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STUDY OF THE POTENTIAL ANTIPSORIATIC EFFECTIVENESS AND SAFETY OF THE COMBINATION OF NAFTALAN OIL WITH SALICYLIC ACID IN CELLULAR TEST SYSTEMS

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The aim *of this work was to evaluate the combination of purified naftalan oil (PNO) with salicylic acid (SA) for topical application as a potential effective antipsoriatic ointment and to study the mechanism of its action using specific in vitro cell models.*

Material and methods. *The effectiveness and safety of the following dosage forms were studied: ointment with 10 % PNO and 3 % SA (PNO-SA), cream with 0.064 % betamethasone dipropionate (BD), and ointment with 3 % SA. Cell viability of original HaCaT human keratinocytes and modified HaCaT/P was determined by colorimetric methods, namely by crystal violet staining or the MTT test method. The level of apoptosis of cells was evaluated by flow cytometry. Production of pro-inflammatory cytokines IL-8 and IL-1β was measured by the ELISA method.*

Results. *It was shown that the sensitivity of cells with a psoriasis-like phenotype (HaCaT/P) to PNO increases statistically significantly compared to control cells, which was confirmed by both the cell viability and the MTT test. to obtain a result with inhibition of cells with psoriasis-like characteristics, HaCaT/P requires a smaller concentration of the drug compared to a similar effect on conditionally normal cells – HaCaT. This may indicate the relative safety of the proposed medicinal product (combination) in parallel with the conditions of its effectiveness concerning pathologically changed cells. The results of flow cytometry showed that the new PNO-SA complex causes a statistically significant increase in the percentage of cells in all phases of apoptosis compared to control cells. Finally, a statistically significant decrease in the production of IL-8 by cells with psoriasis-like characteristics – the HaCaT/P line was shown in the presence of the PNO-SA combination compared with control cells. In addition, there was a significant decrease in the level of IL-8 production in cells in the presence of the combination compared to SA and the comparator BD. However, in terms of its effect on IL-1β production, the PNO-SA combination proved to be inactive.*

Conclusion. *Our proposed combination (PNO-SA), which suppresses pro-inflammatory IL-8 by more than 2 times (by 67.4 %) compared to the control, is a promising potential option for local treatment of psoriatic lesions. We speculate about the future demand of this combination in the clinical setting because along with high efficiency (in terms of viability indicators – the number of living cells, the level of apoptosis) towards pathologically changed keratinocytes, it shows a low toxicity profile towards normal healthy keratinocytes*

Keywords*: purified naftalan oil, salicylic acid, betamethasone dipropionate, psoriasis, human keratinocytes, cell viability, apoptosis, interleukins*

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1. Introduction

Psoriasis is an immune-mediated chronic inflammatory skin disease affecting skin and nails [1]. The average global prevalence of this disease is about 11 %, while genetic, environmental and immunological factors play a key role in the pathogenesis and progression of the disease [2]. More often, it manifests itself as raised, well-demarcated plaques that peel. Histologically, psoriatic lesions develop because of premature hyperproliferative activity of keratinocytes due to long-term inflammation, which causes acanthosis, hyperkeratosis, and violation of the anatomy of the epidermal layers [3].

One of the key roles in developing psoriasis is played by cytokines, and therapy targeting interleukins is considered an effective treatment method for patients with moderate to severe forms of this disease. It is known that IL-8 stimulates excessive proliferation of keratinocytes in psoriasis and is associated with neutrophil accumulation, sustaining the inflammatory process [4]. At the same time, the IL-1β signalling pathway is significantly important in the pathogenesis of chronic autoimmune inflammatory skin diseases, including psoriasis. However, the detailed cellular and molecular mechanisms of this process are still being investigated [5].

Recent studies show the complexity of the pathogenesis of psoriasis, and at the same time, there are many insufficiently studied causes of occurrence, mechanisms of development and features of successful treatment of the disease. This lack of knowledge can be seen in the limitations of current treatment options, as well as in the varying

efficacy and toxicity of treatments between patients [6]. Herewith, local treatment of psoriasis plays a predominant role, both as monotherapy and in combination with systemic interventions in cases of widespread disease. Topical drugs include keratoplastic and keratolytic, glucocorticosteroids, synthetic analogues of vitamin D3, tar preparations, and phytopreparations of topical application [7]. Betamethasone cream is one of the common drugs for treatment: it shows its effectiveness in treating scalp hair and plaque psoriasis on the skin of the upper and lower extremities. Corticosteroids, which include betamethasone, have always been the mainstay of topical psoriasis treatment [8]. However, concerns about long-term side effects have prompted a search for alternatives [9].

