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The impact of ketoprofen on viability and melanization process in normal melanocytes HEMn-DP

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ABSTRACT

The aim of this work was to examine the impact of ketoprofen on melanogenesis in cultured human normal melanocytes (HEMn-DP). The WST-1 assay was used to detect ketoprofen cytotoxic effect. It has been demonstrated that ketoprofen induces the loss of melanocytes viability in a concentration–dependent manner. The value of EC_{50} was found to be ~ 1 mM. It has also been shown that ketoprofen causes inhibition of tyrosinase activity and reduces melanin content in human epidermal melanocytes. The demonstrated inhibitory effect of ketoprofen on melanization process in melanocytes *in vitro* may explain the potential role of melanin biopolymer in the mechanisms of undesirable phototoxic effects of this drug *in vivo*, as a result of its accumulation in pigmented tissues.

Keywords: ketoprofen, melanocytes, melanogenesis, tyrosinase

INTRODUCTION

Nonsteroidal anti-inflammatory drugs are commonly used in the treatment of pain and inflammatory conditions. However, their use is restricted by the high incidence of adverse effects, particularly in the gastrointestinal tract and kidneys. Gastrointestinal damage includes multiple small lesions, gastric and duodenal ulcers, perforated ulcers, and severe bleeding of the upper gastrointestinal tract [1]. It is also recognized that nonsteroidal anti-inflammatory drugs induce photosensitivity, defined as exaggerated or abnormal cutaneous reactions to light [9]. This adverse reaction seems to be reported more commonly with topical formulations, which may be because of the higher concentrations of drug in the skin. Among the different topical formulations of nonsteroidal anti-inflammatory drugs, ketoprofen has often been associated with photosensitivity reactions [1, 9].

Ketoprofen has been widely used for the treatment of inflammatory diseases and musculoskeletal injury. A lot of cases of the photosensitivity attributed to ketoprofen, such as erythema, edema and pigmentation, were reported [4, 9]. The photosensitivity is classified into phototoxicity and photoallergy [1]. The mechanism of the photosensitivity by ketoprofen has not been obviously revealed.

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Melanin is synthesized in the melanosomes in melanocytes, which produce melanin by a process that involves the transformation of tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosinase and the subsequent transformations of L-DOPA into melanin [14]. Two types of melanin are synthesized within melanosomes: eumelanin and pheomelanin. Eumelanin is a dark brown-black insoluble polymer, whereas pheomelanin is a light red-yellow sulphur-containing polymer [6, 8]. The quantity of melanin in various organs has been shown to vary with age, eg. age-related reduction of melanin in the hair, epidermis, retinal pigmented epithelial cells, and certain areas of the central nervous system [19].

Previously, we have documented that ketoprofen forms stable complexes with model synthetic melanin [3]. The aim of this work was to examine the effect of ketoprofen on viability and melanization process in cultured human epidermal melanocytes (HEMn-DP).

MATERIALS AND METHODS

Chemicals. L-3,4-dihydroxyphenylalanine (L-DOPA) and synthetic melanin were purchased from Sigma-Aldrich Inc.(USA). Ketoprofen was obtained in the form of solution – Ketonal (0.1 g/2ml) from Sandoz GmbH (Poland). Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254, gentamicin, amphotericin B and human melanocyte growth supplement-2

(HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A.(Poland).

Cell culture. The normal human melanocytes (HEMn-DP, human epidermal melanocytes, neonatal – dark pigmented, Cascade Biologics, USA) were grown according to the manufacturer's instruction. The cells were cultured in M-254 medium supplemented with HMGS-2, penicillin (100 U/ml), gentamicin (10 μg/ml) and amphotericin B (0.25 μg/ml) at 37°C in 5% CO_2. All experiments were performed using cells in the passages 5-7.

Cell viability assay: The viability of melanocytes was evaluated by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO_2 for 48 h. Then the medium was removed and cells were treated with ketoprofen solutions in a concentration range from 0.001 to 10 mM. After 21-h incubation, 10 μl of WST-1 were added to 100 μl of culture medium in each well, and the incubation was continued for three hours. The absorbance of the samples was measured at 440 nm with a reference wavelenght of 650 nm, against the controls (the same cells but not treated with ketoprofen) using a microplate reader UVM 340 (Biogenet, Poland). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

*Measurement of melanin co*ntent. The melanocytes were seeded in a 35 mm dish at a density of $1x10^5$ cells per dish. Ketoprofen treatment in concentration range from 0.01 mM to 1 mM, began 48h after seeding. After 24h of incubation, the cells were detached with trypsin/EDTA. Cell pellets were placed into Eppendorf tubes, dissolved in 100 μl of 1 M NaOH at 80°C for one hour, and then centrifuged for 20 min at 16000xg. The supernatants were placed into a 96-well microplate, and absorbance was measured using microplate reader at 405 nm – a wavelength at which melanin absorbs light [15]. A standard synthetic melanin curve (0 to 400 μg/ml) was performed in triplicate for each experiment. Melanin content in ketoprofen treated cells was expressed in pg/cell and also as the percentage of the controls (untreated melanocytes).

