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Effect of pinocembrin on thymocyte proliferation and death

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ABSTRACT

Introduction: Cell volume regulation is critical for cellular proliferation and death. Pinocembrin effectively suppresses the volume regulation in thymocytes under hypoosmotic stress by blocking the volume-sensitive anion channel. This study aims to evaluate the effects of this flavonoid on thymocyte proliferation and death.

Methods: Thymocytes were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, and the cell number was determined by cloud-based automated cell counting (Corning). Necrotic and apoptotic cell death were evaluated by propidium iodide- and annexin V-staining, respectively.

Results: Pinocembrin at 10–50 μM caused suppression of primary cultured thymocyte proliferation with a half-maximal effect of 28.4 ± 0.2 μM. The cell counts did not fall below the control level at the doses of 100–150 μM. The fraction of spontaneously necrotic cells was ~26% of the total population and increased to ~51% in the presence of dexamethasone. The fraction of spontaneously apoptotic cells increased by this glucocorticoid from 3.6% to 16.7%. Pinocembrin protected thymocytes from necrosis both in spontaneous and dexamethasone-induced death, reducing the fraction of necrotic cells by ~40–50% at 150 μM. Pinocembrin attenuated dexamethasone-induced apoptotic death, reducing the fraction of annexin-positive cells to the control (spontaneous) level.

Conclusion: Our results suggest that pinocembrin arrests thymocyte proliferation without essential killing. Under conditions of massive death (e.g., during inflammation, when the level of glucocorticoids increases sharply both physiologically and as a result of pharmacotherapy), pinocembrin protects immuno-competent cells from necrotic and apoptotic death.

Implication for health policy/practice/research/medical education:
This paper provides experimental evidence of the antiproliferative activity of pinocembrin on thymocytes and the protection of cells against necrotic and apoptotic death, both spontaneous and glucocorticoid-induced. These results have implications for the pharmacodynamics of pinocembrin and therapeutic strategies when using pinocembrin-based pharmaceuticals..

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Introduction

The bone marrow and thymus are two primary lymphoid organs responsible for the production of the whole T-cell repertoire. Precursor cells migrate from the bone marrow to the thymus to form a pool of immature lymphocytes, thymocytes, which proliferate, differentiate, and undergo positive selection for T cell receptors and negative selection for auto reactivity (1-5). Apoptosis is the fate of most thymocytes, only a small portion is released into the bloodstream subsequently reaching the secondary lymphoid organs as peripheral T-lymphocytes.

Although the thymocytes have a fairly variable set of cell surface markers, such as clusters of differentiations (CD4, CD8, CD25), it is plausible that basic cellular physiological machinery is the same for the whole population. Thus, the thymocytes possess a fully functional cell volume regulation (CVR) system (6-13). Patch-clamp studies have revealed the presence of two volume-regulated anion channels important for the CVR response to the hypoosmotic stress: the volume-sensitive outwardly

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rectifying Cl^- channel (VSOR) and the maxi-anion channel of Maxi-Cl phenotype (14). In addition to its involvement in the CVR, the VSOR serves as a pathway for releasing glutathione from thymocytes into the extracellular matrix (15), whereas the Maxi-Cl channel is known as a pathway for adenosine triphosphate (ATP) and other signaling molecules (16-18).

CVR and the related ion channels play a pivotal role in cell survival and death (19-21). Therefore, low molecular weight modulators of these ion channels may represent new pharmacological tools to ameliorate immunological consequences of thymic involution, both the age-related one and the one induced by infectious diseases and malnutrition (5,22-24).

Pinocembrin (5,7-dihydroxyflavanone) (25) is the main component of flavonoid extracts of licorice (*Glycyrrhiza glabra* L.) (26, 27) and tarragon wormwood (*Artemisia dracunculus* L.) (28). It is also one of the principal bioactive components of propolis (29). Pinocembrin is a widespread natural product that can be isolated from the leaves, roots, buds, fruits, seeds, bark, rhizome, twigs, shoots, and inflorescences of 71 plants belonging to 34 families (30).