The future treatment of psoriasis shows promising new trends: new biological agents targeting new pathways, including interleukin inhibitors. Small molecule inhibitors like RORγt inhibitors and ROCK2 inhibitors provide additional treatment options. Combination therapies, including biologics with methotrexate, may improve treatment response [9].

Also, salicylic acid (SA) ointment, which is used due to its keratolytic effect, is one of the options for the local treatment of psoriatic lesions. Both as monotherapy and in combination with other topical agents, SA helps soften and remove psoriatic scales. It also increases the effectiveness of topical corticosteroids or coal tar by enhancing their absorption [10].

Purified naftalan oil (PNO), derived from naftalan crude oil, is reported in the literature to have anti-inflammatory and regenerative properties. Studies have demonstrated its effectiveness in dermatological conditions such as eczema and psoriasis. However, the mechanism of action of PNO and the pharmacological effects of its combinations with other active pharmaceutical ingredients remain insufficiently studied [11].

Questions remain about increasing the effectiveness of the action, the duration of the effective therapeutic action, and reducing the side effects, which, in combination, will lead to the optimization of the treatment and the improvement of the lives of those suffering from this chronic disease.

The aim of the study was to evaluate a combination of PNO with SA for topical application as a potential effective antipsoriatic agent and to study the mechanism of its action using specific *in vitro* cell models.

2. Planning (methodology) of research

The study design comprised the following stages.

Stage 1. Determination of original normal HaCaT cell viability and modified subline HaCaT/P by colorimetric methods in the media where PNO, SA, or comparator were added.

Stage 2. Evaluation of the level of apoptosis in cells by flow cytometry in the media where PNO, SA, or comparator were added.

Stage 3. Determination of production of pro-inflammatory cytokines IL-8 and IL-1β by modified HaCaT/P subline cells in the media where PNO, SA, or comparator were added.

3. Material and methods

3. 1. Cell lines and culture

Immortalized human keratinocytes of the original HaCaT line and HaCaT keratinocytes induced by IL-6/IL-8/TNF-α were used in the work. Cell lines were received by the Bank of Cell Lines from Human and Animal Tissues of RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (Certificate No. AH 41 dated February 19, 2009). Cells of the original line were cultured in RPMI 1640 nutrient medium (Biowest, France) with 10 % fetal bovine serum (FBS) (Biowest, France) and 1x penicillin-streptomycin (Biowest, France). The studied cells were cultivated in a humidified atmosphere with 5 % CO_2 at 37 °C. Changes to the medium and reseeding cells were carried out according to standard methods. Cells were subcultured after they reached 80–90 % of the monolayer using Versen's solution (Biowest, France) and trypsin (Biowest, France). Cells in the exponential phase of growth were used for research.

Modified cells were cultured in DMEM nutrient medium (Biowest, France) with 10 % FBS and 1x penicillin-streptomycin with the addition of IL-6 – 0.8 ng/ml (Sigma, USA), IL-8 – 0.07 ng/ml (Sigma, USA) and TNF-α – 1 ng/ml (Sigma, USA) for 105 days. The studied cells were cultivated in a humidified atmosphere with 5 % CO_2 at 37 °C.

3. 2. Studied compounds and materials

The studied compounds:

– ointment with 10 % purified naftalan oil and 3 % salicylic acid (PNO-SA) (State Scientific Institution "Institute of Single Crystals" of National Academy of Sciences of Ukraine);

– cream with 0.064 % betamethasone dipropionate (BD) (State Scientific Institution "Institute of Single Crystals" of National Academy of Sciences of Ukraine);

– ointment with 3 % salicylic acid (SA) (State Scientific Institution "Institute of Single Crystals" of National Academy of Sciences of Ukraine);

– solvents and additional substances: dimethyl sulfoxide (DMSO) (1264ML250, neoFroxx, Germany), acetone, ethyl alcohol.