Tyrosinase activity assay. Tyrosinase activity in HEMn-DP cells was determined by measuring the rate of oxidation of L-DOPA to dopachrome according to the method described by Kim et al. [11] and Busca et al. [2], with a slight modification. The cells were cultured at a density of $1x10^5$ cells in a 35 mm dish for 48 h. After 24-h incubation with ketoprofen (concentration range from 0.01 mM to 1 mM) cells were lysed and clarified by centrifugation at 10000xg for 5 min. A tyrosinase substrate L-DOPA (2 mg/ml) was prepared in the same lysis phosphate buffer. Portions of 100 μl of each lysate were put in a 96-well plate, and the enzymatic assay was initiated by the addition of 40 μl of L-DOPA solution at 37°C. Absorbance of dopachrome was measured every 10 min for at least 1.5 h at 475 nm using a microplate reader. Tyrosinase activity was expressed in μmol/min/ml and μmol/min/mg protein and also as the percentage of controls.

Statistical analysis. In all experiments, mean values of at least three separate experiments performed in triplicate ± standard deviations (S.D.) were calculated. The results were analyzed statistically with the Student's t-test using GraphPad Prism 5.04 Software (USA).

RESULTS

The effect of ketoprofen on melanocytes viability is presented in Fig. 1. It has been demonstrated that at a relative low drug concentration (0.001 mM or 0.01 mM) the loss in cell viability is not observed. Treatment of melanocytes with ketoprofen in concentration from 0.1 mM to 10 mM for 24 h has led to the loss of cell viability by 22% to 98%, respectively. The value of EC_{50} (the concentration of a drug that produces loss in cell viability by 50%) is about ~ 1 mM.

The effectiveness of melanization process was esti-

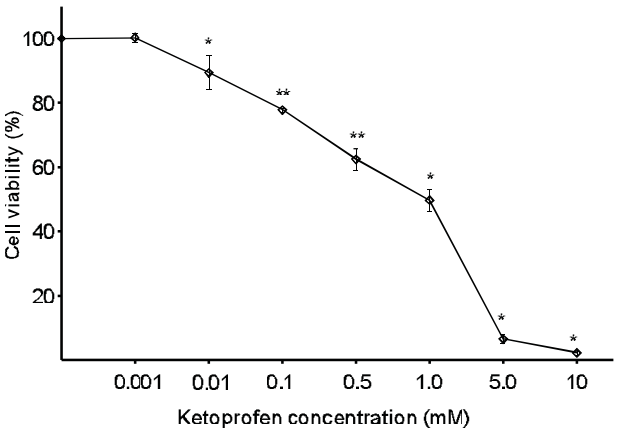

Fig. 1. The effect of ketoprofen on viability of melanocytes. Cells were treated with various ketoprofen concentrations (0.001–10 mM) and examined by WST-1 assay. Data are expressed as % of the controls. Mean values ± SD from three independent experiments performed in triplicate are presented.
* $p<0.05$ *vs.* the control samples, ** $p<0.005$ *vs.* the control samples

mated by measuring the melanin content and cellular tyrosinase activity in melanocytes treated with ketoprofen in concentrations EC_{50} as well as 10-fold and 100-fold lower, for 24 h. After performing a calibration curve, the mela-

nin content per cell was determined as 53.8 to 59.9 pg/cell for melanocytes treated with drug and 60.7±1.7 pg/cell for the control sample (Tab. 1). The obtained results, recalculated for culture ($1x10^5$ cells), were finally expressed as a percentage of the controls (Fig. 2). Ketoprofen in concentration 0.01 mM had no effect on melanin content. Treatment of cells with drug at concentration 0.1 and 1 mM for 24 h reduced melanin production in a concentration-dependent manner, by about 8% and 11%, respectively.