Pinocembrin exhibits a variety of biological activities, including antioxidant, antimicrobial, anti-inflammatory, anticancer, neuro- and cardioprotective (30). As an antioxidant, pinocembrin is implicated in preventing neurodegeneration in Parkinson's disease (31) and is regarded as a neuroprotective phytochemical in experimental ischemic stroke (32). Due to its ability to suppress endothelial cell apoptosis, pinocembrin is promising in the treatment of atherosclerosis (33).

Previously, pinocembrin was demonstrated to effectively suppress the CVR under hypoosmotic conditions by blocking the VSOR (34). Given the key roles of the CVR and VSOR in cell proliferation and death (18,20,21), we have set on the identification of the effects pinocembrin produces on the proliferation and death of thymocytes. As the immune cells have never been considered as a target for pinocembrin, our findings may have implications on the immunity protection in inflammation and therapy with glucocorticoids.

Materials and Methods

Drugs and reagents

Pinocembrin was purchased from LATOXAN (France; prod. No. L6069), dexamethasone (DX) from Biomol (Germany), and HEPES from Dojindo (Kumamoto, Japan). Pinocembrin and DX were added from concentrated (1000x) solutions to dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%, and at this concentration, the solvent did not significantly affect the results.

Cells

Experiments were performed on 6-8-week-old white laboratory rats kept on a custom diet balanced for nutrients. Thymocytes were isolated as described elsewhere (14, 34-37) with some modifications. Briefly, the animals were anaesthetized with diethyl ether and sacrificed by decapitation. All the following procedures were performed in aseptic conditions under a laminar hood. The thymi were dissected, carefully washed with the RPMI-1640 medium (Sigma-Aldrich) containing $NaHCO_3$, glutamine, antibiotics (penicillin and streptomycin), minced with fine forceps in the same medium, and passed through a 100 μm-nylon mesh. The suspension (in a total volume of ~5 mL) was centrifuged at 400 *g* for 5 minutes. Then, the pellet was resuspended in ~5 mL of the same medium, centrifuged at 400 *g* for 5 minutes, and resuspended in 1 mL of the RPMI-1640 medium, subsequently added with 10% fetal bovine serum (FBS). The cell concentration in the suspension was determined in a Corning Cell Counter (Corning Inc., USA) using cloud-based automated cell counting. Cell suspensions contained not more than 5% of damaged cells (normally 1–3%) as assayed by trypan blue exclusion. The suspension was diluted in the same medium to the required nominal concentration (1×10^6 cells/mL) for a total volume to be 10–30 mL, according to the experimental protocol. Immediately before the experiments, insulin (5 μg/mL) was added to the cells, and the baseline number of cells was counted using the Corning Cell Counter (0 hours). Then, the thymocytes (1 mL) were placed into a 24-well plate, incubated at 37 °C, 5% CO_2, and the number of cells was determined after 24 hours incubation.

Propidium iodide (PI, Sigma Aldrich Cat# P2667), a marker of necrosis, and annexin V FITC (AnnV, EXBIO Cat# EXB0029, Czech Republic), a marker of apoptosis, were used to determine cell death type. Following the 24-hour incubation, 100 μL of thymocyte suspension was transferred into a 96-well microplate. PI (1.5 μL of 1.5 mM solution in phosphate buffered saline, PBS) or AnnV (5 μL of the manufacturer's ready-to-use solution) was added to the wells according to the manufacturer's protocol and incubated for 10 minutes in the dark at room temperature. The number of PI-positive and AnnV-positive cells was determined using an ICX41F fluorescence microscope (Sunny Optical Technology, China), a HY-2307 digital camera, and the S-EYE software (Shenzhen Hayear Electronics, China). Images were analyzed using ImageJ freeware (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018).

Data analysis

The dose-response data were approximated using a Hill equation of the following form:

$$P = P_{min} + (P_{max} - P_{min}) / (1 + (C/C_{50\%})^h) \quad (1)$$

where P is the Proliferation index (%); P_{min} and P_{max} are the minimal and maximal values of P, respectively; C

is the concentration of the substance in μM; $C_{50\%}$ is the concentration of the substance rendering a half-maximal inhibitory effect (μM); h is the Hill coefficient.

