Topically applied azaphenothiazines inhibit contact sensitivity to oxazolone in mice

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Topically applied azaphenothiazines inhibit contact sensitivity to oxazolone in mice

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Running title: Immunosuppressive azaphenothiazines

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Summary. In this work we investigated the efficacy of two topically applied azaphenothiazine derivatives, 9-chloro-6-acetylaminobutylinobenzo[3,2-b][1,4]thiazine (compound 4) and 6-chloroethylureidoethylquinob[3,2-b;2';3'-e][1,4]thiazine (compound 5), in the amelioration of inflammatory symptoms of contact sensitivity (CS) to oxazolone in mice, in relation to the commercial ointment Protopic® (tacrolimus), the reference drug. The compounds were administered 24 h following elicitation of CS and, 24 h later, the parameters of inflammation, such as ear edema, blood composition, leukocyte level, numbers of cells in the draining lymph nodes, histological picture of the inflamed tissue, and the morphometric analysis, were analyzed. The study showed that the effectiveness of the studied azaphenothiazines applied as a 0.1% ointment was comparable to the reference drug regarding suppression of the inflammatory process, when all the investigated histological parameters are taken into account.

Key words: Contact sensitivity, Oxazolone, Azaphenothiazines, Protopic®, Mice

Introduction

An increase in the incidence of inflammatory and autoimmune diseases has created a demand for new classes of immunosuppressive drugs. Presently available immunosuppressors, such as cyclosporine A, tacrolimus and pimecrolimus, which are generally classified as calcineurin inhibitors, are not devoid of undesirable side effects (Fredericks and Holt, 2003; Kim et al., 2010). However, for topical applications, calcineurin inhibitors tend to replace steroids, the side effects of which are more serious (Gutfreund et al., 2013). In general, an increased risk of skin malignancies and lymphomas, following application of calcineurin inhibitors, has not been found, but sun exposure should be avoided during therapy with this class of therapeutics (Prucha et al., 2013). The use of topical tacrolimus in pediatric eczema patients was found to be associated with the development of B-cell acute lymphoid leukemia (Cai et al., 2016), and isolated reports may also arouse concern (Shi et al., 2014). The most recent meta-analyses on the efficacy and safety of application of calcineurin inhibitors (Cury Martins et al., 2015; Siegfried et al., 2016) indicate a low risk of adverse side effects, particularly for short-term therapy. Nevertheless, attempts have been undertaken to replace steroids and calcineurin inhibitors with other immune suppressors with different mechanisms of action (Lebranchu, 2010).
Phenothiazines represent a class of compounds exhibiting various biological activities, such as antipsychotic, antihistaminic, antitussive and antiemetic (Gupta and Kumar, 1988). More recently, new biological properties of these compounds were described, such as antitumor, antibacterial and, associated with them, anti-multidrug resistance (MDR) activity, which was summarized by Jaszczyzyn et al., 2012. Neuroleptic phenothiazines, such as chlorpromazine, are also strong modulators of the immune response. The compound was found to suppress both humoral and cellular immune responses (Ichimura, 1978; Roudebush et al., 1991).

Classical phenothiazines with aminoalkyl substituents are an important source of valuable drugs (Gupta and Kumar, 1988). Modifications of phenothiazines with azine rings led to formation of azaphenothiazines. Such phenothiazines may contain not only a tricyclic ring system but also tetra- and pentacyclic ones with additional nitrogen atoms (from 1 to 4) in aromatic rings (Silberg et al., 2006; Pluta et al., 2009). Recently, we synthesized a series of azaphenothiazines by replacing benzene rings with pyridine and quinoline rings (Zimecki et al., 2009; Pluta et al., 2010; Jeleń et al., 2013; Jeleń et al., 2015; Morak-Młodawska et al., 2015). These investigations involved screening about 150 compounds for their potential biological activities, mostly in in vitro models. Several compounds from that group strongly inhibited mitogen-induced proliferation of human peripheral blood mononuclear cells, tumor necrosis factor alpha production and growth of tumor cell lines. Among these compounds, only five that showed the strongest suppressive actions were chosen to evaluate their suppressive potential in mouse in vivo models. Eventually, in the mouse models of delayed type hypersensitivity to ovalbumin and carrageenan-induced footpad inflammation, we selected only two compounds (Artym et al., 2016) that proved particularly suppressive.