Table 1. The effect of ketoprofen on melanin content in melanocytes

Ketoprofen concentration (mM)	Melanin content ± SD (pg/cell)
Control	60.74 ± 1.66
0.01	59.92 ± 1.28
0.1	56.28 ± 1.79
1.0	53.79 ± 1.64

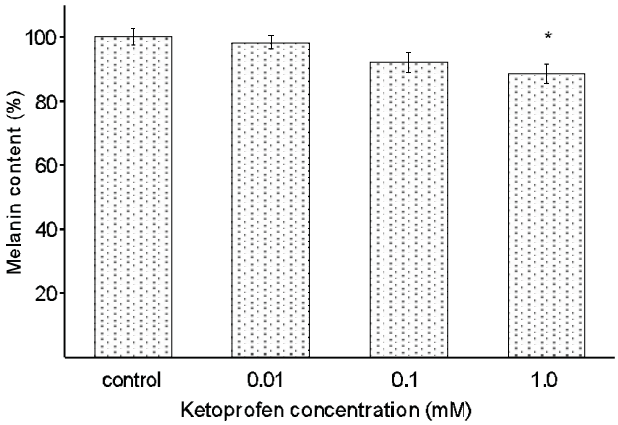

Fig. 2. The effect of ketoprofen on melanin content in melanocytes. Cells were cultured with 0.01, 0.1 or 1 mM of ketoprofen for 24 h and melanin content was measured as described in Materials and Methods. Results are expressed as percentages of the controls. Data are mean ± SD of at least three independent experiments performed in triplicate.
* $p<0.05$ *vs.* the control samples

Tyrosinase activity in HEMn-DP cells treated with ketoprofen also decreased in a manner correlating well with the inhibitory effect on melanin production (Tab. 2, Fig. 3). After 24-h incubation with ketoprofen, tyrosinase activity was suppressed to 85% at 0.1 mM and to 80% at 1 mM compared with control. Ketoprofen in concentration 0.01 mM had slight effect on the cellular tyrosinase activity.

Table 2. The effect of ketoprofen on tyrosinase activity (the rate of oxidation of L-DOPA to dopachrome) in melanocytes

Ketoprofen concentration (mM)	Tyrosinase activity ± SD	
	(μmol/min/ml)	(μmol/min/mg protein)
Control	1.3649 ± 0.0369	2.4201 ± 0.0654
0.01	1.5544 ± 0.0306	2.1954 ± 0.0432
0.1	1.2773 ± 0.0204	2.0602 ± 0.0328
1.0	1.4487 ± 0.0300	1.9446 ± 0.0402

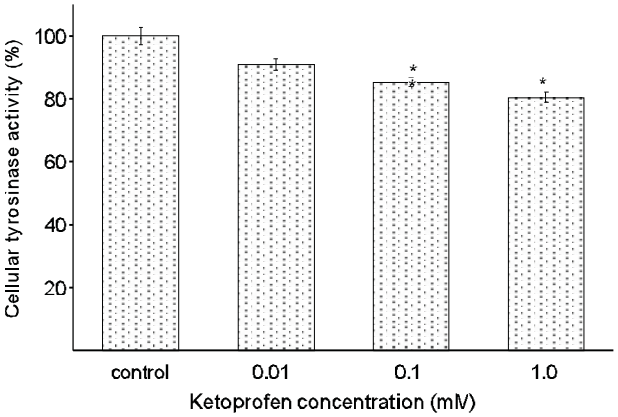

Fig. 3. The effect of ketoprofen on tyrosinase activity in melanocytes. Cells were cultured with 0.01, 0.1 or 1 mM of ketoprofen for 24 h and tyrosinase activity was measured as described in Materials and Methods. Results are expressed as percentages of the controls. Data are mean ± SD of at least three independent experiments performed in triplicate.
* $p<0.05$ *vs.* the control samples

DISCUSSION

When exposed to sunlight, the ketoprofen-applied skin exhibits eczematous dermatitis [4, 9], and this photocontact dermatitis is characterized by a prolongation of photosensitivity at the applied sites. The action spectrum is ultraviolet A light, similar to other photoallergic drugs [20]. There are many references published on this adverse effect after application of topical ketoprofen gel [1] or containing ketoprofen strips [18]. Moreover, some cases of photodermatitis caused by systemic ketoprofen were described [5]. In humans, repeated daily passive topical administration of a ketoprofen gel for 3 days achieved detectable ketoprofen in the synovial fluid of the knee. Ketoprofen has also been measured in venous blood flow from the application sites following cathodic iontophoresis in humans [16].