3. 3. Determination of cell viability of original HaCaT and modified subline HaCaT/P by colorimetric methods

Twenty-four hours after reseeding, the studied cells were removed from the substrate using Versen's solution and the number of cells in the suspension was assessed using trypan blue (A0668, Applichem, Germany) in a Horyaev chamber (Voles, Ukraine). Cells were plated on a 96-well plate (SPL, Korea) at a concentration of 0.8×10^4 cells/well. Cells were cultured in a humidified atmosphere at 5 % CO_2 and 37 °C for 24 hours. After the investigated compounds, solvents, and auxiliary substances were added to the cells, the cells were incubated at 5 % CO_2 and a temperature of 37 °C for another 24 hours. After incubation, the results (number of live cells) were counted visually (direct microscopy method) and colorimetrically

by staining live cells with crystal violet (405831000, ThermoScientific, USA) or MTT test method (A2231, Applichem, Germany). After the end of the incubation, the nutrient medium was removed from all wells of the plate, and 50 µl of crystal violet solution was added to all wells (the dye was diluted in 70 % methanol to a final concentration of 5 mg/ml). After incubation for 10 minutes, the dye was removed from all wells and washed with cold water. After that, the dye was eluted with ethyl alcohol.

In the MTT test, after incubation of cells with the drugs, 10 µl of MTT solution (5 mg/ml 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide in phosphate-salt buffer) was added to all wells of the plate and was placed in a CO_2 incubator for 3 hours, after which the nutrient medium was removed from all wells of the plate and DMSO was added.

With both cell staining methods, the results were recorded using a spectrophotometer at an excitation wavelength of 540 nm. The number of living cells (X) in each well of the plate was expressed as a percentage, calculated by the formula:

$$X = \frac{A_1 \cdot 100\ \%}{A_0},$$

where A_0 is the average value of the optical density in the wells of the negative control, and A_1 is the average value of the optical density in the well of the experimental group.

The degree of cell viability after 24 h of incubation for the tested sample, positive and negative controls was compared with intact cells. IC_{50} (concentration that causes cytotoxic effect in 50 % of cells) was determined by the method of non-linear regression analysis.

3. 4. Evaluation of the level of apoptosis in cells by flow cytometry

The number (%) of cells in the early, middle and late stages of apoptosis was determined by their double staining with Annexin V and propidium iodide (PI) by flow cytometry. Cells were planted in the wells of a 6-well plate (SPL, Korea) in DMEM nutrient medium with 10 % FBS at a concentration of 1.5×10^5 in a volume of 5 ml/well. After 24 hours, the studied drugs were added to the cells and the cells were incubated in a CO_2 incubator at 37 °C in a humid atmosphere for another 18 hours. After the end of incubation, the number of keratinocytes was counted and stained according to the manufacturer's protocol of the Annexin V FITC Apoptosis Detection Kit (Dojindo, Japan). Samples were analyzed using a DxFLEX flow cytometer (Beckman Coulter, USA). Annexin V-FITC staining was analyzed using the FITC channel (light filter 525/40 nm BP), and PI staining was analyzed using the ECD channel (light filter 610/20 nm BP). During the cytofluorimetric study, the gating strategy was to gate the cells as wide as possible by cutting off only cellular debris on the FSC-A/SSC-A dot raft and excluding doublets from the analysis using the FSC-A/FSC-H dot raft. Apoptosis/necrosis parameters were evaluated using a four-quadrant type gate on the FITC-A/ECD-A dot. An average of 25,000 events were analyzed in the study. The results obtained were analyzed using the CytExpert computer program for DxFLEX.

3. 5. Production of pro-inflammatory cytokines IL-8 and IL-1β by modified HaCaT/P subline cells

Cells were plated at a concentration of 1.5×10^5 cells/well in the wells of a 6-well plate in a complete DMEM nutrient medium in the presence of 10 % FBS and 1x penicillin-streptomycin. In 24 hours, the nutrient medium was changed in all wells of the tablet, and the tested compounds were added to the corresponding wells of the tablet. After that, they were placed in a CO_2 incubator and cultivated for another 24 hours under standard conditions. At the end of the incubation, the culture medium was collected from all the wells of the plate and centrifuged for 5 minutes at 1000 rpm, the supernatant liquid was collected and stored at –20 °C for further determination of the cytokine level. Before analyzing the concentration of cytokines, the samples studied were thawed at room temperature. The level of IL-8 (Human Interleukin 8 ELISA Kit, Fine Test, Wuhan Fine Biotech Co., Ltd., China) and IL-1β (Human Interleukin 1β ELISA Kit, Fine Test, Wuhan Fine Biotech Co., Ltd., China) in the culture medium from HaCaT/P cells was determined by the ELISA method, which was carried out according to the manufacturer's instructions.

