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STUDIES OF NEW PURINE DERIVATIVES WITH ACETIC ACID MOIETY IN HUMAN KERATINOCYTES

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Abstract: Recently we described a group of purine derivatives based on theophylline structure with acetic acid moiety. Studies in a group of these compounds demonstrated their analgesic and anti-inflammatory properties. Taking into account wide spectrum of theophylline derivatives activity and searching for their new properties. the aim of the study was to evaluate safety of newly synthesized derivatives in human keratinocytes model. The effect of new purine derivatives with acetic acid moiety: 2-(8-methoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl) acetic acid and 2-(1,3-dimethyl-2,6,8-trioxo-9H-purin-7-yl) acetic acid on proliferation rate and the ability of keratinocytes to migration was carried out. The results clearly demonstrate that purine derivatives with acetic acid moiety did not affect basic keratinocytes functions. Our compounds do not inhibit cells proliferation rate as well as their ability to migration. It can be therefore concluded that new purine derivatives with acetic acid moiety are safe *versus* normal cells. This observation opens up additional prospects in searching for their new applications.

Keywords: theophylline, keratinocytes, safety, proliferation, cell migration

Theophylline (1,3-dimethyl-7H-purine-2,6-dione) is a xanthine, belonging to the group of purine alkaloids. Naturally occurs in cocoa beans, tea leaves as well as in coffee grains and cola seeds (1). This alkaloid was firstly discovered at the end of the nineteenth century in tea leaves and since beginnings of the twentieth century is produced synthetically on a large scale (2, 3). Theophylline is the only compound among methylxanthines currently used in the treatment. Due to its immunomodulatory, anti-inflammatory, bronchoprotective and spasmolytic properties is used in bronchial asthma and chronic obstructive pulmonary disease (COPD) therapy (4-6). Theophylline is also known for its diuretic properties. Its involvement in the extension of vascular and airway smooth muscle has been also described in the literature (2).

Conducted for many years research, has shown that this methylxanthine affects many processes occurring inside the cells. However, its specific mechanism of action is still unknown. It has been shown that theophylline is a non-specific phosphodiesterase inhibitor as well as an antagonist of the adenosine receptors (7). Immunomodulatory function of this alkaloid is manifested mainly by increasing secretion of IL-10 by peripheral blood mononuclear cells (8), enhancing PPARγ level in eosinophils (9) and induction of neutrophils and T lymphocytes apoptosis (2). Theophylline used at low doses causes also activation of histone deacetylases, and thereby can enhance the anti-inflammatory effect of corticosteroids (10).

Over recent years in the search for new drugs the most important are modifications of previously known structures. Alterations of parent theophylline core fit very well into this "drug repurposing" trend. Due to the interesting properties and chemical structure, this compound is modified for many years (11). In the course of our search for new biologically active compounds based on theophylline structure

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we described a group of purine derivatives with acetic acid moiety (12). Among the evaluated compounds, 2-(8-methoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl)acetic acid (purine-2,6-dione derivative, **1** and 2-(1,3-dimethyl-2,6,8-trioxo-9H-purin-7-yl)acetic acid (purine-2,6,8-trione derivative, **2** (Fig. 1) have been tested for anti-inflammatory and analgesic activity in the *in vivo* models. Compound **1** decreased neutrophils count and inhibited intensity of early vascular permeability in rats. This derivative displayed also high activity in the zymosan-induced peritonitis. Furthermore, this compound significantly inhibited TNF-α production in plasma of rats with endotoxemia (13). Compounds **1** and **2** displayed also analgesic activity confirmed in two applied *in vivo* models: the writhing syndrome and the formalin tests (12, 13, our unpublished data). Considering interesting pharmacological properties of our new compounds we decided to check their safety in the *in vitro* model.

Our study was aimed to assess the cytotoxic effect of new purine derivatives with acetic acid moiety in human keratinocytes model. We evaluated also the influence of evaluated compounds on keratinocytes ability to migration, one of the basic parameters which characterize phenotype of these cells.