Contact sensitivity (CS), manifested as sensitizer-elicited skin inflammation, constitutes a worldwide problem (Oppel and Schnuch, 2006; Wolf et al., 2013). One of the experimental models related to this type of immune reaction is contact skin sensitivity to oxazolone in mice (Noonan and Halliday, 1978).

The aim of this investigation was to assess the potential therapeutic value of these compounds, applied topically in the form of an ointment, in the amelioration of contact sensitivity in this model, with a particular emphasis on histological investigations.
Materials and Methods

Mice
BALB/c female mice, 8-10 weeks old, delivered by the Institute of Laboratory Medicine, Łódź, Poland, were used for the study. The mice were fed a commercial, pelleted food and water ad libitum. The local ethics committee at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw approved the study (permission # 64/2015 and 79/2015).

Reagents
9-chloro-6-acetylaminobutylquinobenzo[3,2-b][1,4]thiazine (compound 4) (Jeleń et al., 2013) and 6-chloroethylureidoethylidiquino[3,2-b;2’;3’-e][1,4]thiazine (compound 5) (Pluta et al., 2010) were synthesized as previously described. We decided to preserve their denotation as #4 and #5 since they correspond to the same compounds described in the preceding article (Artym et al., 2016). The commercial ointment Protopic® (0.1% tacrolimus) originated from Astellas, Ireland, DMSO, oxazolone, acetone, Evans blue, Giemsa, May-Grünwald, hematoxylin, eosin and formalin were from Sigma-Aldrich. The ointment base consisting of vaseline and lanolin (50/50 v/v) was purchased in a pharmacy store. The compounds were thoroughly distributed in the ointment by 1 h manual stirring of 1 mg of compounds with 1 g of the ointment base to obtain 0.1% concentration.

The antibodies used for the immunostaining were as follows: rat anti-mouse Ly6G antibody (component of antigen Gr-1, clone RB6-8C5, Thermo Fisher Scientific), rabbit anti-mouse F4/80 antibody (clone SP115, Thermo Fisher Scientific), rat anti-mouse Langerin/CD207 antibody (clone 929F3.01, Novus Biologicals), and rabbit anti-mouse CD3 antibody (clone SP7, Thermo Fisher Scientific). We used ImmPRESS REAGENT KIT anti-rabbit Ig (Vector) and ImmPRESS REAGENT KIT anti-rat IgG, mouse adsorbed (Vector) as a detection system. ImmPACT™ DAB (Vector) and ImmPACT™ NovaRED™ (Vector) were used as chromogens.

Contact sensitivity to oxazolone
The test was performed according to Noonan and Halliday (Noonan and Halliday, 1978) with some modifications. Mice were shaved on the abdomen (2×2 cm area) and after 24 h 100 µl of 0.5% oxazolone in acetone was applied. The contact sensitivity reaction was elicited 5 days later by application of 50 µl of 1% oxazolone in acetone on both sides of the ears. The ear
edema was measured 48 h later using a spring caliper (Scheme 1). This group of mice will be referred to as the “positive” control. The mice that received only the eliciting dose of oxazolone and the vehiculum 24 h later will be termed the “background” (BG) group. This group of mice will develop only a nonspecific irritation reaction to oxazolone, dissolved in acetone. The results showing the ear edema are presented as an antigen-specific increase of ear thickness, i.e., background ear thickness of mice, given only the eliciting dose of the antigen (non-specific irritation response), is subtracted from the responses measured in sensitized mice. The results are presented as a mean value of ear thickness measured in 8 mice (both ears measured) and expressed in mm ± standard error (SE).