Data were analyzed using Origin 8 software (OriginLab, Northampton, MA, USA). Pooled data were given as the mean ± SEM of n observations. Comparisons between the two experimental groups were made using the unpaired Student's t test. Differences were considered statistically significant at $P < 0.05$.

Results

At 0 hours, the thymocytes were seeded at the nominal density of 1 × 10^6 cells/mL (actual cell numbers were measured immediately before cell culturing and plotted in Figure 1A). After 24 hours culturing, the thymocytes in the control group increased their number up to (1.48 ± 0.12) ×10^6 cells/mL (n = 6) (Figure 1A). The Proliferation index, defined as the percent increase in cell number after 24 hours culturing relative to the initial value at 0 hours, was estimated to be 142 ± 5% (n = 6). The proliferation of thymocytes was found significantly suppressed after their 24 hours culturing with pinocembrin at the concentration of 10–25 μM and completely arrested at one of 50 μM (Figure 1A, B). A small but significant reduction in the cell number (~10%) could be seen at 50 μM; however, with 100–150 μM pinocembrin in the medium, the number of cells after 24 hours of culture did not differ significantly from the number of cells at 0 hours (Figure 1A, B). The findings suggest that pinocembrin arrests the proliferation of thymocytes without inducing their death. Approximation of the dose-response data at the range of 0–50 μM with Equation (1) (solid line in Figure 1B) yielded a half-maximal effective concentration of pinocembrin $C_{50\%}$ = 28.4 ± 0.2 μM (Hill coefficient being 4.5).

The optical counting in the bright field (data of Figure 1) did not discriminate between live and dead cells. Therefore, in the next series of experiments, we assayed the effect of 24-hour incubation of pinocembrin on the relative number of necrotic and apoptotic thymocytes. In the control experiments (with no flavonoid added), we found that the proportion of spontaneously necrotic PI-positive cells in the total population was 25.5 ± 3.2%, while the proportion of AnnV-positive (apoptotic) cells was 3.6 ± 1.2 % (Figure 2A, B). The fraction of PI-positive cells was down by half at the maximal tested concentration of pinocembrin (150 μM), as compared to the control group (Figure 2A). Meanwhile, pinocembrin did not significantly change the proportion of apoptotic AnnV-positive cells (Figure 2A) over the entire range of concentrations tested (10–150 μM). Based on these observations, we concluded that pinocembrin protected the thymocytes from necrotic but not apoptotic spontaneous cell death.

In addition to the systemic glucocorticoid secreted by adrenal glands, the thymus produces glucocorticoids locally, and thus, thymocytes are heavily affected by these hormones in differentiation and maturation (2). In the present study, we used a synthetic glucocorticoid, DX, known for inducing the death of immunocompetent cells. Actually, it is widely used as an effective medication for the treatment of inflammatory and autoimmune disorders, being recommended for the treatment of patients with coronavirus disease 2019 (COVID-19) (38). Here we showed that 1 μM DX caused a dramatic increase in the fraction of necrotic PI-positive cells in the total population up to 50.8 ± 7.2 % and increased the proportion of AnnV-positive (apoptotic) cells up to 16.7 ± 1.3 % (Figure 3). In the combined presence of DX (1 μM) and pinocembrin, the fraction of PI-positive cells decreased considerably by ~41 % at the maximal tested concentration of pinocembrin (150 μM), as compared to the value found in experiments with DX alone (Figure 3). The fraction of AnnV-positive (apoptotic) cells in the presence of pinocembrin decreased even more profoundly by ~68% for 10 μM. The fraction of apoptotic (AnnV-positive) cells became indistinguishable