EXPERIMENTAL

Isolation and culture of primary human skin keratinocytes

The human keratinocytes were isolated from skin fragments derived from plastic surgeries with the consent of the Jagiellonian University Bioethics Committee (No.: KBET/72/B/2008) and informed, written consent was obtained from all study participants. Isolation procedure was performed according to the protocol described by Drukała et al. (14). Cells were cultured in a defined, serum-free, KGM-Gold medium (Lonza) at 37°C temperature, 5% CO_2 atmosphere and 95% humidity. Culture medium was changed every 48 h. For the experiments, cells between 3-7 passages were used. For monitoring cells morphology Nikon Eclipse TS100 phase contrast microscope was used.

Purine derivatives with acetic acid moiety synthesis

Purine-2,6-dione (**1**) and purine-2,6,8-trione (**2**) derivatives were synthesized as described previously (13, 15).

Compounds preparation

Compounds **1** and **2** were diluted in the culture medium from stock solution (1 mg/mL prepared in

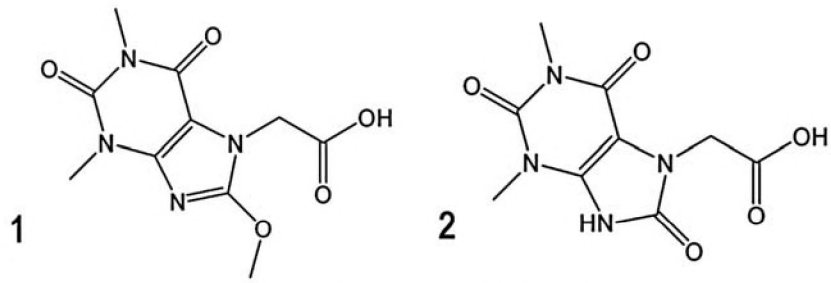

Figure 1. Structures of investigated compounds **1** and **2**

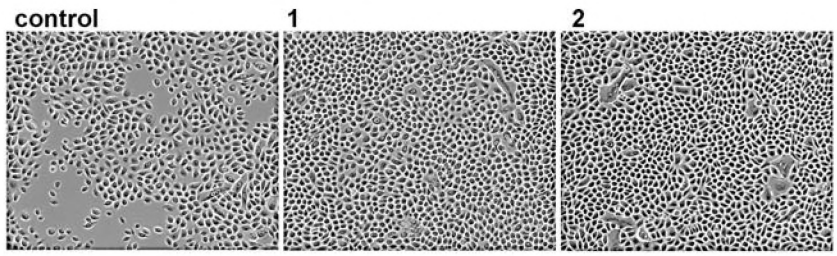

Figure 2. Morphology of keratinocytes after 72 h incubation with **1** or **2** at concentration 200 μM in comparison to control conditions. Pictures were taken using inverted bright field, microscope with IMC contrast