Application of the compounds
The compounds were applied in the form of a 0.1% ointment, topically, on both sides of the ears (total volume of 50 µl per one ear), 24 h after administration of the eliciting dose of oxazolone (the peak of the inflammatory reaction, Scheme 1). Protopic® as a reference drug (0.1% ointment) was used in its commercially available form in a similar fashion.

Determination of lymph node cell numbers
Superficial parotid, mandibular, accessory mandibular lymph nodes were isolated, homogenized by pressing against a stainless screen into PBS and re-suspended in PBS containing 0.2% Trypan blue. The total number of cells was determined using a light microscope and Bürker’s hemocytometer. Mice treated only with the eliciting dose of antigen served as a background control.

Determination of circulating leukocyte number and blood picture
Mice were subjected to isoflurane anesthesia and bled from the retro-orbital plexus, followed by cervical dislocation. The number of blood leukocytes was determined by dilution of blood in Türk’s solution and by counting the cells in a hemocytometer. Blood smears were prepared on microscope glass, dried and stained with Giemsa and May-Grünwald reagents. The smears were subsequently reviewed histologically. Up to 100 cells were counted on two glasses. The circulating leukocyte numbers were presented per 1 mm³ and the blood cell compositions as a percentage of a given cell type.
Histological analysis

The mice auricles were fixed in 4.0% neutral buffered formalin, dehydrated in an alcohol series, cleared in xylene and embedded in paraffin. The paraffin blocks were sliced in Micron HM310 microtome into 5 µm sections. The paraffin sections were stained with hematoxylin and eosin (H&E) to identify the cellular infiltrates (data not shown) and with toluidine blue in order to detect mastocytes in the connective tissue of auricles. To identify the cellular infiltrates observed in H&E stained sections, the immunohistochemical analysis was carried out. Neutrophils were detected with rat anti-mouse Ly6G antibody (component of antigen Gr-1, clone RB6-8C5), macrophages with rabbit anti-mouse F4/80 antibody (clone SP115), Langerhans cells with rat anti-mouse Langerin/CD207 antibody (clone 929F3.01), and T lymphocytes with rabbit anti-mouse CD3 antibody (clone SP7). The primary antibodies were detected using ImmPRESS REAGENT KIT anti-rabbit Ig and ImmPRESS REAGENT KIT anti-rat IgG, mouse adsorbed according to manufacturer instructions. Antigen retrieval was performed using sodium citrate buffer pH -6 (for: Ly6G, F4/80, CD3) and Tris/EDTA buffer pH 9 (for CD207) for 20 minutes at 97°C in water bath. Slides were incubated in 3% H₂O₂ solution to quench endogenous peroxidase activity and blocked in 2.5% normal goat blocking serum. Sections were incubated for 1 h at room temperature with antibodies at final dilutions: Ly6G 1:800, CD3 1:500, F4/80 1:300, CD207 1:500. ImmPACT™ DAB and ImmPACT™ NovaRED™ were used as chromogens. Sections were counterstained with Meyer's Hematoxylin. In all immunohistochemical staining, to exclude non-specific reactions, negative controls were included.

Quantitative analysis of labeled cells (neutrophils, macrophages, T cells, mastocytes and Langerhans cells) was achieved by counting positively stained cells in 8 random high-power fields for each animal. From every group, 8 animals were analyzed. In addition, the cross-sectional area of the pustules in the epidermis was measured and expressed in µm². The measurements were performed on both sides of auricles, from the base toward the apex. Quantitative and morphometric analyses were performed in the light microscope Nikon Eclipse 80i with the aid of imagine software NIS-Elements at 100× magnification.

Statistics: The results are presented as mean values ±SE. Brown-Forsyth’s test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey’s test to estimate the significance of the differences between groups. Nonparametric data were evaluated with the Kruskal-Wallis analysis of variance, as indicated
in the text. Significance was determined at p<0.05. Statistical analysis was performed using STATISTICA 7 for Windows.

