group		Expression of caspase 3 in epidermis				
		(-)	1 (+)	2 (++)	3 (+++)	Totally
Control group	Number	113	182	5	0	300
	%	37.67%	60.67%	1.67%	0.00%	
Experimental group I	Number	69	83	121	27	300
	%	23.00%	27.67%	40.33%	9.00%	
Experimental group II	Number	105	97	84	14	300
	%	35.00%	32.33%	28.00%	4.67%	

(−) negative reaction, 1 (+) weak reaction, 2 (++) the average response, and 3 (+++) a strong reaction

Table 3: Correlation between groups for presence of p53 protein expression

Investigated groups	Control group and experimental group I	Control group and experimental group II	Experimental group I and experimental group II
Chi-quadrat test	P<0.0001	P<0.0001	P=0.1005

Table 4: Expression and intensity of p53 protein immunohistochemistry reactions

Investigated group		Expression of p53 protein in epidermis				
		(-)	1 (+)	2 (++)	3 (+++)	Totally
Control group	Number	272	28	0	0	300
	%	90.67%	9.33%	0.00%	0.00%	
Experimental group I	Number	92	25	63	120	300
	%	30.67%	8.33%	21.00%	40.00%	
Experimental group II	Number	74	18	42	166	300
	%	24.67%	6.00%	14.00%	55.33%	

(−) negative reaction, 1 (+) weak reaction, 2 (++) the average response, and 3 (+++) a strong reaction

epidermal layer (Fig. 10). Most cells showing strong expressions of the p53 protein (over 55%) were found in sections derived from experimental group II (Table 4). In experimental group II, varying intensities of immunohistochemistry reactions were observed. IHC reaction was visible mainly in the cell nuclei and cytoplasm of some keratinocytes. A strong expression of the p53 protein, assessed at 3 (+++), was especially observed in the cells from the basal layer of the epidermis (Fig. 11).

Discussion

Cladribine is a relatively low toxic drug and generally well tolerated (Gasperini *et al*., 2010). It may damage healthy cells of an organism, particularly those characterized by intense divisions, e.g. the epidermis. The literature shows that the main side effect of 2-CdA treatment is myelosuppression, while skin reactions are rare (Chubar and Benett, 2003). Rossini *et al*. (2004) present a case report of patients who had histopathological changes in their skin images after 2-CdA treatment. The main side effects were seen in the form of soreness and redness of the skin, lumps, spots, erythema and itching.

In our study, H&E samples from experimental group I showed thinning of the epidermis. Moreover, the reproductive cells of the basal layer were loosely arranged and were smaller in number as compared to the control group. This may indicate cell mortality resulting from the application of the drug.

The results of this study are confirmed by Epstein and Schubert (1999). They found that cytostatic drugs and radiation therapy directly affected the proliferation of epithelial cells, reduced the number of divisions in the basal layer of the epidermis and released IL-1 and TNF-α which led to the destruction of the epithelium and the development of inflammation. This can result in, the thinning of the epithelium and its loss in extreme cases (Epstein and Schubert, 1999). Similar results were also presented by Karolewska *et al*. (2004) who evaluated changes in the oral mucosa of children in the course of leukemia and its treatment.

In the present study, to assess the potential recovery capacity of the epidermis, the animals from experimental group II were decapitated four weeks after administration of the last dose of the drug. H&E staining showed no changes in epidermis construction. Based on the work of Baroni *et al*. (2012), it can be assumed that regeneration occurred during the four-week break in 2-CdA therapy, and that the total renewal of the epidermis takes 28 days on average. A similar example is given by Rossini *et al*. (2004) where the patient was treated with cladribine. After administration of the drug, distinct changes were seen in the skin image; however, after therapy cessation, side effects subsided.

The 2-CdA mechanism of action is based on the

induction of apoptosis (Nomura *et al*., 2000). Our research shows that apoptosis induced by 2-CdA comes about as a result of caspase 3 stimulation, leading to DNA fragmentation (Ola *et al*., 2011). Stimulated caspase 3 activity is necessary to initiate DNA fragmentation and create a bulge on the cell membrane (Végran *et al*., 2011).