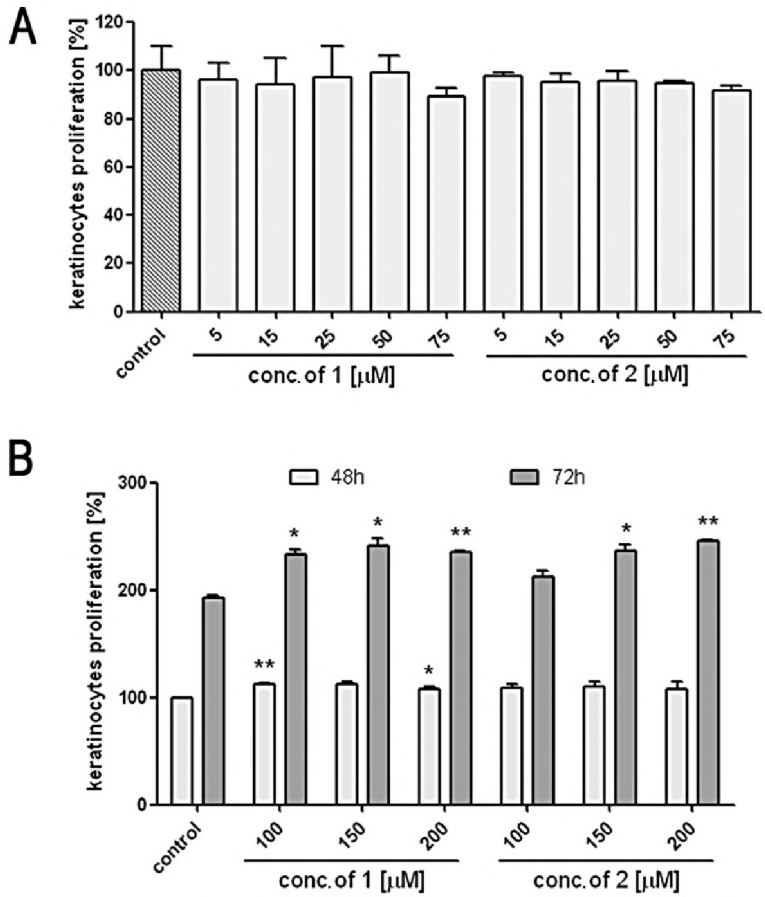

Figure 3. The effect of investigated compounds **1** or **2** on keratinocytes proliferation rate. Cells were cultured with growing concentrations (5-75 μM – A and 100-200 μM – B) of **1** or **2** and counted after 48 h (A) and 48/72 h (B) of incubation. Bars represent mean value of cells proliferation expressed in percentages with S.D. Experiments were run in duplicates.* $p < 0.05$; ** $p < 0.01$

dimethyl sulfoxide, DMSO; Sigma-Aldrich) to the working concentration: 5, 15, 25, 50, 75, 100, 150, 200 μM). The medium containing an amount of DMSO equivalent to the highest applied **1** and **2** concentration (0.5‰) was taken as a solvent control. At this concentration, DMSO did not exert an effect on human keratinocytes basic features and functions like morphology, proliferation, viability and motility (data not shown).

Proliferation assay

For proliferation assay, cells were seeded into 12-well plates at an initial density of 5×10^3 cells/cm^2 (10×10^3 cells/cm^2 at lower compound concentrations), in KGM-Gold medium and culture for 24 h in standard conditions. After 24 h, the culture medium was replaced with a fresh one containing **1** or **2**. Unstimulated cells represented an experiment control. Cells were incubated with tested compounds for 24 and 72 h and then harvested by trypsinization and counted using Scepter™ 2.0 Cell Counter (Millipore).

Time lapse-monitoring of movement of individual cells

Keratinocytes were seeded into 12-well plates at a density of 5×10^3 cells/cm^2 and cultured in KGM-Gold medium in standard conditions. After the cells reach the appropriate density, culture medium was replaced with medium containing compounds **1** or **2** at 5, 15, 25, 50 or 75 μM concentrations and then cultured for 24 h. After that time, cells migration was recorded for 90 min at 90 s time intervals, using the LEICA DMI 6000B microscope. The quantitative analysis of cells movement parameters was performed using the Hiro 1.0.0.4 software (16). For each data point 50 cells were analyzed. The cell trajectories were presented in circular diagrams with the starting point of each trajectory situated at the plot center.

Table 1. Movement parameters of human keratinocytes in control condition and after 1 or 2 treatment. Values represent means with SD. CME = coefficient of movement efficiency - it means total length of cell displacement to total length of cell trajectory ratio.