Results

Determination of ear thickness

Fig. 1 presents the effects of treatment of the ears with the investigated compounds during the maximal inflammatory reaction to oxazolone (24 h following elicitation of the reaction). Protopic® was used as a reference drug. The antigen-specific increase of ear thickness was shown by subtracting the ear thickness of mice treated only with the eliciting dose of oxazolone (representing only a nonspecific irritation). The effects of compound 4 and 5 were strongly inhibitory (p<0.001). The suppressive action of compound 5 (by 56.8%) was stronger in comparison to its counterpart (by 42.6%) and both compounds were more effective than Protopic® (19.2% inhibition, p<0.05).

Effects of the compounds on the circulating blood parameters and lymph node cell numbers

Although contact sensitivity to oxazolone was elicited locally, it had a profound, stimulatory effect on the number of circulating blood leukocytes (Fig. 2) caused by an increase of the myelocytic cell content (Fig. 3). Although the effect of Protopic® on the blood leukocyte number was minor, the investigated compounds lowered the leukocyte levels to the background values (Fig. 2). The elicitation of contact sensitivity to oxazolone induced a strong output of the myeloid cell types into circulation, represented mainly by band cells and eosinophils (Fig. 3). The content of these cells was lowered by Protopic® (not statistically significant effect). The azaphenothiazines were more effective, particularly compound 5 (its suppressive action on the content of neutrophils, bands and eosinophils was significant – p<0.05). A significant, suppressive action of compound 4 was found only in the case of eosinophils (p<0.05).

On the other hand, the suppressive effect of compound 4 on the number of cells in the draining lymph nodes (Fig. 4) was stronger than that of compound 5 and Protopic® and was statistically significant (p<0.001).
Histological investigations

Fig. 5-9 illustrate the effects of the studied azaphenothiazines and the reference drug, applied topically at the time of maximal intensity of the inflammatory process, on the cellular and extracellular matrix changes. The slides were stained for the Ly6G neutrophil marker, CD3 lymphocyte T marker, F4/80 macrophage marker, CD207 Langerhans cell marker and toluidine blue – mastocyte marker.

The histological picture of auricles of naive mice show a presence of single Ly6G-positive cells (neutrophils), located mainly in the lumen of blood vessels, single F4/80-positive cells (macrophages) in the dermal and subdermal regions, as well as rare CD3-positive T cells, occurring both in the epidermis as well as in the dermis (Fig. 5, 6, 7). Single Langerhans cells were observed in the spinosus cell layer of the epidermis (Fig. 8).

In the auricles of control mice with full-blown inflammation (the sensitized mice following application of the eliciting dose of oxazolone), a severe inflammatory process, involving the epidermis and the dermis, was observed. Histological analysis revealed abundant inflammatory cell infiltrations in the dermis as well as in the subcutis. Dilation and hyperemia of the blood vessels and extravasations were registered. In addition, widened spaces between the collagen fibers were observed, indicating an edema. This was confirmed by histometric measurements, demonstrating an increase of auricle thickness. The immunohistochemical stainings showed that the cell infiltrations in the dermis consisted predominantly of neutrophils and not numerous T lymphocytes and macrophages (Fig. 5, 6, 7). In the control group of mice with full-blown inflammation, the neutrophil numbers were highest in comparison to other groups. Toluidine blue staining demonstrated a decrease in mastocyte content in comparison to naive mice (Fig. 9). In addition, the immunohistochemical analysis revealed a slight decrease in the number of Langerhans cells (Fig. 8). Along the whole epidermis, small vesicles and numerous, large, neutrophil-containing pustules occurred (Fig. 5). Locally, particularly near the ear base, epidermal necrosis was observed.

The BG group (the mice exposed once to oxazolone) presented a moderate inflammatory state of the dermis. The inflammatory changes occurred in both the epidermis and dermis, but the changes were smaller than in the control, “positive” group. Neutrophils, T lymphocytes and macrophages in the dermis were also less numerous (Fig. 5, 6, 7). In the dermis, an edema was sustained. In the epidermis, focal spongiosis, vesicles and pustules were found. The pustules in the epidermis were less numerous in comparison to mice with full-blown inflammation (Fig. 5). Crusts were also registered. The above described inflammatory
changes are probably a result of nonspecific irritation (irritant contact dermatitis; ICD) caused by acetone and the first exposure to oxazolone (Gittler et al., 2013).