3. 6. Statistical data processing

Statistical processing of research results was performed using Excel 2016 software by calculating the average value of the studied indicators (M) and standard deviation (SD). Student's t-test was used to assess significance levels of differences in mean values between groups. Calculations were performed using STATISTICA 6.0 software. Differences with a probability of at least 95 % ($p<0.05$) were considered reliable.

4. Results

We analyzed the effect of the tested drugs on the viability of cells of the original line of normal keratinocytes – HaCaT and modified by long-term action of cytokines to obtain a model of keratinocyte cells with psoriasis-like characteristics/changes – HaCaT/P. Long-term modification of cells with the help of cytokines was carried out by the employees of the Cell Bank in R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine and kindly provided for further studies of this experimental model. In this scientific work, a new potential antipsoriatic combination is proposed, which includes PNO, which can be used for local application in combination with SA. PNO is a substance of natural origin, a highly purified fraction of naftalan oil, a transparent substance containing naphthenic hydrocarbons. Unlike crude naftalan oil, PNO is the result of deeper processing of naftalan oil, which is formed after the removal of potentially carcinogenic and allergenic fractions of petroleum hydrocarbons; it is devoid of dyes and has significantly improved technological properties.

At the first stage, we conducted a comparative study of the effect of the above-mentioned combination (PNO-SA) with betamethasone on indicators of viability (number of living cells and metabolic activity) of control (HaCaT) and modified (HaCaT/P) keratinocytes. At the same time, the effect on the cells of auxiliary solvents for the purity of the experiment was also evaluated, which is presented in the tables below (Tables 1–6). It should be added that as a comparator for the effectiveness of the action in this scientific work, we used a well-known drug with antipsoriatic action – BD.

As a result, it was shown that the sensitivity of cells with a psoriasis-like phenotype (HaCaT/P) to PNO increases statistically significantly compared to control cells (Table 3). An increase in PNO concentration led to increased toxicity toward keratinocytes, while its effect on cells with a psoriasis-like phenotype was more pronounced across the entire concentration range.

Table 1

Viability of human keratinocytes of the HaCaT line after their treatment with purified naftalan oil+salicylic acid combination and betamethasone dipropionate (staining – crystal violet)

Concentration of SA, %	Number of living cells, %	Concentration of PNO, %	Concentration of SA, %	Number of living cells, %	Concentration of BD, %	Number of living cells, %
3.0	0.8±0.3	10.0	3.0	0.2±0.2	1.0	72.0±3.4
1.5	4.8±1.8	5.0	1.5	0.4±0.4	0.5	74.5±2.8
0.8	6.3±2.0	2.5	0.8	0.4±0.2	0.25	76.9±4.6
0.4	49.4±4.5	1.25	0.4	0.6±0.2	0.13	85.6±2.4
0.2	76.1±4.3	0.62	0.2	10.9±1.6	0.06	99.2±3.3
0.1	86.8±0.8	0.31	0.1	76.9±1.8	0.03	101.5±1.3
0.05	93.2±4.7	0.16	0.05	100.0±2.5	0.015	101.1±1.6
Concentration of DMSO, %	Number of living cells, %	Concentration of ethyl alcohol, %	Number of living cells, %	–	Concentration of acetone, %	Number of living cells, %
10.0	74.2±2.8	10.0	64.0±3.1	–	1.0	96.6±4.3
5.0	91.6±1.6	5.0	82.1±3.0	–	0.5	96.6±1.9
2.5	100.1±0.3	2.0	92.5±1.6	–	0.25	97.9±1.4
1.25	101.0±0.9	1.0	101.7±2.4	–	0.13	100.3±3.7
0.62	101.3±0.8	0.5	99.1±0.9	–	0.06	100.9±1.8
0.31	101.3±1.2	0.25	100.8±2.1	–	0.03	100.6±2.6
0.16	100.5±0.7	0.13	100.6±0.3	–	0.015	98.7±0.9

Table 2

Viability of human-modified keratinocytes (HaCaT/P line) after their treatment with purified naftalan oil+salicylic acid combination and betamethasone dipropionate (staining – crystal violet)

