

Ketoconazole-p Aminobenzoic Cocrystal Exhibits a Potent Anti-inflammatory Effect on the Skin of BALBc Mice

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Abstract

Fungal infections are a growing global health problem. Therefore, our group has designed and characterized a novel cocrystal formulation starting from ketoconazole and para-amino benzoic acid, named KET-PABA aiming to improve the bioavailability, biocompatibility, and efficiency of the parent drug. The cocrystal showed improved physical properties, such as stability in suspension, solubility, as well as antimycotic efficiency as compared to ketoconazole. The current study investigated the local possible side effects induced on BALBc mice skin by the application of KET-PABA cocrystal. KET-PABA proved to be safe, without signs of skin sensitization as shown by the mouse ear sensitization test (MEST), or histopathology. KET-PABA induced a potent anti-inflammatory effect through the inhibition of proinflammatory cytokines such as IL1 α , IL1 β , IL6 and TNF α , and other inflammation promoters such as NRF2, compared to the vehicle. KET-PABA had no effect on the levels of the anti-inflammatory cytokine IL10, or proinflammatory enzyme COX2 and had minimal effects on the activation of the NF κ B pathway. Overall, KET-PABA application induced no sensitization, moreover, it induced an anti-inflammatory response. Based on the improved antimycotic effect versus ketoconazole and the anti-inflammatory action, KET-PABA cocrystal has the potential to be an efficient drug in the treatment of cutaneous mycosis.

Introduction

Ketoconazole (KET) is a lipophilic imidazole which inhibits non-specifically the P450-dependent enzyme lanosterol 14- α -demethylase and prevents the synthesis of ergosterol, leading to the inhibition of fungal growth and replication. (Deng et al. 2017) Even though ketoconazole is an efficient antifungal agent, its use was restricted by the general side effects, such as hepatotoxicity, endocrine disturbances, and drug interactions to topical use (Choi et al.2019).

Ketoconazole is indicated in the treatment of superficial fungal infections, such as Malassezia, Candida and dermatophyte infections. Reported local side effects of topical ketoconazole are allergic contact dermatitis and photoallergic contact dermatitis (Choi et al. 2019). The clinical diagnosis must be confirmed by patch test or photo-patch test.

Recent research is focused on improving topical ketoconazole delivery, water solubility, and protection against light degradation, in order to provide good thermodynamic stability, increased percutaneous permeability, selective skin deposition, and prolonged effect (Choi et al. 2019).

The discovered formulas include drug loaded micelles (Ketoconazole-loaded Y shaped monomethoxy poly (ethylene glycol) block-poly (ϵ -caprolactone) micelles) (Deng et al. 2017), nanostructured lipid carriers, liposomes and niosomes, and microemulsion (Choi et al. 2019).

Another way to increase the solubility and bioavailability of ketoconazole is crystal engineering by the formation of a salt with oxalic acid, or cocrystal formation with para-aminobenzoic acid (PABA), fumaric acid, adipic acid, succinic acid, and nicotinamide (Martin et al. 2020).

Lately, there has been an increased interest in benzoic acids and their derivative, due to their chemical and biological properties. PABA is a precursor for the biosynthesis of folic acid and is a compound present in plants, fungi, and some parasites. PABA is absent in the human body, which does not have the pathway of folic acid (Kluzk et al. 2002).

PABA is also found in food, as part of the vitamin B complex, ensuring an antioxidant effect. PABA has multiple indications in medicine (Crisan et al. 2014) based on its antibacterial, antifungal and antiparasitic properties. Also, PABA has anticoagulant, antimutagenic, fibrinolytic, immunomodulating, photoprotective effects. It is used in sunscreen creams, where it functions as an UVB shield and it can quench reactive oxygen species generated by UV exposure (Hu et al 1995). It is also used in local anesthetics such as procaine (Mackie et al. 1999). Also, the potassium salt of PABA is used to treat connective tissue diseases such as dermatomyositis and scleroderma (Sharma et al. 2013).

PABA is stable in solid and liquid state, and is soluble in water, in methanol and in ethanol (Kluzk et al. 2002). The aminogroup of PABA ensures the transport of PABA and its derivatives, and reduces toxicity (Martin et al. 2020). Several studies that focus on improvement of oral drug delivery and skin targeting of PABA using liquid crystal formulations were published (Kadhun et al. 2016).

However, the topical use of PABA has rendered sensitization reactions, in the form of contact allergic dermatitis, or contact photoallergic dermatitis (Thune et al. 1984, Greenspoon et al. 2013). Also, cross-reactivity between PPD and PABA was reported (LaBerge et al. 2011). Since ketoconazole has also been involved in initiating allergic contact dermatitis (Choi et al. 2019), the current study is focused on the evaluation of the local immunological reactions after topical application of the ketoconazole-PABA cocrystal.

In a previous study we showed that the newly synthesized cocrystal ketoconazole with PABA, KET-PABA proved several advantages: safe pharmacological profile, both *in vitro* and *in vivo*, as compared to ketoconazole, with a good crystal stability under ambient conditions, increased solubility, significantly enhanced antifungal activity compared to ketoconazole or PABA alone, and an antioxidant effect (Martin et al. 2020).