COMPOUND	COMPOUND CONCENTRATION [μM]	MOVEMENT PARAMETERS				
		Total length of cell trajectory [μm]	Average speed of cell movement [μm/min]	Total length of cell displacement [μm]	Average rate of cell displacement [μm/min]	CME coefficient of movement efficiency
Control	0	227.46 ± 8.62	2.57 ± 0.10	147.53 ± 10.27	1.67 ± 0.12	0.63 ± 0.03
1	5	230.66 ± 10.30	2.61 ± 0.12	138.17 ±11.23	1.56 ± 0.13	0.59 ± 0.03
	15	220.90 ± 8.42	2.50 ± 0.10	135.42 ± 10.57	1.53 ± 0.12	0.60 ± 0.03
	25	239.99 ± 8.12	2.71 ± 0.09	158.69 ± 8.09	1.79 ± 0.09	0.66 ± 0.03
	50	234.18 ± 10.83	2.65 ± 0.12	157.06 ± 10.06	1.78 ± 0.11	0.65 ± 0.03
	75	237.63 ± 8.62	2.69 ± 0.10	156.31 ± 10.07	1.77 ± 0.11	0.65 ±0.03
2	5	232.82 ± 9.27	2.63 ± 0.11	166.60 ± 9.69	1.88 ± 0.11	0.70 ± 0.03
	15	247.88 ± 7.68	2.80 ± 0.09	159.04 ± 9.57	1.80 ± 0.11	0.63 ± 0.03
	25	255.78 ± 9.06	2.89 ± 0.10	180.39 ± 8.25	2.04 ± 0.09	0.71 ± 0.02
	50	231.24 ± 8.18	2.61 ± 0.09	150.49 ± 8.55	1.70 ± 0.10	0.66 ± 0.03
	75	220.81 ± 9.13	2.49 ±0.10	133.72 ± 7.59	1.51 ± 0.09	0.63 ± 0.03

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, USA). Comparisons of parameters between experimental conditions were performed using Student's t-test for unpaired data. Experiments were run in triplicates. The results are presented as the means with standard deviation. Statistical significance was assumed as $p < 0.05$ or $p < 0.01$.

RESULTS

Purine derivatives with acetic acid moiety do not affect the viability and morphology of human skin keratinocytes

Compounds **1** and **2** were previously demonstrated to possess analgesic properties (12, our unpublished data). The derivative **1** showed anti-inflammatory activity in *in vivo* tests (13). Therefore, we decided to investigate whether these new purine derivatives with acetic acid moiety may also affect the cell basic functions and features in *in vitro* experiments. In order to evaluate their safety, human skin keratinocytes cultures were used. Microscopic observation of cells after addition of the studied compounds showed that neither **1** nor **2** had an effect on keratinocytes morphology. As illustrated in Figure 2, compounds administrated in the highest tested concentration, after 72 h long culture did not affect cells phenotype. There were also no differences in the cells morphology after 24, 48 and 72 h of incubation with analyzed derivatives. Moreover, we found that new purine derivatives with acetic acid moiety were not toxic to human skin keratinocytes (data not shown).

Purine derivatives with acetic acid moiety slightly affect the proliferation rate of human skin keratinocytes

A number of compounds tested for toxicity appear to have no effect on cell viability. On the other hand, the same substances may affect the rate of cell proliferation, causing loss of division capacity. Since we found that the investigated compounds turned out non-toxic towards epithelial cells, we decided to explore their impact on keratinocytes proliferation. Performed experiments have shown that analyzed derivatives

used at lower concentrations (5-75 μM) did not affect keratinocytes proliferation rate. After 48 h of incubation, both compounds did not cause statistically significant changes in ability to cell divisions (Fig. 3A). Keratinocytes proliferation rate after 48 h slightly increased after incubation in higher concentration (100-200 μM) of purine derivatives with acetic acid moiety but only in case of purine-2,6-dione this effect is statistically significant (Fig. 3B). Further prolongation of keratinocytes culture in the presence of tested compounds for 72 h led to an increase in proliferation rate. Three days long cells incubations in the higher concentrations of purine-2,6-dione and purine-2,6,8-trione resulted in proliferation promoting effect (Fig. 3B). As illustrated in Fig. 3B, this effect is statistically significant for both compounds.