Concentration of SA, %	Number of living cells, %	Concentration of PNO, %	Concentration of SA, %	Number of living cells, %	Concentration of BD, %	Number of living cells, %
3.0	0.3±0.2	2.0	3.0	0.5±0.1	1.0	3.6±0.8
1.5	0.6±0.1	1.0	1.5	0.7±0.1	0.5	42.8±4.5
0.8	2.3±1.7	0.5	0.8	0.5±0.3	0.25	54.7±3.5
0.4	36.8±6.2	0.2	0.4	2.1±2.7	0.13	68.5±1.6
0.2	75.8±1.5	0.13	0.2	59.7±7.6	0.06	73.8±1.0
0.1	93.7±5.7	0.06	0.1	95.9±3.5	0.03	97.6±2.3
0.05	98.7±2.0	0.03	0.05	99.0±2.6	0.015	98.7±2.4
Concentration of DMSO, %	Number of living cells, %	Concentration of ethyl alcohol, %	Number of living cells, %	–	Concentration of acetone, %	Number of living cells, %
2.0	86.4±1.0	10.0	58.9±3.1	–	1.0	82.6±2.1
1.0	99.8±1.6	5.0	76.5±4.1	–	0.5	94.3±3.1
0.5	100.0±0.5	2.0	93.1±2.4	–	0.25	98.1±0.4
0.2	98.5±1.2	1.0	96.7±3.0	–	0.13	98.7±0.3
0.13	99.4±1.2	0.5	97.6±2.1	–	0.06	99.9±1.5
0.06	100.6±0.8	0.25	100.0±0.6	–	0.03	100.8±1.6
0.03	100.5±0.7	0.13	100.7±1.3	–	0.015	101.4±2.2

Table 3

IC50 of experimental test samples according to the number of living cells (proteins, DNA)

Test sample (drug)	PNO	SA	BD
HaCaT (control cells), IC_{50}, %	0.39±0.01	0.40±0.02	Not defined
HaCaT/P (modified cells), IC_{50}, %	0.13±0.01*	0.33±0.04	0.41±0.07

*Note: * – changes in indicators are statistically significant, p<0.05.*

Table 4

Respiratory activity of human keratinocytes of the HaCaT line after their treatment with purified naftalan oil+salicylic acid combination and betamethasone dipropionate (MTT-test)

Concentration of SA, %	Respiratory activity of cells, %	Concentration of PNO, %	Concentration of SA, %	Respiratory activity of cells, %	Concentration of BD, %	Respiratory activity of cells, %
3.0	0.4±0.2	10.0	3.0	0.2±0.2	1.0	55.6±3.2
1.5	0.6±0.3	5.0	1.5	0.5±0.1	0.5	73.6±1.9
0.8	13.3±1.5	2.0	0.8	0.9±0.1	0.25	84.7±1.7
0.4	47.8±1.6	1.0	0.4	2.4±0.5	0.13	89.2±6.2
0.2	68.5±4.0	0.5	0.2	14.0±0.3	0.06	93.9±3.8
0.1	78.4±1.8	0.25	0.1	64.1±5.1	0.03	96.6±3.2
0.05	91.1±6.8	0.13	0.05	83.3±1.8	0.015	99.3±1.5
Concentration of DMSO, %	Respiratory activity of cells, %	Concentration of ethyl alcohol, %	Respiratory activity of cells, %	–	Concentration of acetone, %	Respiratory activity of cells, %
10.0	72.0±3.6	10.0	44.5±4.3	–	1.0	98.8±2.0
5.0	86.8±1.4	5.0	74.5±2.9	–	0.5	99.9±0.7
2.0	95.8±1.5	2.0	86.6±3.6	–	0.25	99.9±2.0
1.0	101.7±2.8	1.0	99.8±1.8	–	0.13	101.2±0.8
0.5	98.2±0.7	0.5	99.4±0.9	–	0.06	100.8±1.0
0.25	98.1±3.0	0.25	100.1±0.7	–	0.03	99.7±1.1
0.13	98.8±3.7	0.13	100.9±1.2	–	0.015	101.8±0.4

Table 5

Respiratory activity of human-modified keratinocytes (HaCaT/P line) after their treatment with purified naftalan oil+salicylic acid combination and betamethasone dipropionate (MTT-test)