Therefore, this study aims to evaluate possible local side effects, such as allergic contact dermatitis initiation or another type of local inflammatory reaction, in response to topical application of the cocrystal, as well as ketoconazole and PABA on the skin of BALBc mice. A specific test (Mouse Ear Swelling Test) was used, combined with histopathology exam and the measurement of pro and anti-inflammatory cytokines and inflammation mediators. Overall, the cocrystal proved to be safe on mouse skin, moreover the anti-inflammatory effects of the cocrystal were more important when compared to the parent drug- ketoconazole.

Materials And Methods

Materials

Ketoconazole, commercial form, was purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China) and *p*-aminobenzoic acid was purchased from Merck Romania SRL, (affiliate of Merck KGaA, Darmstadt, Germany); dinitrochlorobenzene (DNCB) from Sigma Aldrich Co, Ethyl acetate used for KET-PABA extraction from serum samples was purchased from Chimopar Trading SRL (Bucharest, Romania), HPLC-grade acetonitrile was purchased from VWR Chemicals (France) and formic acid from Cristal R Chim SRL (Bucharest, Romania). Ultrapure water was prepared using a Milli-Q Ultrapure purification system (Millipore, USA). Hematoxylin–eosin (HE), Sigma Aldrich Co, St. Louis, Missouri, USA; antibodies against: nuclear factor kappa B (NFκB), phosphorylated NFκB p65 (Ser536) (93H1) (pNFκB), from Cell Signaling Technology, Inc, Danvers, USA; cyclooxygenase 2 – (COX2), β actin and secondary peroxidase-linked antibodies (Santa Cruz Biotechnology, Delaware Ave, Santa Cruz, USA); Supersignal West Femto Chemiluminiscent substrate (Thermo Fisher Scientific, Rockford IL, USA); ELISA kits for IL1 α, IL1β, IL6, Quantikine ELISA Immunoassay kit,(R&D Systems, Inc, Minneapolis, USA); Bradford total protein concentration assay (Biorad, Hercules, California, USA), bovine serum albumin and reagents for spectrophotometry (Sigma Aldrich Co, St Louis, Missouri, USA), standard food for lab animals – mice, was purchased from Cantacuzino Institute, Bucharest, Romania.

Other solvents and reagents used were of analytical grade, purchased from commercial suppliers.

Characterization of the KET-PABA solid form

KET-PABA was prepared, as a white powder sample, by solvent drop grinding (SDG) method performed by mechanical grinding using a Retsch MM400 ball milling (Martin et al. 2020). Its solid form structure was confirmed by comparing the experimental X-ray powder diffraction (XRPD) pattern with the calculated one. The XRPD measurements were performed in the 3-40° range, in steps of 0.02°, at room temperature using a Bruker D8 Advance powder diffractometer with CuKα1 radiation ($\lambda = 1.54056 \text{ \AA}$). The calculated XRPD pattern was obtained based on its crystal structure determined from single-crystal X-ray diffraction (Martin et al. 2020). Single crystal X-ray data collection was done using Oxford Diffraction SuperNova dual-wavelength diffractometer and CrysAlisPro program (Agilent 2010), Olex2 program (Dolomanov et al 2009) was used for crystal structure solution and refinement, SHELXS97 (Sheldrick 1997) for structure solution and SHELXL (Sheldrick 2008) for full-matrix least-square refinement on F₂. The crystal structure was confirmed by solid state NMR (measurements performed on Bruker Avance III wide bore spectrometer operating at ¹H Larmor frequency of 500.13 MHz) and computed chemical shifts (using NMR CASTEP module of Materials Studio Package, v5.5 [Biovia Materials Studio]).

Powder dissolution data for KET-PABA revealed a 10-fold solubility increase in deionized water that determines a 6.7-fold oral bioavailability improvement compared to pure KET. The stability tests performed under accelerated (climatic chamber at 40 °C/75% RH for 6 months) and long-term ambient conditions (12 months) showed no structural changes of the KET-PABA solid form (Martin et al. 2020).

Biological studies

All lab animal experiments were approved by ethics committee of the University of Medicine and Pharmacy Cluj-Napoca and the Veterinary Health Directorate, Romania (authorization number 67/ 06 06 2017). The lab animals were humanly treated and sacrificed under anesthesia.

Mice

Animals (6-8 week old female) BALBc mice (Tordesillas et al. 2014) were purchased from Cantacuzino Institute, (Bucharest, Romania). During the experiments, the animals were kept at humidity 65%, 21°C, day/night cycles of 12 h, fed with standard food and water ad libitum. The food of the animals was supplemented with vitamin A (250UI/g) to enhance the sensitivity of the epicutaneous sensitization test (Dunn et al. 1990, Hussain et al. 2011, Tordesillas et al. 2014).

Epicutaneous sensitization test

The test was done according to the MEST protocol. Animals were randomly divided into five groups, 7/group: group 1- control - vehicle (alcohol 70%), group 2- ketoconazole, group 3 -KET-PABA, group 4- PABA and group 5- DNCB dinitrochlorobenzene (DNCB, Sigma). Animals were anesthetized and abdominal fur was carefully shaved, without abrasion of the skin. All animals were administered subcutaneously, in the right flank 30 µl/mouse of Freund's complete adjuvant (Thermo Scientific™, Waltham, Massachusetts, United States) to increase the immunogenicity of the tested substance (Stewart et al. 2006). All animals in a group were marked on the back with a nontoxic dye. Then animals were exposed to the tested substances, each in a concentration of 100 µg/ml solved in ethanol 70%. 100 µl solution/mouse was spread thinly on the shaved abdominal skin to dry; solution was applied 1x/day for 6 consecutive days. On day 7, the thickness of the ears was measured with an electronic caliper, which served as the initial ear thickness, then mice were challenged with 50 µl/mouse of the same solution on the left ear pinnae, rechallenge was done on day 14. Ear thickness was measured at 24h and 48h after each ear application.