Purine derivatives with acetic acid moiety have no effect on human skin keratinocytes migration

Because increased proliferation activity of epithelial cells is associated with changes in the migration activity of these cells, in the next step we decided to explore the impact of purine derivatives with acetic acid moiety on keratinocytes motility. Keratinocytes in relation with their function in the epithelium, and particularly in the process of wound healing, are cells that possess high migratory activity. Also in our experimental model this effect has been confirmed. The movement trajectories of individual cells under control conditions are presented in a circular diagram (Fig. 4Aa). Surprisingly, we observed that our compounds did not change keratinocytes motility. Cells incubated in the presence of **1** (Fig. 4Ab-f) or **2** (Fig. 4Bg-k) in concentrations ranging from 5 to 75 μM showed no changes in migration activity. The analysis of keratinocytes movement parameters under the action of evaluated derivatives revealed that there cannot be observed any differences between various experimental conditions. Table 1 shows the individual results of quantitative analysis parameters such as length of cells trajectory, displacement, average speed of cells movement, rate of cells displacement and the most

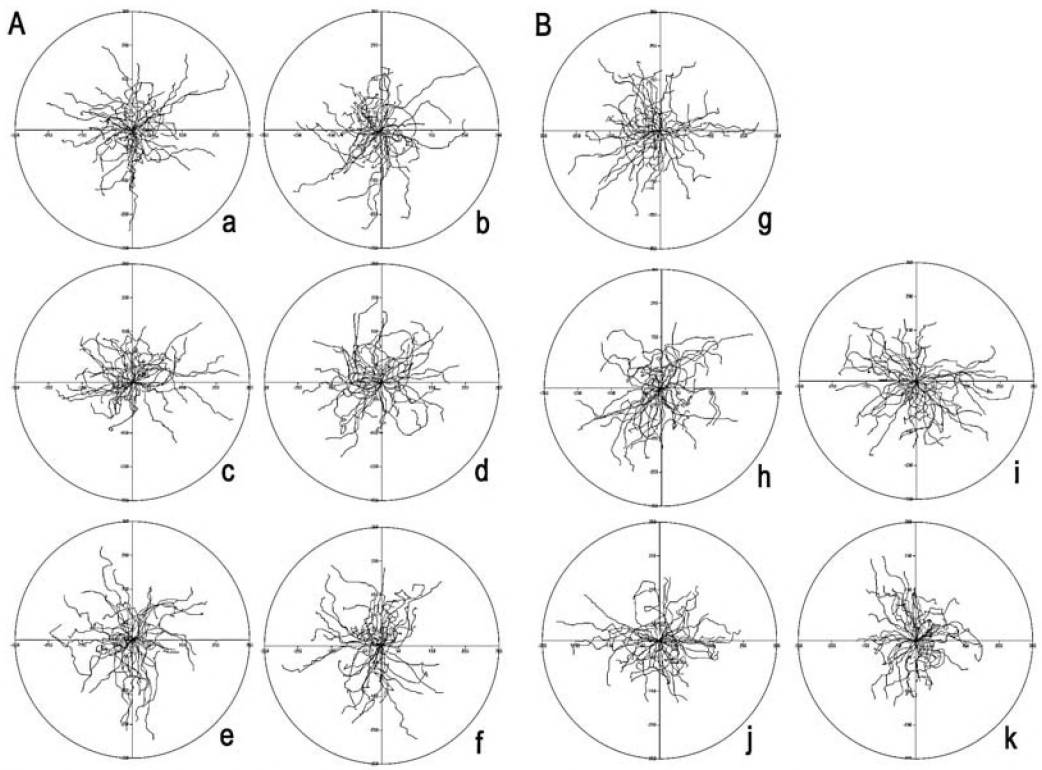

Figure 4. The influence of investigated compounds **1** or **2** on keratinocytes migration activity. Cells were cultured with growing concentrations (5-75 μM) of **1** (A) or **2** (B) and single trajectories of individual cells were analyzed. Circular diagrams represents cells migrating after **1** or **2** treatment in 5 μM (b,g) 15 μM (c,h), 25 μM (d,i), 50 μM (e,j), 75 μM (f,k) concentration and in control conditions (a). The beginning of single cell trajectory is marked on the graph at the point 0.0 of the coordinate system. 50 cells were analyzed in each experimental condition

imaging – coefficient of displacement efficiency. Thus, may be concluded that the effects of **1** and **2** on keratinocytes proliferation rate were not paralleled by changes in migration activity. The increased proliferation rate after **1** or **2** treatment did not correlate with human skin keratinocytes migration activity.