In the group of mice treated with Protopic® a distinct silencing of the inflammatory process was observed in comparison to the respective control. H&E staining revealed moderate inflammatory infiltrations within the dermis (data not shown). Immunostaining showed a decrease in the number of neutrophils and macrophages and a small increase of T lymphocytes (Fig. 5, 6, 7). Protopic® was efficient in diminishing the frequency and magnitude of pustules in the epidermis, where mainly small pustules were observed (Fig. 5).

Compounds 4 and 5 were most efficacious in diminishing the inflammatory infiltrations in the dermis. The compounds caused statistically significant drops in the numbers of neutrophils and macrophages as compared to the control, “positive” group (Fig. 5, 7). On the other hand, the content of Langerhans cells slightly increased in relation to the control group (Fig. 8). Both compounds diminished the intensity of changes in the epidermis. Compound 4, however, was more efficient in decreasing the area size of pustules and compound 5 in limiting their numbers.

The results, depicted in Table 1, indicate that a significant infiltration of neutrophils occurred already upon the first encounter with the antigen (oxazolone), but it was almost doubled upon second exposure to oxazolone. The suppressive effects of the compounds in terms of neutrophils content may be described as follows: Protopic® (by 24.4%), compound 4 (by 30.5%) and compound 5 (by 30.7%). It is also clear that the inflammatory process also attracts lymphocytes. A very low level of lymphocytes in naive mice was increased in mice treated once with oxazolone and further (by 2-fold) upon elicitation of the CS. However, the studied compounds did not alter the content of this cell type. Langerhans cell content was increased in BG mice, as compared to naive mice, but returned to initial values in the “positive” control mice and mice treated with Protopic®. The azaphenothiazines, in particular compound 4, caused an increase in this parameter in comparison to “positive” control mice.

The mastocyte content dropped in the BG and control mice, with an additional decrease in mice treated with Protopic® and compound 4 and 5. The occurrence of macrophages was increased in mice exposed to oxazolone (BG and “positive” control group) with some decrease in azaphenothiazine-treated mice.

Table 2 presents the effects of the compounds on the number and magnitude of pustules in the epidermis, as very relevant parameters of the dermal inflammatory process. The pustules were categorized according to their area, expressed in µm², and pustules of 10^4 µm² were most frequent. The pustules already appear in mice exposed to a single dose of antigen, which in
this case may be regarded as an irritating agent, together with its solvent (acetone). Nevertheless, in the case of mice treated twice with the antigen, the number of most frequent pustules rose by almost 4-fold. The effects of the studied compounds and the reference drug were differential. Protopic® did not lower the number of small pustules (up to \(10^3 \, \mu m^2\)), but the occurrence of bigger pustules was more markedly suppressed. The treatment with azaphenothiazines led to a decrease of the number of pustules in a \(10^3 \, \mu m^2\) area.

Discussion

The results of this study revealed a high efficacy of compounds 4 and 5, applied as a 0.1% ointment, in reducing inflammatory phenomena associated with the effectual phase of the CS to oxazolone in mice. The compounds were selected not only because of their strong immunosuppressive actions but also on the basis of low toxicity towards human peripheral blood lymphocytes: compound 4 (Jeleń et al., 2013) and compound 5 (Pluta et al., 2010). The anti-inflammatory effects of the compounds were stronger or at least comparable to those of Protopic®, the reference compound, when various parameters inherent in the inflammatory process are taken into account. Besides reduction of ear thickness, other indices of the inflammation, such as the number of lymphocytes in the draining lymph nodes, blood composition, the number of infiltrating neutrophils in the perivascular zone of the ear connective tissue, and the number of pustules, were correlated with the inhibition of ear swelling.