Calculation

$$\% \text{ ear thickness} = 100 \cdot (A - B) / B,$$

A = ear thickness of the treated ear, B = initial ear thickness.

Mice were sacrificed afterwards, and the ear tissue was collected for further investigations.

Histopathology

Samples- left ear pinna were fixed in 10% buffered neutral formalin and embedded in paraffin. Sections were made at 4 µm and the slides were stained by Hematoxylin–Eosin (HE) method. Slides were examined under a microscope Olympus BX 51 and images were taken with an Olympus UC 30 digital camera and processed by a special image acquisition and processing program: Olympus Stream Basic Olympus Stream Basic (Shinjuku, Tokyo, Japan).

ELISAfig

Ear sample homogenates were prepared as previously described by David et al. (David et al. 2014). The protein content in tissue homogenates was measured by Bradford method (Noble et al. 2009). IL1 α , IL1 β and IL6 Quantikine ELISA Immunoassay kit was used. Ear homogenate samples were treated according to manufacturer's instructions; readings were done at 450 nm with correction wavelength set at 540 nm, using an ELISA plate reader.

Western Blot

Lysates (20 μ g protein/lane) were separated by electrophoresis on SDS PAGE gels and transferred to polyvinylidene difluoride membranes, using Biorad Miniprotean system (BioRad). Blots were blocked and then incubated with antibodies against: IL10, STAT3, COX2, then further washed and incubated with corresponding secondary peroxidase-linked antibodies. Proteins were detected using Supersignal West Femto Chemiluminescent substrate and a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software (Biorad). β actin was used as a protein loading control.

Statistical methods

The statistical difference between treated and control groups were evaluated by two-way ANOVA, followed by Bonferroni posttest, using GraphPad, the statistical significance of the treatments inside a certain group was tested using one way ANOVA and Dunnet's multiple comparison tests; results were considered significant for $p < 0.05$. Statistical package used for data analysis was Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.

Results

Epicutaneous sensitization

Clinical observation of the treated mice in the vehicle, KET, KET-PABA and PABA groups did not detect any ear surface alteration or swelling. The animals seemed unaffected by the exposure. However, in the DNCB group, the animal's abdomen became red, and some small crusts appeared on the surface, starting from day 6; on the ears, there was redness, edema and the mice showed a slight sensitivity to touch at 24 and 48h after the challenge and maintained the same aspect after rechallenge. The clinical observations were sustained by the quantitative measurements of the ear thickness (Fig 1.). Vehicle group showed no statistical differences between the measured values ($p > 0.05$, one way ANOVA). Data show that in the groups treated with KET, KET-PABA and PABA there was no significant ear swelling, compared to vehicle. Interestingly KET seemed to diminish ear thickness, compared to the initial value and to the vehicle. There was a strong statistically significant difference between the initial ear thickness and the values after challenge and respectively rechallenge ($p = 0.0009$, one way ANOVA). KET-PABA application induced a slight, not significant increase in the ear thickness after challenge and at 24h after rechallenging, however, at 48h the values were like those of the vehicle ($p > 0.05$, one way ANOVA). In the

PABA group, ear thickness decreased after the challenge and afterwards they slightly increased, however, not significantly compared to the initial value ($p > 0.05$, one way ANOVA). The DNCB group showed a strong statistically significant increase in the ear thickness ($p < 0.0001$, one way ANOVA). The most important increase was found after the rechallenge at 24h, afterwards the ear thickness diminished; however, it was still significant, when compared to the initial value. There was a strong statistically significant difference ($p < 0.0001$) showed by two-way ANOVA between the groups. Bonferroni post test showed significant ($p < 0.001$) differences between the DNCB group and all the other groups.

Histopathology analysis

In the DNCB treated group there were signs of moderate inflammation. The ear skin lesions showed epidermal spongiosis, with mild acanthosis, slight dermal edema, and a perivascular dermal inflammatory infiltrate, consisting of a moderate number of lymphocytes and plasma cells (Fig 2.). All these modifications are consistent with the aspect of contact dermatitis. The epicutaneous sensitization measurements along with the histopathology results prove that contact dermatitis was induced in the positive control group. Therefore, this group can be used for comparison with the other experimental groups. No histological changes were seen in the control - vehicle treated group, Ketoconazole, KET-PABA and in the PABA treated group (Fig 2.).

Inflammatory markers

Pro-inflammatory cytokines IL1 α , IL1 β and IL6 from the treated mouse ear tissue homogenates were determined by ELISA (Fig 3.).

IL1 α and IL1 β levels were slightly decreased by KET, KET-PABA and PABA, not significantly compared to vehicle. The most important decrease was in the case of KET-PABA. In the DNCB group, there was an important, significant increase compared to the vehicle and all the other groups (Fig 3.). There were significant differences between the groups as shown by one way ANOVA ($p = 0.0001$ for IL1 α and IL1 β).