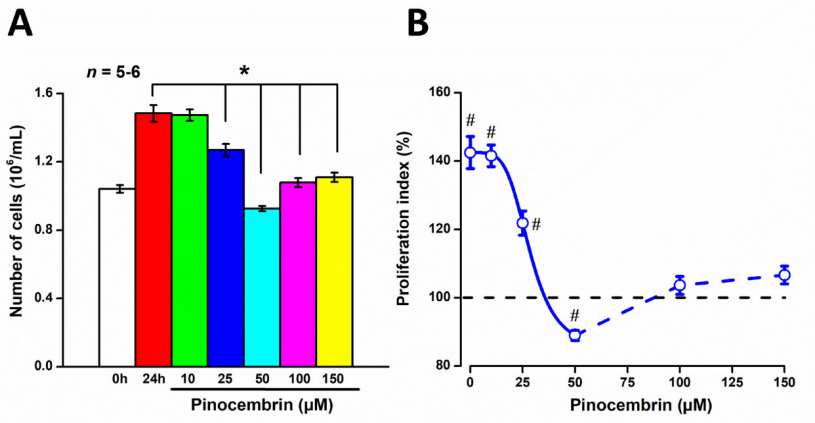

Figure 1. Dose-dependent effect of pinocembrin on thymocyte proliferation. (A) The absolute number of cells in the suspension immediately before and after 24 h of cell incubation in the absence (Control) or presence of pinocembrin at the indicated concentrations. **(B)** Dose-dependence of the Proliferation index (defined as the percentage change in the cell number in suspension after 24 h incubation relative to the initial cell number). The solid line is a fit to Equation (1) with parameters given in the text. * Statistically different relative to the control group (24 h with no drug, red column in panel A) at $P < 0.05$ (Student's t test). # Statistically different relative to 0 h (white column in panel A) at $P < 0.05$ (Student's *t-test*). *n:* number of experiments.