Many drugs are known to be markedly accumulated and retained for a considerable time by pigmented tissues and the retention of these compounds is proportional to degree of melanin pigmentation. The ability of melanins to bind different drugs is probably of the greatest biological importance. Melanin binding may lead to the accumulation of drug *in vivo* in melanin-rich tissues (e.g. skin), which may prolong the action of the drug and influence its phototoxic adverse effects. Drug–melanin binding is a phenomenon that has been observed with structurally and pharmacologically unrelated drugs following administration [13]. Previously, we have documented that ketoprofen forms stable complexes with synthetic DOPA-melanin characterized by the association constants $K_1 \sim 10^5\ M^{-1}$ and $K_2 \sim 10^4\ M^{-1}$ [3].

Human melanocytes function as a pivotal protective barrier against ultraviolet radiation and oxidative stress by generating the radical-scavenging pigment melanin [10].

By inhibiting or significantly restricting drug access to cell receptors, melanins protect organism against undesirable drugs side effects. However, long-term exposure and slow release of drugs or its melabolites from bonds may increase the level of noxious substances stored on melanin, what may cause degeneration in the melanin-containing cells (especially in the eye, ear, skin and brain) and surrounding tissues [7, 12, 13].

The aim of the present study was to investigate the effect of ketoprofen on melanin formation (closely related to pigmentation) in HEMn-DP melanocytes.

Melanogenesis takes place in the melanosomes. These cytoplasmic organelles are produced by specialized cells – melanocytes - which are located on the basal lamina and project their dendrites into the epidermis. The melanocytes use their dendrites to maintain an intimate contact with surrounding keratinocytes and to transfer the melanosomes to them. The melanin production may continue even after the transfer of melanosomes to keratinocytes [8, 14]. Whether this has any metabolic consequences for the hosting cells is not known. Pigment production (melanogenesis) involves a chain of enzymatic and non-enzymatic reactions leading to the formation of phenolic and indolic intermediates that are characterized by their ability to polymerize [8]. The key enzyme is tyrosinase to initiate the active pigmentation machinery. Tyrosinase is a glycoprotein located in the membrane of the melanosome, a minifactorial vesicle inside the melanocyte. Tyrosinase catalyses the first two steps of melanin production: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of this o-diphenol to the corresponding quinone, L-dopaquinone [6].

In this study we have used the culture of normal human epidermal melanocytes as an *in vitro* experimental model system. Since tyrosinase is a major regulator of melanin synthesis, we have examined a direct effect of ketoprofen on the activity of this enzyme. Ketoprofen in concentration 0.1 mM and 1 mM decreased the tyrosinase activity in melanocytes by 15% and 20%, respectively. An analysis of melanin formation in cells cultured in the presence or absence of a drug showed that ketoprofen in concentrations 0.1 mM and 1 mM suppressed the melanin content to 92% and 89%, respectively. It has also been stated that ketoprofen in concentration 0.001 mM does not significantly affect the viability of melanocytes as well as melanin synthesis and tyrosinase activity. Higher drug concentrations (from 0.01 mM to 10 mM) have led to the loss of cell viability in a concentration-dependent manner. The value of EC_{50} was determined to be ~ 1 mM.

The therapeutic ketoprofen concentration in serum is described as 1 to 20 μg/ml, i.e. 0.005 to 0.1 mM [17]. The concentrations found in this study to have an inhibitory effect on melanogenesis are similar or about 10-fold higher than the concentration normally observed *in vivo* after systemic drug administration. Previously, we have demonstrated that ketoprofen undergoes a specific interaction with melanin, what may lead to the accumulation of this drug in melanin-reach tissues. Slow release of ketoprofen from bonds may bild up high and long-lasting level of this drug and cause the prolonged exposure of melanin containing cells and surrounding tissues to the toxicity of the tested nonsteroidal anti-inflammatory drug [3].

The demonstrated inhibitory effect of ketoprofen on melanization process in melanocytes *in vitro* may explain the potential mechanism for the undesirable side effects of this drug *in vivo*, as a result of drug accumulation in pigmented tissues. In addition, we have demonstrated, that the inhibitory effect of ketoprofen on melanogenesis is probably due to its direct inhibition of tyrosinase activity.

CONCLUSION

The demonstrated effect of ketoprofen on melanization process in melanocytes *in vitro* may explain the potential role of melanin biopolymer in the mechanisms of phototoxic effect of this drug *in vivo* as a result of its accumulation in melanin – rich tissues.

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