IL6 was only decreased by KET-PABA (Fig 3.), while the other substances increased it, however, the only significant increase was for DNCB. One way ANOVA showed significant differences between the groups ($p = 0.011$).

The anti-inflammatory cytokine IL10 level was measured by Western Blot. IL10 showed a lower level in the mice treated with KET-PABA compared to controls, although not significantly. The other groups showed a slight increase, but only DNCB was significant (Fig 4.). These modifications were not significant between the groups ($p = 0,074$, one way ANOVA)

The level of COX2, a proinflammatory enzyme was found strongly increased by DNCB, as expected, leading to the local inflammation (Fig 4.). In the other groups the modifications were not significant, with a slight increase in the ketoconazole and PABA group and decrease in the KET-PABA group. The difference between the groups treated with ketoconazole and KET-PABA were significant ($p = 0.012$).

Overall, one-way ANOVA showed that these modifications were significant between the treated groups ($p < 0.0001$).

TNF α was significantly increased in the case of ketoconazole and DNCB, showing a proinflammatory effect of these substances (Fig 4.). Moreover, there was a significant difference between the levels of TNF α in the groups treated with ketoconazole versus KET-PABA ($p=0.03$) and the one-way ANOVA showed a strongly significant difference between the groups ($p < 0.0001$).

NRF2 level showed a similar trend with that of COX2, it was increased slightly by ketoconazole and PABA and decreased by KET-PABA (Fig 4.). There was a significant difference between the effects of ketoconazole and KET-PABA on NRF2 expression ($p=0.0007$). Overall, the groups showed a significantly different effect on NRF2 ($p < 0.0001$, one-way ANOVA).

NF κ B was statistically significant increased by exposure to ketoconazole and DNCB. There was a significant difference between the groups, as showed by one way ANOVA ($p < 0.0001$). The level of the phosphorylated, active form - pNF κ B was increased in all treated groups, compared to controls (Fig 4.). The effect was significant only in the case of DNCB. One way ANOVA showed a weak significance, $p=0.0108$, between the groups. The ratio of activated form pNF κ B / versus total protein NF κ B was increased by KET-PABA and PABA exposure, however not significantly compared to the vehicle. This is explained by the lower level of NF κ B protein in the two groups and the higher level in the ketoconazole and DNCB groups. The ratio pNF κ B /NF κ B showed significant variation between groups ($p=0.0023$, one way ANOVA).

Discussion

The current study aims to evaluate the possible unwanted reactions of the cocrystal, when applied to the skin. Considering the sensitizing potential of the two active components (ketoconazole and PABA), local skin testing was performed with each individual component, and with the cocrystal compound using the Mouse Ear Swelling Test (MEST). MEST protocol evaluates contact sensitization by a quantitative method and data generated by this test are approved by US FDA (Gad et al. 1986). This was followed by the histopathology tissue assessment and measurement of pro and anti-inflammatory cytokines and inflammation mediators from the ear tissue by ELISA and Western-Blot to have a better image of the underlining mechanisms.

Topical ketoconazole is the first-line therapy, according to current guidelines, for seborrheic dermatitis and pityriasis versicolor (Choi et al. 2019), but it is also approved by the United States FDA for the treatment of candidiasis and dermatophyte infections.

Different studies showed benefits of ketoconazole in many skin disorders: primarily as an efficient topical antimicrobial drug, but also in the treatment of androgenetic alopecia by inhibiting 5 α -reductase; the treatment of atopic dermatitis and psoriasis by decreasing *Malassezia* colonization, which is considered a trigger factor (Choi et al. 2019), by anti-inflammatory effect, due to inhibition of 5-lipoxygenase, and by

restoring the skin barrier due to inhibition of hyperproliferation; the treatment of acne by decreasing the sebum levels, due to inhibition of androgenesis.

Ketoconazole has also anti-parasitic properties, with indication in the treatment of cutaneous leishmaniasis, and anti-bacterial properties, with indication in the treatment of staphylococcus blepharitis and folliculitis (Choi et al. 2019).

In the present study we evaluated the sensitizing potential of three compounds - KET, PABA and KET-PABA, the last one resulted by co crystallization. The study was performed on BALBc mice because their skin is immunologically like that of humans. This is a commonly used model for epicutaneous sensitization tests of newly developed substances (Tordesillas et al. 2014).

To study the contact sensitization, several animal models have been used, initially the guinea pig and then the mouse. Of these, the most validated test is mouse ear swelling test (MEST) (Garrigue et al. 1994). MEST is based on the method described by Gad and al. and by Thorne for evaluating the potential to cause sensitization in mice, to different substances (Gad et al. 1986, Thorne et al. 1991).

In time, the initial protocol was improved to increase the sensitivity of the test for detection of contact sensitizers. The factors used to increase the sensitivity were the removal of the stratum corneum by tape-stripping, the stimulation of the immune system of the animals by increasing reactivity due to vitamin oral A supplementation, and Freund's adjuvant injection, methods that have been reported to have an effect of stimulating epicutaneous sensitization to moderately allergenic substances (Stewart et al. 2006, Tordesillas et al. 2014, Dunn et al. 1990). The advantages of this method turned out to be effective, less expensive, and objective and the data collected more accurate for predicting the response in humans (Gad et al. 1986).