To assess the intensity of the apoptosis in the animals' epidermis, we evaluated the expression of caspase 3. Statistical analyses showed significant correlation in caspase 3 expression between the control group and experimental group I ($P<0.0001$). In experimental group I, 77% of the cells showed positive caspase 3 expression. Also in this group, 9% of the cells revealed a most intense expression of this enzyme, assessed at 3 (+++). Moreover, the strong nuclear expression of this protein indicates the duration of the apoptosis process. A significant difference also appeared between both experimental groups ($P<0.0012$). However, the difference in the caspase 3 expression between the control group and experimental group II was statistically negligible ($P=0.4971$). It should be noted that animals of experimental group II were decapitated four weeks after the administration of the last dose of the drug, which may suggest tissue renewal. In addition to these results, morphological analyses revealed no significant changes in the epidermis of rats in experimental group II.

In both experimental groups, caspase 3 expression was visible in the cytoplasm and nuclei of epidermal cells in the form of a dark brown granulation, mainly in the basal layer. This may indicate that apoptosis occurred in the germ layer. Caspase 3 is an enzyme which directly activates nuclear endonucleases (Robak *et al*., 2006; Chmielewski *et al*., 2008).

In 2004, Rogalińska *et al*. estimated the process of apoptosis, *in vivo*, in lymphocytes isolated from the blood of patients with B-CLL. In their study, the active form of caspase 3 was discovered in the material gained from all patients. It should be mentioned; however, that in monotherapy, the level of activity was lower in comparison with groups of patients treated according to schemes CC and CMC. Investigations conducted by Conrad *et al*. (2008) also demonstrated that the apoptosis induced by 2-CdA in the Jurkat cell line passed on dependence of caspase-3.

Jędrych *et al*. (2013) carried out an investigation on the expression of the cell proliferation marker, Ki-67, and the expression of caspase 3 in ovarian surface epithelial cells. Authors found that the expression of caspase 3 in OSE cells increased after cladribine treatment, suggesting that 2-CdA induces apoptosis within the examined tissue. Moreover, the expression of Ki-67 in the tissue was significantly lower in the experimental group as compared to the control group ($P<0.05$). The obtained results thus suggest that cladribine can inhibit OSE cell proliferation in rats.

One of proposed mechanisms of activity of 2-CdA in cells is based on the inhibition of the synthesis, replication and repair of DNA. This action leads to an accumulation of cracking in the DNA structure and activation of apoptosis as controlled by the protein p53 action (Szmigielska-Kapłon and Robak, 2001; Bastin-Coyette *et al*., 2011).

The p53 protein is maintained at low concentrations in normal cells. When it comes to DNA damage, the concentration of p53 protein increases in the cell nucleus, as this regulates the cell cycle. This protein stops the cell cycle at the G1, G2 and S phase checkpoints, allowing the DNA structure to be repaired before de-oxyribonucleic acid synthesis and before the cell enters into mitosis. It should be noted that, in addition to the primary function of this protein, it is associated with preventing the duplication of damaged genetic material. Moreover, it is also involved in DNA repair, hence allowing further cell proliferation (Bargonetti and Manfredi, 2002; Dworakowska, 2005; Sengupta nd Harris, 2005; Bai and Zhu, 2006; Sznarkowska *et al*., 2010). The p53 protein mechanism of action is different in normal cells from cancer cells. In the latter, it induces apoptosis, acting as the basis of cytostatic drug activities.

In order to analyze the process of apoptosis, and assess potential recovery mechanisms in the investigated material, the expression of the p53 protein was examined. Statistical analysis of the results of the research showed a significant correlation ($P<0.0001$) between the incidence of p53 protein expression in the skin of rats of the control group and experimental group I, and the control group and experimental group II. In contrast, no correlation was shown in the intensity of p53 protein expression between experimental groups I and II ($P<0.1005$). This suggests that the level of activity of the enzyme in the two experimental groups was maintained at a similar level.

In experimental group I, in nearly 70% of the analyzed cells, an IHC nuclear reaction was observed. Since p53 protein is not active in normal cells, its visible expression in the nuclei of keratinocytes can provide evidence of ongoing processes of genetic material repair or apoptosis. Based on light microscopy morphological analysis, it is possible to confirm that the process of death occurs in the epidermis where there is a clear skin damage. It should be noted that the animals in this group were sacrificed 24 h after the last dose.

In experimental group II, more than 75% of the epidermal cells showed an apparent IHC reaction. Furthermore, in this group, most cells (more than 55%) revealed a strong p53 nuclear expression. Since H&E staining revealed no changes in the skin structure of this group, the displayed p53 expression may indicate the presence of the repair process (Bargonetti and Manfredi, 2002; Sznarkowska *et al*., 2010), as the p53 protein regulates the cell cycle. Regarding the DNA structural irregularity, the p53 protein may initiate apoptosis in addition to controlling the repair of genetic material (Budanov and Karin, 2008).