Concentration of SA, %	Respiratory activity of cells, %	Concentration of PNO, %	Concentration of SA, %	Respiratory activity of cells, %	Concentration of BD, %	Respiratory activity of cells, %
3.0	1.1±0.5	2.0	3.0	0.6±0.2	1.0	5.4±1.5
1.5	1.3±0.2	1.0	1.5	0.3±0.3	0.5	43.1±0.6
0.8	17.7±3.5	0.5	0.8	0.9±0.1	0.25	53.7±2.0
0.4	69.3±7.8	0.2	0.4	4.7±0.9	0.13	74.2±5.2
0.2	87.6±0.1	0.13	0.2	61.2±2.2	0.06	83.5±4.9
0.1	91.1±0.8	0.06	0.1	92.4±2.1	0.03	99.3±2.5
0.05	99.4±1.4	0.03	0.05	100.5±1.7	0.015	101.3±1.3
Concentration of DMSO, %	Respiratory activity of cells, %	Concentration of ethyl alcohol, %	Respiratory activity of cells, %	–	Concentration of acetone, %	Respiratory activity of cells, %
2.0	78.2±4.5	10.0	43.2±2.3	–	1.0	81.7±2.9
1.0	89.6±1.9	5.0	68.1±2.5	–	0.5	93.0±1.7
0.5	95.4±1.2	2.0	77.3±3.5	–	0.25	95.4±1.1
0.25	97.4±1.4	1.0	93.0±0.9	–	0.13	98.2±0.4
0.13	98.3±1.7	0.5	95.1±1.8	–	0.06	99.2±0.5
0.06	99.1±0.8	0.25	96.8±0.7	–	0.03	100.2±0.6
0.03	101.2±1.8	0.13	99.7±1.1	–	0.015	101.0±0.7

Table 6

IC50 of experimental test samples according to according to the respiratory activity index

Test sample (drug)	PNO	SA	BD
HaCaT (control cells), IC_{50}, %	0.41±0.02	0.41±0.02	Not defined
HaCaT/P (modified cells), IC_{50}, %	0.13±0.01*	0.50±0.02*	0.39±0.07

*Note: * – changes in indicators are statistically significant, p<0.05.*

According to the results of the indicators of metabolic (respiratory) activity in the MTT test, similar to the results of cell viability, namely the number of living cells, it was shown that HaCaT/P cells are characterized by a higher sensitivity to PNO-SA. This fact is extremely interesting and promising because to obtain a result with inhibition of cells with psoriasis-like characteristics, HaCaT/P requires a smaller concentration of the drug compared to a similar effect on conditionally normal cells – HaCaT. This may indicate the relative safety of the proposed medicinal product (complex) in parallel with the conditions of its effectiveness in relation to pathologically changed cells.

To further evaluate the effectiveness and detail the mechanism of action of the proposed PNO-SA combination on keratinocytes modified by cytokines (HaCaT/P),

the parameters of apoptosis in cells after the action of the tested complex were evaluated in comparison with SA and the comparison drug – BD (Fig. 1).

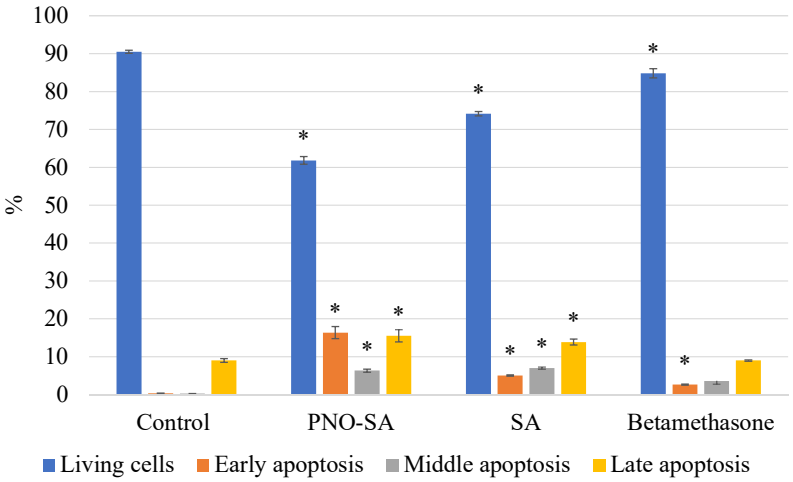

Fig. 1. Number of HaCaT/P in different phases of apoptosis (%): * – changes in indicators are statistically significant relative to the control group, $p<0.05$

According to the results of flow cytometry, it was shown that the new PNO-SA combination causes a statistically significant increase in the percentage of cells in all phases of apoptosis compared to control cells ($p<0.05$). At the same time, the % of cells in early apoptosis after the action of the PNO-SA combination is significantly different from other variants for treating cells with drugs – SA and the comparison drug BD.