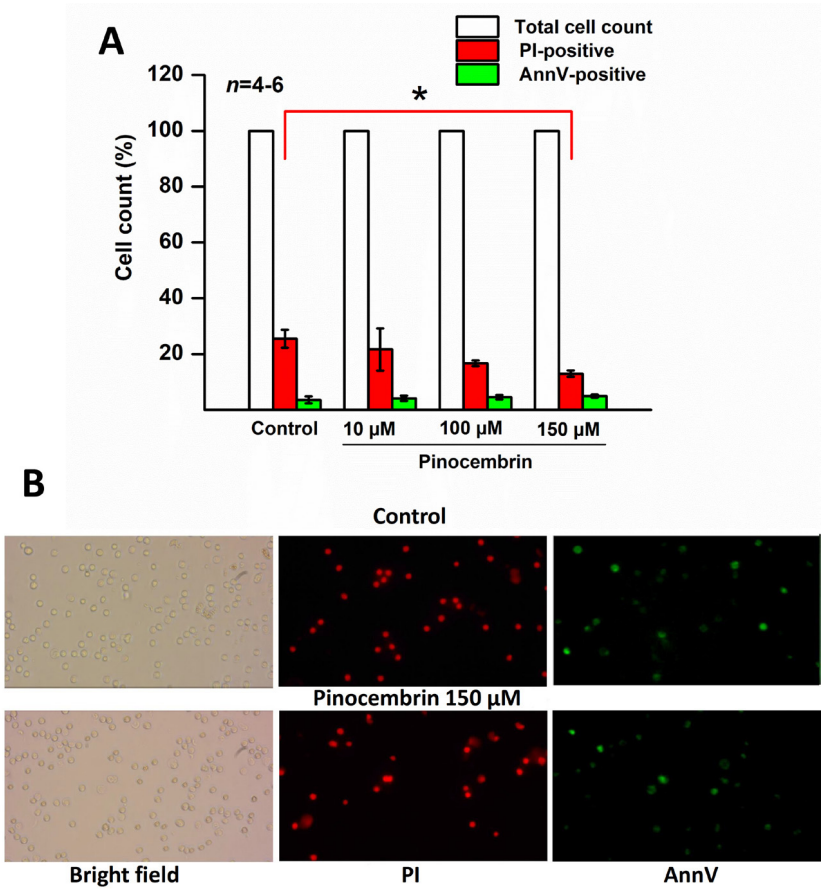

Figure 2. Effect of pinocembrin on the relative numbers of necrotic propidium iodide-positive and apoptotic annexin V-positive cells in primary cultured thymocytes. (A) The relative number of PI-positive and AnnV-positive cells in the suspension of thymocytes after 24 h culture in the absence (Control) and presence of pinocembrin at the indicated concentrations. These data are derived from original photographs similar to those shown in (B). The percentage of AnnV- and PI-positive cells was calculated relative to the total number of cells visible in the bright field. **(B)** Original photos of PI and AnnV staining of thymocytes in the control (no drug added) and in the presence of pinocembrin (150 μM, 24 h). *Statistically different relative to the control group (24 h with no drug) at $P < 0.05$ (Student's *t-test*). *n:* number of experiments. PI: propidium iodide; AnnV: annexin V.

from the control value (with no DX) in the presence of pinocembrin at 100 μM (Figure 3). Based on these results, we can conclude that pinocembrin effectively protects thymocytes from glucocorticoid-induced cell death.

Discussion

Our results indicate that pinocembrin dose-dependently suppresses the division of primary cultured thymocytes at the concentration range of 10–50 μM. The half-maximal dose (IC_{50}) of 28.4 μM obtained by approximation the dose-response data (Figure 1B) to the Hill equation is close to the IC_{50} of 39.1 μM observed earlier for the suppression of the regulatory volume decrease (RVD) process in thymocytes (34). This would strongly suggest that the inhibition of thymocyte CVR is the mechanism underlying the inhibitory effect of pinocembrin on thymocyte proliferation. The most likely molecular target for the activity of the flavonoid is VSOR channel, which was largely blocked by the bath application of 100 μM pinocembrin in the patch-clamp experiments (34).

Interestingly, a small but significant reducing effect of pinocembrin on the cell density (~10%) was observed at its low concentration (50 μM), being indistinct at higher ones (up to 150 μM). We suppose that pinocembrin's effect on cell proliferation and death is bidirectional. CVR is necessary for cell division (19-21), and its blockage should arrest the cell proliferation process as we have actually observed in our experiments. In addition, CVR inhibition should promote necrotic cell death due to uncontrolled swelling called necrotic volume increase (NVI) (20, 21). We believe that NVI is specifically responsible for ~10% reduction in cell number under the effect of pinocembrin at the concentration of 50 μM. However, cell death was not evident at higher doses of pinocembrin (100–150 μM). Moreover, pinocembrin exhibited a clear protective effect on necrotic cell death when applied at 150 μM alone or in combination with DX. The mechanism underlying the protection is not clear. The well-known antioxidant properties of pinocembrin (30,39) could be related to the pinocembrin's protective effects against necrosis, e.g., by suppressing the lipid peroxidation which labializes the lipid matrix of the cellular membrane. We hypothesize that similar to gossypol (36) as well as to glycyrrhizic acid and its derivatives (40), pinocembrin may intercalate