Therefore, in our test we used oral vitamin A supplementation and Freund's adjuvant, to increase the test sensitivity. Moreover, a positive control group using DNCB and a negative control, using vehicle alone were employed to have reference points for the assessment of the developing of an inflammatory reaction.

The MEST test was negative for all tested substances, and shown no epicutaneous sensitization of mice to KET, PABA, and KET-PABA. Interestingly, KET seemed to diminish ear thickness, compared to the initial value and the vehicle, although the difference was not statistically significant.

The histopathology results (epidermal spongiosis, with mild acanthosis, slight dermal edema, and a perivascular dermal inflammatory infiltrate) confirmed that contact dermatitis was induced in the positive control group (DNCB). This group was effective as a positive control for contact sensitization and served as a reference for the other test groups. No histological changes were seen in the vehicle and in the other test groups.

Evaluation of contact sensitization is necessary for the development of a new drug as part of the biocompatibility testing and relies on *in vitro* and *in vivo* tests in animals. Contact sensitization is a T-cell

mediated (type IV) delayed hypersensitivity reaction which results from exposure and sensitization of a genetically susceptible host to an allergen, followed by re-exposure to that allergen that will trigger an inflammatory reaction (Garrigue et al. 1994).

There are two phases in the development of contact sensitization—the sensitization phase and the elicitation phase. In the sensitization phase, haptens, or the unprocessed allergens, penetrate the skin and then are taken up by the dendritic cells. The dendritic cells then migrate via lymphatics to the regional lymph nodes where they present the leucocyte antigen-complex to naive antigen-specific T lymphocytes. This process leads to expansion of these T cells, that will immigrate into circulation and then to the skin. The migration of dendritic cells from the skin to the lymph nodes is regulated by different factors, including the cytokines (Sebastiani et al. 2002). In allergic contact dermatitis the inflammatory infiltrate comprises CD4+ and CD8+ T lymphocytes, monocytes, and dendritic cells, and in early phase, neutrophils are also present. Leukocyte attraction is controlled by cytokines. (Sebastiani et al. 2002). The inflammation is mediated by members of IL1 family and other cytokines. (Lauritanoa et al. 2020). The IL1 family includes two molecules, IL1 α and IL-1 β , responsible for regulating immune reactions and the inflammatory response (Lauritanoa et al. 2020). The important role of these molecules in contact hypersensitivity was demonstrated in studies of mice deficient in IL1 α and IL1 β genes, and it has been showed that the IL1 α has a more critical role. IL1 β is produced by monocytes, macrophages, Langerhans cells and dendritic cells, and IL1 α is released by keratinocytes. (Ho et al. 2019).

Also, studies showed in an animal model of allergic contact dermatitis that alternatively activated macrophages accumulate in the skin, and these macrophages can exacerbate the contact hypersensitivity, by producing high levels of IL1 β , IL6, and TNF α . (Suzuki et al. 2017). IL6 is a well characterized cytokine, and it is known that acts as a pro-inflammatory cytokine.

In our study KET-PABA had the most important anti-inflammatory effect exerted by the stronger decrease of IL1 α and IL1 β , compared to the vehicle and to KET and PABA and the inhibition of IL6.

In contact hypersensitivity in mice, mast cells may also attenuate the inflammation by producing anti-inflammatory cytokines, such as IL10 (Lauritanoa et al. 2020). Another source of IL10 are the epidermal keratinocytes. IL10 acts on monocytes, macrophages, and dendritic cells, inducing inhibition of expression of class II MHC and inhibit the production of T cell-stimulating cytokines, such as IL1, IL6 and IL12. (Ho et al. 2019). Studies showed that the absence of IL10 predispose mice to exaggerated contact sensitivity responses. In our model, KET-PABA had no effect on IL10, while KET, PABA and DNCB increased it. This correlated with the decrease of all the other pro-inflammatory cytokines such as IL1 α and β , IL6, or the lack of augmentation of the other inflammatory promoters: COX2, TNF α and NRF2. The data suggest that the increase of the IL10 was no longer necessary, since IL10 is secreted to alleviate and modulate an inflammatory state. In the presence of KET-PABA, the treated skin showed a normal status, similar to that of the vehicle for most of the inflammatory molecules or even the inhibition of the IL1 and IL6 compared to the vehicle. Overall, data showed a significant anti-inflammatory effect exerted by the local application of the KET-PABA. This effect could have additional beneficial clinical applications in the

case of the treatment of a cutaneous mycotic infection especially in the presence of an acute inflammatory reaction to the pathogen.

NFκB signaling pathway is involved in inflammation, and TNFα, IL1β, IL6, COX2, and NOS2 are biomarkers for inflammation, which can be induced by dermatitis (Li et al. 2016).

The cytokines, particularly tumor necrosis factor alpha (TNFα), which is secreted by epidermal keratinocytes, plays an important role in inflammation in the skin. TNFα induces inflammation by activation of cytokines IL6 and IL1β, and by increasing the influx of inflammatory cells via induction of expression of adhesion molecules such as endothelial leukocyte adhesion molecule 1 and intercellular adhesion molecule 1 (ICAM1) on neighboring endothelial cells (Zhang et al. 2014).