The appearance of respective cell types at the time of maximal inflammatory changes was typical and involved predominantly neutrophils and lymphocytes, normally not present in naive mice, consistent with other observations (Engeman et al., 2004). In fact, we should not expect significant differences in the density of cell types involved in antigen recognition and migration of specific T cells, since the analyzed skin sections relate to the diminishing effectual phase of CS reaction. Neutrophils represent a major cell type in all inflammatory processes, including delayed type hypersensitivity reactions (Lee et al., 1988), being a source of inflammatory mediators (neutrophil proteases). And indeed, the content of neutrophils (Table 1) was directly associated with the therapeutic effects of the investigated compounds. The increased density of Langerhans cells in the BG mice may be explained by nonspecific irritation by the antigen vehicle (acetone) and oxazolone itself (Gittler et al., 2013). The number of Langerhans cells was decreased in “positive” control mice indicating their crucial role in antigen recognition and subsequent migration to draining lymph nodes (Hitzler et al.,
On the other hand, moderately increased numbers of Langerhans cells in mice treated with compounds 4 and 5 (Table 1) may suggest a role of this cell type in suppression of the inflammatory reaction mediated by antigen-specific T cells (Hitzler et al., 2012). There was no difference in the macrophage content between mice exposed once and twice with oxazolone, suggesting that the role of macrophages may be restricted to production of some proinflammatory mediators (Ferreri et al., 1991) during contact sensitivity. Likewise, a decrease in the mastocyte content in the sensitized mice indicates their role in the process of CS, although their presence during the whole process is relatively constant (Kerdel et al., 1987). The effects of the studied compounds on cell composition in the dermis and other parameters of blood and lymph nodes were differential, and are probably associated with a different mechanism of action. Tacrolimus, for example, blocks expression of several T-cell activators, including IL-2, IL-4 and tumor necrosis factor α (TNF α) (Tocci et al., 1989). Thus, the early T cell activation steps are inhibited, which may also be the case for the recruitment of antigen-specific T cells. Interestingly, tacrolimus, as well as the azaphenothiazines, did not inhibit infiltration of lymphocytes to the inflammatory site. It is probable that the infiltrating lymphocytes belong to several subtypes and also include nonspecific T cells (a bystander recruitment). Our previous studies revealed that compounds 4 and 5, both classified as azaphenothiazines, may also exhibit different mechanisms of action. Compound 5 was strongly antiproliferative with regard to tumor cell lines (Pluta et al., 2010) and elevated expression of caspase 3, Fas and Bcl2 in Jurkat cells (Artym et al., 2016) indicated apoptosis as a major mechanism of its action. Compound 4, in turn, demonstrated strong antiproliferative action in mitogen-stimulated lymphocytes and inhibition of lipopolysaccharide-induced TNF α in blood cell cultures (Jeleń et al., 2013), but was devoid of apoptotic action (unpublished data) and did not lower viability of human blood lymphocytes (Jeleń et al., 2013). The inhibition of TNF α production by compound 4 could explain its suppressive effects in a CS model since TNF α produced by keratinocytes upon contact with sensitizers is crucial for activation of Langerhans cells (Haas et al., 1992).

In terms of potential mutagenicity of the investigated compounds, some data in the literature indicate that they may be devoid of such properties. For example, phenothiazines with a half mustard (chloroethylureidoalkyl) substituent (also present in compound 5) were not mutagenic (Motohashi et al., 2000). Of interest, they even enhanced natural killer (NK) cell activity, possibly via direct stimulation of NK cells or large granular lymphocytes, suggesting their role in the immunologic defense against malignancy (Petri et al., 1996). In another report
(Motohashi et al., 2006), the authors showed that tetracyclic phenothiazine reduced the induced mutation by 27%, as it is a more potent antimutagenic agent than chlorpromazine.