Galmarini *et al*. (2003) noted that the inactivation of p53 and $p21^{WAF1}$ proteins influences the sensitivity of dividing cells as related to the cytotoxicity of 2-CdA. These results also indicate that the cells containing wt-p53 are more sensitive and susceptible to the effects of 2-

CdA than are cells p53-/- and $p21^{WAF1}$-/-. These results confirm those obtained in our experiment. Similar results were also obtained by other researchers. Pettitt *et al*. (1999) investigated the mechanism of death induced by purine analogues in non-dividing lymphocytes. Based on their research, it was found that p53-knockout cells are more resistant to 2-CdA than are cells with wild forms of the protein. Moreover, it was noted that the death of cells under the influence of inter alia 2-CdA takes place either with the participation of the p53 protein or in-dependently.

Confirmation of our results regarding the activity of p53 protein after 2-CdA may be found in Gartenhaus *et al*. (1996), who examined the expression of the p53 protein in the apoptosis of CLL cells induced by 2-CdA. They found a high correlation between the expression of p53 protein and apoptotic cell death in CLL cells induced by 2-CdA.

Based on the statistical analysis of our results, there was no correlation between p53 protein expressions of the experimental groups. However, the relationship between protein expression of the control group and the two experimental groups was significant at the $P<0.0001$ level. Moreover, the number of positive immuno-precipitates in both experimental groups was similar. The p53 protein is known to be active in cells when major damage of the genetic material occurs, inhibiting the cell cycle. Nevertheless, it can also be activated in cells undergoing repair processes (Dworakowska, 2005; Sengupta and Harris, 2005; Bai and Zhu, 2006).

The results of the present study show that cladribine administration in the standard treatment dosage schema of MS in humans, induces changes in the epidermis images of test animals. Furthermore, on the basis of morphological analysis and p53 protein expression, it can be concluded that the four-week break in drug administration contributes to the regeneration of skin epithelial tissue of rats. Thus, the obtained results can significantly contribute to proper management therapy regarding the dose and route of administration so as to minimize its adverse effects. It is suggested that this experiment be supplemented by further clinical studies.

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مقاله کامل:

بیان پروتئین p53 و مطالعه هیستومورفولوژیکی اپیدرم در حیوانات آزمایشگاهی پس از تجویز کلادریبین

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(دریافت مقاله: 11 تیر 1392، پذیرش نهایی: 3 آذر 1392)

کلادریبین به عنوان داروی ضد نئوپلاسم و سرکوب کننده ایمنی و ضد التهاب عمل می‌کند. هدف این پژوهش مطالعه اثرات احتمالی این دارو بر اپیدرم می‌باشد. آزمایش بر روی موش صحرایی نژاد ویستار انجام گردید. گروه شاهد غذای استاندارد و آب دریافت کردند. گروه آزمایشی I تحت درمان کلادریبین مطابق با الگوی درمانی مولتیپل اسکلروز انسانی قرار گرفت. 24 ساعت پس از افزودن دوز دارو نمونه‌گیری جهت ارزیابی میکروسکوپی و هیستوشیمیایی انجام شد. دارو در گروه آزمایشی II نیز مطابق با روش مورد استفاده در گروه آزمایشی I تجویز گردید با این تفاوت که نمونه‌گیری 4 هفته بعد از آخرین تجویز دارو انجام شد. پس از اتمام دوره آزمایشی نمونه‌های پوست تمام موش‌ها تحت مطالعه هیستومورفولوژیک و ایمونوهیستوشیمیایی قرار گرفت. آنالیز نمونه‌های بافتی رنگ شده با هماتوکسیلین و ائوزین، تغییرات قابل مشاهده‌ای را در الگوی اپیدرم نشان داد. لایه‌های بافتی در مقایسه با گروه شاهد بسیار نازک‌تر شده بود. تفاوت حضور پروتئین p53 در گروه شاهد با دو گروه آزمایشی از نظر آماری معنی‌دار بود ($P<0.0001$). افزودن کلادریبین در گروه‌های آزمایشی سبب تغییر چهره بافتی اپیدرم می‌شود. بر اساس یافته‌های مورفولوژیکی و بیان پروتئین p53 می‌توان نتیجه گرفت که وقفه 4 هفته‌ای در تجویز دارو موجب کمک به بازسازی بافت اپیتلیوم پوست موش صحرایی می‌شود.

واژه‌های کلیدی: کلادریبین، اپیدرم، آپوپتز، پروتئین p53، کاسپاز 3