TNFα can also activate the nuclear factor κB (NFκB) signaling pathway, leading to chronic inflammation in the skin. NFκB regulates the expression of many genes that are involved in the initiation of the inflammatory response. Also, the synthesis of cytokines, such as TNFα, IL1β, IL6, and IL8, and the expression of cyclooxygenase 2 (COX2) are mediated by NFκB (Tak et al. 2001). During the sensitization phase, dendritic cells are activated, migrate to the draining lymph nodes, and induce the priming of hapten-specific T cells (Kissenpfennig et al. 2005). NRF2 is induced in response to contact sensitizers in dendritic cells and keratinocytes (Emter et al. 2010).

In our study, NRF2 has shown an important increase in the DNCB group, suggesting the activation of the sensitization phase, in the presence of the substance, along with the other markers like COX2 and the cytokines. In the KET and PABA groups, NRF2 also showed a slight increase, correlated with the COX2 and TNFα increases. Interestingly, in the KET group, TNFα showed a stronger induction, an effect observed by us *in vitro*, on endothelial (HUVEC) cells but not on dermal fibroblasts (HDFa) (Martin et al. 2020).

This effect correlated with the increase of the IL6. Overall data show that KET had a slight pro-inflammatory effect, but it was modulated by the presence of the increased anti-inflammatory cytokine IL10 which led to the suppression of the inflammation. This rendered the negative MEST result as well as the lack of morphopathology changes in the ear tissue. In the KET-PABA group, there was no NRF2 response, which suggests that the substance did not elicit any inflammatory reaction.

NRF2 plays a major role in the control of both phases of ACD: sensitization and elicitation. During the sensitization phase, NRF2 influence the irritation potency of the contact sensitizers, and, has the role of signaling the danger (Ali et al. 2013). NRF2 deficiency leads to an increased inflammatory response during the elicitation phase, suggesting that NRF2 may also play a role in the regulation of inflammation through a defect in regulatory T cells function (Josefowicz et al. 2012).

The immune response which appears in allergic contact dermatitis involves the oxidative and inflammatory pathways. The NRF2/Keap1 pathway is a major regulator of cellular oxidative and

electrophilic stress, and is activated in the different skin innate immune cells including epidermal Langerhans cells and dermal dendritic cells, but also in keratinocytes.

Both Nrf2 and NFκB pathways are activated by oxidative stress. NRF2 antagonizes NFκB activation through IKK proteasomal activation and HO-1 activity end-products. NFκB downregulates NRF2 via p65-mediated CBP deprivation as well as p65-induced Keap1 nuclear translocation. Nrf2 and NFκB are regulated antagonistically: if NRF2 predominates, it decreases inflammation and oxidative stress through activation of antioxidant enzymes; if NFκB predominates, it leads to pro-inflammatory mediators' secretion and maintains the oxidative stress (Helou et al. 2019).

In the present study, there is a strong NFκB induction in the ketoconazole and DNCB groups, further leading to a proinflammatory environment. KET-PABA had no effect on NFκB level. Interestingly, in the case of the activated form, p NFκB, the effects were only statistically significant in the case of DNCB.

NFκB is a key transcription factor of M1 macrophages and induces the transcription of pro-inflammatory mediators such as IL6, TNFα, and COX2 (Wang et al. 2014).

Prostaglandins play a key role in the generation of the inflammatory response. They are produced during the acute phase of the inflammation. Prostaglandin production depends on the activity of enzymes that contain both cyclooxygenase and peroxidase activity and which exist as distinct isoforms referred to as COX1 and COX2.

COX2 is induced by inflammatory stimuli, and is the most important source of prostaglandin synthesis. It has been suggested that COX2 have a dual role in the inflammatory process, initially contributing to the onset of inflammation and later in supporting resolution of this process (Ricciotti et al. 2011).

In our study, KET-PABA induced the decrease of COX2 and IL6, compared to the vehicle, while KET and PABA slightly increased them. This is consistent with the inhibition of the NRF2 and IL1 and the lack of NFκB activation and suggests an intrinsic anti-inflammatory effect of the cocrystal on the skin of BALBc mice. In our study the contact sensitization test was negative for all substances tested -Ketoconazole, PABA, and cocrystal compound KET-PABA. Knowing the allergenic potential of PABA, reported in the literature (Aronson 2016, Glaser 2016) multiple, high-dose skin applications were used, but no contact allergy was found.

However, the allergenic potential of PABA is very small, and an extremely large number of cases are needed for validation.

Compared to animals, in humans the sensitivity is more variable, and it is highly difficult to predict what proportion of the human population will be sensitized. The compound obtained by co crystallization (KET-PABA) is composed by two active components, KET and PABA, each of them having different properties.

A negative finding does not guarantee that this compound will not be a sensitizer in humans, although we expect it to be at most a weak sensitizer.

Another problem that could raise is the cross-sensitization, meaning that one component of the compound is not distinguished as different by the educated lymphocytes.