We have also taken into account the possibility that compounds 4 and 5 may contain substituents potentially responsible for sensitization of the immune system. Compound 4 has the acetyloaminobutyl group, predominantly responsible for its biological activity, and the chlorine atom in the benzene ring, which is less important for biological activity and rather acts as an enhancer of the main substituent activity. A chlorine atom is present in other sensitizers like dinitrochlorobenzene and tetrachlorosalicylaniline, but in these compounds the nitro group is responsible for sensitization (Sachs et al., 2001). Compound 5 has the chloroethyloureidoethyl substituent, which is not present in typical sensitizers. In summary, the substituents at the thiazine nitrogen atom, which condition biological activities, are not present in major sensitizers.

The mechanisms of CS suppressions by various compounds and proteins are differential and may involve: inhibition of the proinflammatory cytokine production (Cho et al., 2010A; Pinto et al., 2010; Jung et al., 2013) and NF-kappaB binding activity (Cho et al., 2010B), immune deviation (Zimecki et al., 2012) and blocking T cell-antigen presenting cell interaction by binding to CD4 molecule (Sugiura et al., 2010).

In this study we also evaluated the efficacy of Protopic®, the reference therapeutic for the investigated azaphenothiazines. Protopic® contains tacrolimus, a calcineurin inhibitor, as the active compound. The suppressive effects of Protopic® on skin histological changes were strong and comparable to those of the azaphenothiazines. On the other hand, the drug had some undesirable effects on the systemic indices, such as blood composition, blood leukocyte numbers and the number of cells in the draining lymph nodes. It seems that several factors could account for such actions of Protopic®. Application of tacrolimus in atopic dermatitis is generally well-tolerated but not devoid of side-effects such as skin irritation, particularly in the first stage of therapy (Rustin, 2007; Remitz and Reitamo, 2009). In addition, topical tacrolimus may transiently induce mast cell degranulation in mouse skin (Ständer et al., 2007), which may account for its lesser efficacy in diminishing skin inflammation, which was also observed in our study, in comparison to the studied azaphenothiazines. Consistent with the transient pro-inflammatory properties of tacrolimus were higher numbers of infiltrating neutrophils in the ear perivascular connective tissue, as compared with the studied azaphenothiazines, no diminution of circulating leukocyte level and lower ability to normalize the distorted blood composition. A possibility to permeate blood circulation, as found in a clinical trial (Draelos et al., 2005), could account for such effects of tacrolimus. Another
limitation for use of topical calcineurin inhibitors is a possibility of disruption of the skin barrier, which may lead to susceptibility to infection (Kim et al., 2010).

In conclusion, this study demonstrated yet another possible therapeutic utility of azaphenothiazines, which, when applied topically, inhibit the effectual phase of contact sensitivity with efficacy comparable or ever better than that of the reference drug tacrolimus in the form of a commercial ointment (Protopic®). Compound 4 appears to be more attractive in this model than compound 5, taking into consideration its slightly better effects on skin inflammation and lack of toxicity towards lymphocytes, as previously shown (Jeleń et al., 2013).

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References


### Table 1

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### Table 2

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<td>7</td>
<td>0</td>
<td>3</td>
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**Figure and table legends:**

**Scheme 1** Experimental design of the test. See Materials and Methods section for a description of the experimental protocol.

**Fig. 1** Effects of the compounds on antigen-specific increase of ear edema.
The mice were sensitized with oxazolone and the CS reaction was elicited after 5 days as described in the Materials and Methods. The compounds and Protopic® were applied topically on the auricles as a 0.1% ointment 24 h after elicitation of the CS. After the next 24 h, ear edema was measured using a spring caliper. The results are presented as mean values from 8 mice (16 determinations) as a mean value of antigen-specific increase in ear thickness and expressed in mm ±SE.
Statistics: * p<0.05; # p<0.001 versus Control

**Fig. 2** Effects of the compounds on the number of circulating blood leukocytes.
The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds, the mice were bled and the blood leukocyte numbers were enumerated as described in the Materials and Methods. The results are presented as mean values of the number of leukocytes per mm$^3$ from 10 mice ±SE.
Statistics: * p<0.05; # p<0.001 versus Control

**Fig. 3** Effects of the compounds on circulating blood cell composition.
The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds, the mice were bled, blood smears prepared, stained with Giemsa and May-Grünsfeld reagents, and blood composition analyzed by a histologist. The following cell types were visualized: bands (immature neutrophils) (B), mature neutrophils (Ne), eosinophils (Eo), lymphocytes (L), and presented as a percentage content.