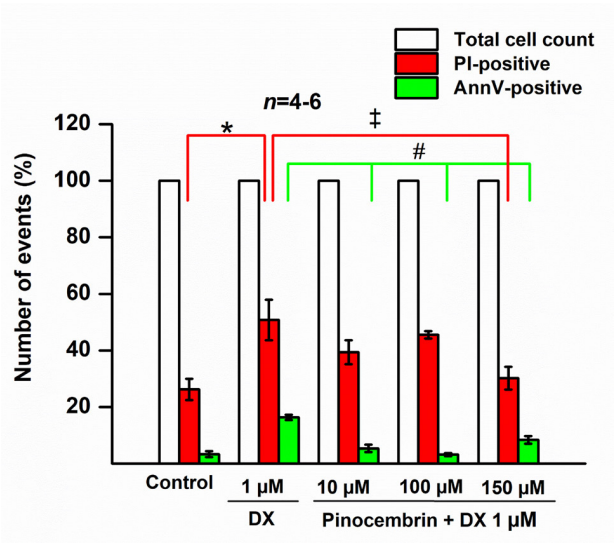

Figure 3. Effect of pinocembrin on dexamethasone (DX)-induced thymocyte death. The cells were cultured for 24 h with no drug added (Control), in the presence of dexamethasone (1 μM) and in the combined presence of dexamethasone (1 μM) and pinocembrin at the indicated concentrations. *Statistically different relative to the control group (24 h with no drug) at $P < 0.05$ (Student's *t-test*). *n*: number of experiments. Symbols ‡ and # denote statistical difference for propidium iodide-positive and annexin V-positive cells, respectively, in the combined presence of dexamethasone and pinocembrin relative to the dexamethasone alone. PI: propidium iodide; AnnV: annexin V.

into the bilayer lipid matrix of the thymocyte plasma membrane and enhance its rigidity and resistivity towards the mechanical distortion.

Apoptotic volume decrease (AVD) is one of the earliest events during apoptosis; the RVD machinery and the underlying ion channels (especially, VSOR) are activated by pro-apoptotic signaling pathways even under normotonic conditions (18,21,41-43). Therefore, the blocking effects of pinocembrin on RVD and VSOR channels are expected to suppress apoptosis as we have actually observed in our experiments for the combined effects of DX and the flavonoid.

Although the antiproliferative action of pinocembrin in our study is consistent with similar effects of the flavonoid demonstrated in HCT116 human colon cancer cells (44), B16F10 and A375 melanoma cells (45), human ovarian cancer SKOV3 cells (46), human prostate cancer PC-3 cells (47), and human lung cancer A549 cells (48), the reduction in cell number in these studies was achieved by promoting apoptosis and/or autophagy. In contrast, we have demonstrated here that antiproliferation by pinocembrin was achieved by CVR and VSOR blockage, whereas apoptosis was suppressed but not promoted by this flavonoid. This difference in apoptosis-related events may reflect the fact that thymocytes, unlike the cancer cells used in the cited papers, are normal, not cancerous.

Pinocembrin was shown to exert a protective effect against amyloid peptide injury of human brain microvascular endothelial by inhibiting MAPK/NF-κB inflammatory pathways (49). Pinocembrin attenuated 6-hydroxydopamine-induced neuronal cell death via Nrf2/ARE pathway (50). In addition, the flavonoid inhibited doxorubicin-induced pyroptosis of cardiomyocytes via the Nrf2/Sirt3 signalling (51). Based on these findings, we hypothesize that the protective effect of pinocembrin on thymocytes may also involve MAPK/NF-κB and/or Nrf2/ARE, but further investigations will be necessary.

The limitation of the present study is related to the use of normal primary cultured thymic cells. It remains to be verified if cancer cells are sensitive to the antiproliferative action of pinocembrin or not. The results might be useful in designing new flavonoid-based therapeutic strategies for protecting immune cells from death during inflammation and upon glucocorticoid medication. Possible anticancer applications of pinocembrin could also be considered.

Conclusion

Our results suggest that pinocembrin arrests thymocyte proliferation without inducing cell death. Under conditions of massive cell death (e.g., during inflammation, when the level of glucocorticoids increases sharply both physiologically and as a result of pharmacotherapy), pinocembrin is able to protect immuno-competent cells from necrotic and apoptotic death.

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Authors' contributions

Conceptualization: Gulnoza Toshtemirova, Sarvinoz Rustamova, Petr Merzlyak, Ranokhon Kurbannazarova and Ravshan Sabirov
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Visualization: Gulnoza Toshtemirova, Sarvinoz Rustamova, Petr Merzlyak
Writing–original draft: Gulnoza Toshtemirova, Sarvinoz

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Writing–review and editing: Petr Merzlyak, Ranokhon Kurbannazarova and Ravshan Sabirov.

Conflict of interests
The authors declare no conflict of interest.

Ethical considerations
All procedures for cell isolation were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental or Other Scientific Purposes (Strasbourg, 1986), following the ARRIVE guidelines (https://arriveguidelines.org/) and were approved in advance by the Bioethics Committee of the Institute of Biophysics and Biochemistry (BEC/IBB-NUU/2019/01-1).

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