Because PABA is widely used in cosmetics, the number of users is high, and the possibility of allergies is obviously increased with the number of applications. Another aspect of PABA use is the occurrence of phototoxic reactions. Because PABA is used in creams as a sunscreen, these reactions can occur as an important part of the reported skin reactions. On the other hand, since PABA functions as a sunscreen, the cocrystal might have a beneficial role in protecting ketoconazole against UV induced degradation and skin phototoxic reactions. However, these hypotheses were not tested in our study. In the current research, the mice were not exposed to UV, so phototoxic reactions were not studied. Further studies are needed to test the appearance of this type of adverse reactions prior to complete the safety profile of the substance. In fact, KET-PABA has been synthesized for a possible use as an antifungal product with topical application, which does not involve concomitant exposure to ultraviolet radiation.

Conclusions

The current study investigated the local possible side effects induced on the skin of BALBc mice by the application of KET-PABA cocrystal. The previously characterized co-crystal showed improved physical properties, such as stability in suspension, solubility, as well as antimycotic efficiency as compared to the parent drug. According to our data, KET-PABA application proved to be safe, without any sensitization effects showed by the MEST study, or the histopathological exam. KET-PABA induced a potent anti-inflammatory effect through the inhibition of proinflammatory cytokines such as IL1 α , IL1 β , IL6 and TNF α , and other proinflammatory inducers such as NRF2. KET-PABA had no effect on the levels of the anti-inflammatory cytokine IL10, or proinflammatory enzyme COX2 and had minimal effects on the activation of the NF κ B pathway. Overall, KET-PABA application led to a better anti-inflammatory response when compared to ketoconazole. Based on the improved antimycotic effect and anti-inflammatory action, KET-PABA cocrystal has the potential to be an efficient drug in the treatment of cutaneous mycosis.

Declarations

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Conflicts of interest

All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

Authors' contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Ethics approval

All lab animal experiments were approved by ethics committee of the University of Medicine and Pharmacy Cluj-Napoca and the Veterinary Health Directorate, Romania (authorization number 67/ 06 06 2017)