DISCUSSION AND CONCLUSION

Theophylline is a drug used in the clinic for over 100 years. Although described adverse effects, mainly due to its narrow therapeutic index, this drug is still intensively studied. Particular interest of many research groups is modifying the main theophylline structure and seeking for its new properties. In accordance with the principle of "an old drug – new applications", scientists are trying to find new possibilities of theophylline derivatives exploitation. In these stream undoubtedly fit searching in the group of purine derivatives with acetic acid moiety.

Newly synthesized and described compounds exhibited anti-inflammatory and analgesic activity in the *in vivo* experiments (12, 13, our unpublished data). This provided a rationale for further exploration of their new biological functions. Because contemporary science moving away from carrying screening with the use of laboratory animals and instead proposing *in vitro* models we decided to check the safety profile of our compounds in human keratinocytes model. These epithelial cells that constitute about 90% of the cells found in the epidermis, are a very good model for safety assessment of new compounds and testing their cytotoxicity (17-19).

Our research has shown that new purine derivatives with demonstrated anti-inflammatory and analgesic activity have proven to be safe in tests carried out in cellular model. The derivative of purine-2,6-dione as well as purine-2,6,8-trione were not cytotoxic towards epithelial cells - keratinocytes. This is very interesting especially in the context of a well-described in the literature effect of theophylline on the keratinocytes viability. Already many years ago, Chopra described that these cells treated with theophylline beginning to enter the apoptosis pathway (20). It was also demonstrated that this methylxanthine, in contrast to other immunomodulators, does not affect the reduction of T-cells-induced keratinocytes apoptosis (21). On the other hand, some research reported that methylxathines and among them theophylline co-administrated with anti-cancer drugs may protect cells by diminishing mutagenic effects of chemotherapeutics (22). Regardless of the described theophylline actions, our studies indicate that modifications within the parent compound structure are translated into its new biological properties and in case of new purine derivatives with acetic acid moiety may result in limiting its toxic effects towards human epidermal cells.

Rapid proliferation is the one of main features of keratinocytes. Moreover, proliferation dynamics and migration rate of these cells are very important for recovery of homeostasis of epithelial tissue during wound healing. Our experiments indicate that new purine derivatives with acetic acid moiety administrated at lower concentrations did not affect keratinocytes proliferation rate as well as its migration efficiency. A number of factors that, depending on therapeutic purpose, may inhibit keratinocytes proliferation as well as those that may enhance it, are described in scientific reports. The search for substances and methods limiting epithelial cell proliferation is carried out in the studies of pathogenesis diseases like psoriasis or atopic dermatitis (23-27). From the wound healing point of view compounds increasing proliferation rate are sought. Experiments conducted in the framework of new purine derivatives with acetic acid moiety safety testing showed that our compounds given in higher concentrations are capable of enhancing keratinocytes proliferation rate. It is well known that not cytotoxic and cytostatic towards epidermis cells compounds, and at the same time slightly inducing rate of cell division, may be potentially used in wound healing therapies (28-30). Taking into account purine-2,6-dione and purine-2,6,8-trione derivatives anti-inflammatory and newly discovered proliferation promoting properties, it appears that these compounds may have potential application in supporting the process of scarring.

During the study it was shown that evaluated compounds are not toxic and do not affect the movement parameters of keratinocytes. However, we were able to identify their proliferation-promoting effect. Considering that these compounds are safe relative to keratinocytes, it seems very interesting their further evaluation particularly in the context of their proliferation-promoting and anti-inflammatory effects.

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