**Fig. 4** Effects of the compounds on the numbers of lymphocytes in the draining lymph nodes.
The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds the draining lymph nodes were isolated, homogenized and lymphocytes counted in a hemocytometer. The results are presented as the number of cells (10$^6$) per organ.
**Fig. 5** Effects of the studied compounds on the neutrophil infiltration of the auricles (staining with anti-Ly6G antibody; 100× magnification; scale bar = 200 µm).

The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds the ears were cut off and subjected to procedures described in the Materials and Methods.

A – auricle of naive mouse (normal appearance), B – auricle of a mouse from the BG group (weak to moderate neutrophil infiltration, small and medium-sized pustules in the epidermis), C – auricle of a mouse from the control group (extensive neutrophil infiltration in the dermis, edema in the dermis and large pustules in the epidermis), D – auricle of a mouse treated with Protopic® (moderate neutrophil infiltration, small pustules in the epidermis), E – auricle of a mouse treated with compound 4 (moderate neutrophil infiltration and small pustules in the epidermis), F – auricle of a mouse treated with compound 5 (moderate neutrophil infiltration, medium pustules in the epidermis); arrowheads indicate pustules.

**Fig. 6** Effects of the studied compounds on the T lymphocyte infiltration of the auricles (staining with CD3 antibody; 100× magnification; scale bar = 200 µm).


**Fig. 7** Effects of the studied compounds on the macrophage infiltration of the auricles (staining with F4/80 antibody; 100× magnification; scale bar = 200 µm).


**Fig. 8** Effects of the studied compounds on the Langerhans cells (LC) number of the auricles (staining with CD207 antibody; 100× magnification; scale bar = 200 µm).

A – auricle of naive mouse (normal appearance), B – auricle of a mouse from the BG group (minimal increase in LC number), C – auricle of a mouse from the control group, D – auricle
of a mouse treated with Protopic®, E – auricle of a mouse treated with compound 4 (minimal increase in LC number), F – auricle of a mouse treated with compound 5 (minimal increase in LC number).

**Fig. 9** Effects of the studied compounds on the mastocytes number of the auricles (staining with toluidine blue; 100× magnification; scale bar = 200 µm).

A – auricle of naive mouse (normal appearance), B – auricle of a mouse from the BG group, C – auricle of a mouse from the control group (minimal decrease in mastocytes number), D – auricle of a mouse treated with Protopic® (minimal decrease in mastocytes number), E – auricle of a mouse treated with compound 4, (minimal decrease in mastocytes number) F – auricle of a mouse treated with compound 5

**Table 1.** The cellular content of main cell types in the auricles. The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds, the ears were cut off and subjected to procedures described in the Materials and Methods. Quantitative analysis of labeled cells (neutrophils, Langerhans cells, mastocytes, T cells and macrophages) was achieved by counting positively stained cells in 8 random high-power fields for each animal. From every group 8 animals were analyzed.

Statistics: * p<0.05; † p<0.001 versus Control

**Table 2.** Effects of the compounds on the number and cross-sectional area of pustules. The mice were treated with oxazolone and the studied compounds as described in Fig. 1. 24 h after application of the compounds, the ears were cut off and subjected to procedures described in the Materials and Methods. The cross-sectional area of the pustules in the epidermis was measured and expressed in µm². The numbers of pustules with indicated areas are presented. The analysis was achieved by counting pustules on both sides of one auricle for each animal. From every group 8 animals were analyzed.
HISTOLOGY AND HISTOPATHOLOGY

0.5% oxazolone
1% oxazolone
0.1% ointment

measurement of the ear edema
blood
lymph node
auricle