IL-8 and IL-1β were studied in the medium of cell culture after exposure to the test samples for a comparative assessment of the effect of the studied drugs on the production of pro-inflammatory cytokines by keratinocytes of the modified HaCaT/P subline (Table 7).

Table 7

Changes in the production of IL-8 and IL-1β by modified keratinocytes HaCaT/P, after their exposure to purified naftalan oil+salicylic acid combination and betamethasone dipropionate

Group	IL-8 production, pg/ml	Changes in IL-8 production	IL-1β production, pg/ml	Changes in IL-1β production
Control	298.3±18.1	N/A	67.88±0.18	N/A
SA (0.2 %)	175.8±13.1*	Decrease by 41.1 %	66.5±9.9	No statistically significant change
PNO-SA (1+0.2 %)	97.2±3.8*	Decrease by 67.4 %	72.33±2.37	No statistically significant change
BD (0.4 %)	114.1 ±5.0*	Decrease by 61.7 %	67.75±1.06	No statistically significant change

*Note: * – changes in indicators are statistically significant relative to each other, $p<0.05$.*

When evaluating the production of interleukins 8 and 1β, a statistically significant decrease in the production of IL-8 by cells with psoriasis-like characteristics – the HaCaT/P line was shown in the presence of the PNO-SA combination in comparison with control cells – without the influence of medicines. In addition, there was a significant decrease in the level of IL-8 production in cells in the presence of the complex compared to SA and the comparator BD. At the same time, there were no changes in the production of another investigated cytokine – IL-1β under the influence of test agents.

Thus, regarding its effect on IL-1β production, PNO proved to be inactive. From the perspective of the current understanding of psoriasis pathogenesis [12], IL-1β is important in the early stages of inflammation initiation, as it stimulates a range of signals that support keratinocyte proliferation and immune cell migration. In contrast, IL-8 is critically involved in the formation of cellular infiltration (primarily neutrophilic) characteristic of psoriatic plaques. It can be concluded that PNO may be effective in reducing predominantly the neutrophilic component of inflammation.

5. Discussion

Psoriasis is thought to come and go in cycles. Such a cycle consists of 3 parts:

1) initiation;
2) perpetuation;
3) resolution.

In the first stage, exogenous factors of various natures disrupt the covering barrier, damage keratinocytes, and initiate an inflammatory reaction [13]. In addition to external factors, the inflammatory reaction can also be caused by internal disturbances. Damaged keratinocytes release numerous inflammatory mediators, including TNF-α and IL 1β, 6, and others. Inflammatory mediators stimulate plasmacytoid dendritic cells, natural killer cells, and macrophages to produce numerous additional mediators that trigger a cascade of sequential reactions. Activation of plasmacytoid dendritic promotes myeloid dendritic cells maturation and production of IL-12, IL-23, and TNF-α, which leads to the activation of T helper 1 and 17 and subsequent secretion of inflammatory cytokines, such as IL-17, -21, -22 and TNF-α. Keratinocytes are then activated by these cytokines and produce antimicrobial peptides, cytokines, and chemokines, contributing to the amplification of inflammation [4, 14].

At the same time, despite the long history of searching for mechanisms of the disease and therapy for patients with psoriasis, the question of increasing the effectiveness of the treatment strategy remains open, and its solution is urgent. In this scientific work, we investigated a complex of drugs as a new potential combination with antipsoriatic action. Namely, we proposed the complexation of PNO with SA. In our opinion, such a combination can be not only effective but also quite safe. Moreover, separately by component, these products (their analogues) have already shown effects on psoriatic lesions [15].

In particular, SA, thanks to its combined anti-inflammatory and exfoliating action, effectively reduce peeling, thickening, itching and erythema. SA is characterized by an antiproliferative effect and increases the effectiveness of other drugs when used in combination by increasing their penetration through the skin when applied topically [16]. There is also data on the antipsoriatic properties of naftalan oil, but they are quite limited because the characteristics of the oil, its purity, etc., often differ [17].

In this scientific work, we used a highly purified fraction of naftalan oil, which is the result of deeper processing and is formed after the removal of potentially carcinogenic and allergenic fractions of petroleum hydrocarbons, for combination with SA. According to the results of indicators of cell viability (number of living cells) and their metabolic (respiratory) activity, it is shown that HaCaT/P cells with a psoriasis-like phenotype (after modification with a cytokine complex) are characterized by a greater sensitivity to PNO-SA than control HaCaT cells. The data show the potential effectiveness of the proposed complex in psoriatic pathology in parallel with low toxicity to normal skin cells. At the same time, according to the results of flow cytometry, it is shown that the new PNO-SA combination proposed by us also causes a statistically significant increase in the percentage of cells in all phases of apoptosis compared to control cells ($p<0.05$). This also confirms a more toxic effect on pathologically changed cells compared to normal ones and shows one of the mechanisms of cell death with psoriasis-like characteristics, namely increased apoptosis under the influence of a PNO-SA combination. The fact that the percentage of cells in early apoptosis after the action of the complex is significantly higher than other variants of cell treatment turned out to be interesting.

