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Topically applied azaphenothiazines inhibit experimental psoriasis in mice

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The therapeutic efficacy of topically applied azaphenothiazine derivatives: 9-chloro-6-acetylaminobutylquinobenzo[3,2-b][1,4]thiazine (compound **4**) and 6-chloroethylureidoethyldiquino[3,2-b;2′;3′-e][1,4]thiazine (compound **5**) in the amelioration of inflammatory symptoms of imiquimod-induced psoriasis in mice was investigated. Clobederm®, containing clobetasol propioniate, served as a reference drug. The application of the compounds led to thinning of the epidermis and reduction of the cell layers. The suppressive actions of the compounds were even stronger with regard to pathological changes of the dermis. The compounds also exerted generalized, anti-inflammatory effects by decreasing the number of circulating leukocytes, lowering subiliac lymph node weight and partially normalizing an altered blood cell composition. The changes in the composition of main cell types in the epidermis and dermis were less affected by the compounds. In addition, both compounds inhibited to a similar degree production of tumor necrosis factor α (TNF α) in human whole blood cell culture. Whereas compound **5** strongly inhibited IL-8 and CXCL10 chemokines in human keratinocytes – KERTr cell line, transfected with poly(I:C), the suppressive action of compound **4** in this model was weak. In addition, compound **5**, but not compound **4**, exhibited at low doses proapoptotic properties with regard to colonic cell lines. In summary, we demonstrated the therapeutic potential of two selected azaphenotiazines in the amelioration of the skin pathology elicited in a mouse experimental model of psoriasis.

1. Introduction

Psoriasis is a chronic inflammatory skin disease, the etiopathogenesis of which is still not fully understood, and is characterized by uncontrolled proliferation of keratinocytes [1]. The role of T cells producing cytokines such as IL-17, IL-22, IL-23 and TNF α in the pathogenesis of this immunological disorder has been well established [2,3].

Treatment of psoriasis involves, among others, major types of immune suppressors such as: calcineurin inhibitors [4–6], methotrexate [7], macrolides [8], steroids [9–11], as well as vitamin D [12], plant products [13], anticytokine antibodies [14–16] or phototherapy [17]. Although calcineurin inhibitors and steroids have successfully been applied as topical pharmaceutics in skin disorders [5,6,10], alternative approaches have also been considered due to their potential side-effects [18,19].

Phenothiazines are a class of compounds that exhibit various biological activities [20,21]. Classical phenothiazines with aminoalkyl substituents are an important source of valuable drugs [20]. Modification of phenothiazines with azine rings leads to the formation of azaphenothiazines [22,23]. Recently, we synthesized a series of azaphenothiazines by replacing benzene rings with pyridine and quinoline rings [24–28]. Several compounds from this group of azaphenothiazines strongly inhibited mitogen-induced proliferation of human peripheral blood mononuclear cells, TNF α production and growth of tumor cell lines. Subsequently, we selected only two compounds [29], which proved to be suppressive in the models of delayed type hypersensitivity to ovalbumin and carrageenan footpad test in mice. These compounds were also effective in the amelioration of contact sensitivity to oxazolone [30].

Abbreviations: TNF α, tumor necrosis factor α; IL, interleukin; LPS, lipopolysaccharide; IMQ, imiquimod; KERTr, human skin keratinocyte cell line; CXCL, C-X-C motif ligand; poly(I:C), polyinosinic:polycytidylic acid; LF, lipofectamine

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The aim of this investigation was to evaluate the potential therapeutic value of these compounds, applied topically in a form of ointment, in the amelioration of symptoms of psoriasis in an imiquimod-induced experimental mouse model, with a particular emphasis on histological investigations. In addition, we investigated the effects of the compounds on IL-8 and CXCL10 production in a keratinocyte KERT cell line and lipopolysaccharide (LPS)-induced TNF α production in human whole blood cell culture.

2. Materials and methods

2.1. 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, pellet food and water *ad libitum*. The local ethics committee at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland, approved the study (permission # 64/2015 and 79/2015).

2.2. Reagents

9-Chloro-6-acetylaminobutylquinobenzo[3,2-b][1,4]thiazine (compound **4**) [26] and 6-chloroethylureidoethyldiquino[3,2-b;2′;3′-e][1,4]thiazine (compound **5**) [25], were synthesized as previously described. The structures of the compounds are presented in Scheme 1. Aldara™, imiquimod (IMQ) 5% containing crème originated from Meda AB (Sweden) and Clobederm® (clobetasol propionate 0.5% ointment) from PharmaSwiss (Czech Republik). Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) from *E. coli* strain O111:B4, Evans blue, Giemsa and May-Grünwald reagents and formalin originated from Sigma-Aldrich. The ointment base (vehiculum), consisting of vaseline and lanolin (50/50 v/v), was purchased in a pharmacy store. The ointment base was supplemented with 1% of DMSO. The compounds were thoroughly distributed in the ointment by manually stirring the compounds with the ointment base for 1 h.

2.3. Experimental design

Mice were shaved on the back (1.5 × 1.5 cm area) (day 0). On days 1–7 the mice ($n = 4$) were treated daily, on the shaved skin, with 50 µl of Aldara™ crème and on days 6–7 (once daily) and 8–9 (twice daily) with vehiculum with 1% of DMSO ("positive" control mice). Mice (n = 4) from the background (BG) group were topically treated with only the vehiculum on days 1–9. The experimental groups included mice (4 per group) treated on days 1–7 with 50 µl of Aldara™ crème and on days 6–7 (once daily) and 8–9 (twice daily) with a reference drug – Clobederm® ointment, compound **4** or compound **5** with a concentration of 0.1% and 1.0% ointment. On day 7 (a maximal expression of psoriatic reaction) skin samples from the BG and "positive" control groups were taken for histological examination. Later in the article we will refer to these groups as "BG" and "control 7 d." On day 10 the mice were bled from the retro-orbital plexus under isoflurane anesthesia and were euthanized by cervical dislocation. The blood was analyzed for determination of blood leukocyte numbers and for determination of blood cell composition. In addition, spleens and draining subiliac lymph nodes were isolated for determination of organ weight. Skin samples were cut off for histological examinations. Later in the article we will refer to this group as "control 10 d." The description of treatment of the experimental groups is depicted in Table 1 and the experimental design in Scheme 2.

2.4. Determination of circulating leukocyte number and blood picture

Mice subjected to anesthesia were bled and the number of blood leukocytes was determined by diluting the blood in Türk's solution and 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 by a histologist. Up to 100 cells were counted on two glasses. The circulating leukocyte numbers were presented per 1 mm^3 and the blood cell compositions as a percentage of a given cell type.

2.5. KERTr cell culture conditions and cell treatment for determination of chemokine production

The human skin keratinocyte cell line, CCD 1106 KERTr (shortly KERTr) was purchased from ATCC. Cells were cultured in a Keratinocyte-SFM serum-free medium (Gibco), supplemented with Bovine Pituitary Extract (Gibco) and Epithelial Growth Factor (Sigma-Aldrich), in concentrations of 0.05 mg/ml and 35 ng/ml, respectively. The cell line was propagated in the culture for no longer than for 3 months by weekly transfer using trypsin-EDTA for cell detachment. Cells were plated in a medium with a density of 10^5/0.5 ml to obtain semi-confluence, and cultured one day prior to transfection with poly (I:C) (polyinosinic:polycytidylic acid) and treatment with the azaphenothiazines. Poly(I:C) is a synthetic analogue of double-stranded viral RNA, which mimics viral infection and is a strong immune stimulant for cytokine/chemokine production in KERTr cells. Before use, compounds **4** and **5** were dissolved in DMSO, placed in an ultrasonic bath and kept at 37 °C for 1 h, with occasional shaking. Directly before transfection/treatments, the culture medium was changed and cells were transfected with 0.2 µg of poly(I:C) using Lipofectamine 2000 Reagent (Invitrogen) and Opti-MEM serum-reduced medium, strictly according to the manufacturer's instructions. Four hours after transfection, the medium was replaced and the studied compounds **4** and **5** were added at 0.5–2 µM and 1–5 µM concentrations, respectively. 20 h after treatment with the compounds, the medium was collected, centrifuged at 300 × *g* for 5 min to remove floating cells and cellular debris. Then, the medium was frozen at −30 °C until use. Appropriate controls, such as untreated cells and cell cultures containing DMSO at concentrations of 0.04% (compound **4**) and 0.1% (compound **5**) were cultured in parallel. Concentrations of the compounds in the culture were selected based on the cell viability assay towards KERTr cells where compound **4** was not toxic up to 2 µM and compound **5** to 5 µM concentration. Compound **4** was used at 0.5–2 µM and compound **5** at 1–5 µM concentration range that corresponded to 0.2–1 µg/ml and 0.5–2.5 µg/ml for compounds **4** and **5**, respectively.

IL-8 and CXCL10 levels in the cell medium were measured using ELISA assays (DuoSet ELISA Development System, R&D Systems), according to the manufacturer's instructions, in 96-well plates. Measurements of each sample were performed in doublets, in 100 µl medium each. The colorimetric reaction was developed with TMB solution (eBioscience) and stopped by adding sulfuric acid. The optical density was read at a wavelength of 450 nm (with correction to 570 nm). Results were obtained from 3 independent experiments.

Scheme 1. The structures of the compounds.

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