It is known that in psoriasis, the balance between cell proliferation and apoptosis in the skin shifts toward proliferation. With an increase in apoptosis, which could potentially be one of the mechanisms of action of the PNO-SA combination, immune cells gain the ability to eliminate immature keratinocytes, providing an opportunity to regulate the excessive proliferation process [18].

But the most impressive, in our opinion, is a significant and statistically reliable reduction of IL-8 in cells with psoriasis-like characteristics under the action of the new combination proposed by us. As mentioned above, cytokines play a key role in the course of the pathological process in psoriasis, and anti-interleukin therapy has become the mainstay of treatment for patients with moderate to severe psoriasis [19]. We investigated the production of IL-8, which is a pro-inflammatory factor and a factor that induces keratinocyte overgrowth in psoriasis. In addition, overexpression of IL-8 in the skin is associated with the accumulation of large numbers of neutrophils and neutrophil infiltration is associated with inflammatory and treatment-resistant psoriasis. IL-8 receptors are expressed on the surface of both keratinocytes and neutrophils, and angiogenesis in psoriatic lesions is accelerated by the cytokine IL-8, which additionally promotes keratinocyte proliferation [19, 20].

Today, there are certain results of anti-IL-8 therapy for psoriasis, in particular, clinical studies regarding the use of Abcream and ABX-IL8, but the results are quite limited; drug registration authorizations are also limited to one country or have not received authorization [21, 22]. In general, anti-IL therapy in the treatment of patients with psoriasis is a promising direction of therapy, but currently, there are many contradictions, and research should be continued to find optimal solutions and drugs and to reveal the mechanism of sensitivity/resistance to therapy [23, 24]. At the same time, the local application of drugs that can control and regulate the production of cytokines by keratinocytes is extremely promising and interesting, since local application usually causes fewer toxic effects on the body in contrast to the systemic effect of drugs. Under the conditions of suppression of pro-inflammatory IL-8 more than 2 times (by 67.4 %) compared to the control, the new complex proposed by us is a promising potential option for the local treatment of psoriatic lesions. Thus, recent data describing the role of cytokines in the etiology of psoriasis have prompted the development of specific interleukin modulators that restore the normal proliferation and function of keratinocytes [13]. Additional local, biological, and intracellular therapeutic strategies are also being enthusiastically explored, one of which, in our opinion, may be the use of the PNO-SA combination.

Practical relevance. The ability of the PNO-SA combination to suppress pro-inflammatory IL-8 by 67.4 % compared to the control and its high efficiency towards pathologically changed keratinocytes, combined with a low toxicity profile towards normal healthy keratinocytes was demonstrated in an array of *in vitro* models, which indicates the prospect of creating a new soft dosage form with this complex for treatment of psoriasis.

Study limitation. This study is limited to using *in vitro* models only, which does not allow us to conclude about the effectiveness and safety of the PNO-SA combination *in vivo*.

Further research prospects. Further studies should be carried out addressing the effectiveness and safety of the PNO-SA combination in the setting of *in vivo* experimental models of psoriasis.

6. Conclusions

The local application of drugs capable of controlling and regulating the production of cytokines by keratinocytes in psoriasis is extremely promising and interesting, as it usually causes a less toxic effect on the body in contrast to the systemic action of drugs. Therefore, our proposed combination (PNO-SA), which suppresses pro-inflammatory IL-8 by more than 2 times (by 67.4 %) compared to the control, is a promising potential option for local treatment of psoriatic lesions. We speculate about the future demand of this complex in the clinical setting because along with high efficiency (in terms of viability in-

dicators – the number of living cells, the level of apoptosis) towards pathologically changed keratinocytes, it shows a low toxicity profile towards normal keratinocytes.

Conflict of interest

The authors declare that they have no conflict of interest related to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available at a reasonable request.

Use of artificial intelligence

The authors confirm they did not use artificial intelligence technologies when creating the current work.

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