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Figures

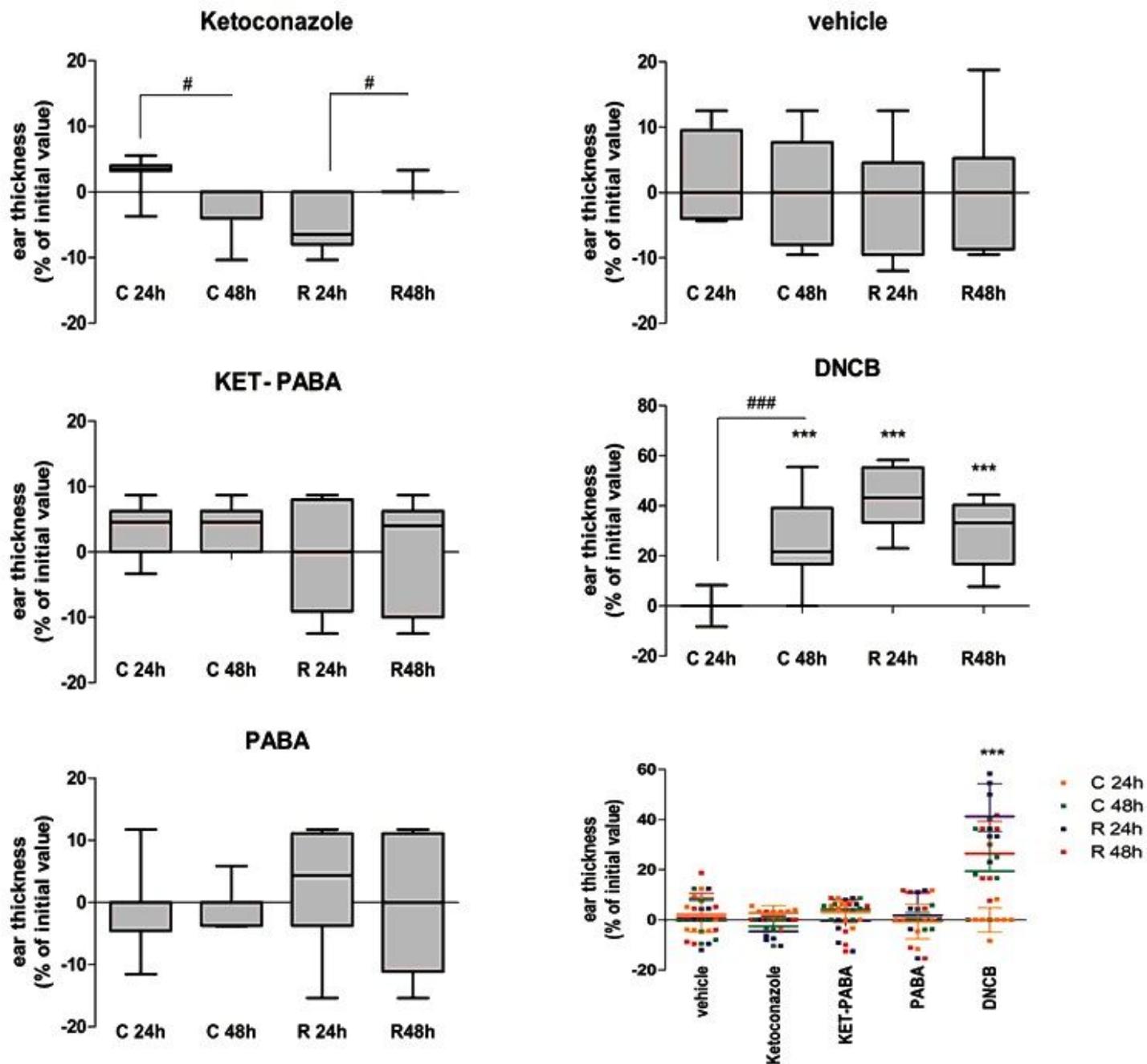


Figure 1

Mouse ear swelling test (MEST). The measurements of the ear thickness (% of initial value) for each group are presented (mean \pm SD, n=7); C24h, C48h = challenge at 24h, respectively 48h from ear skin application, R24h and R48h = rechallenge at 24h respectively 48h from ear skin applications; *** = $p < 0.0001$ compared to the vehicle; # = $p < 0.05$, ### = $p < 0.0001$ between the different time points. Last panel presents the distribution values of the ear thickness within the groups, * = $p < 0.05$ and *** = $p < 0.0001$.

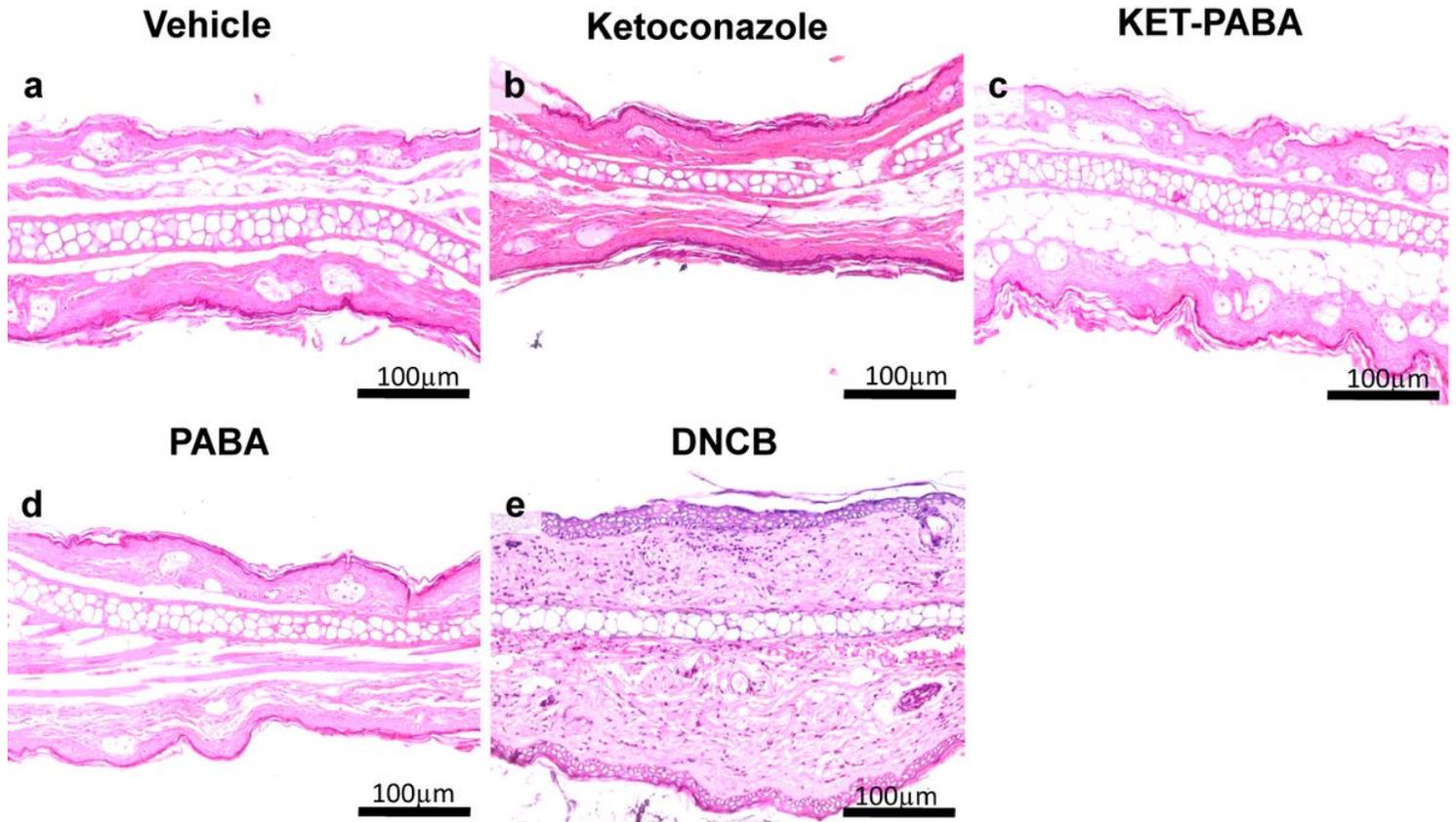


Figure 2

Histological analysis of ear pinna. Vehicle control (A), Ketoconazole (B), Ketoconazole-p-aminobenzoic acid cocystal (C), p-aminobenzoic acid (D) treated mice showed normal histological features. DNCB treated mice exhibited lymphocytes and plasma cells infiltration (E). HE stain, Bar=100µm.

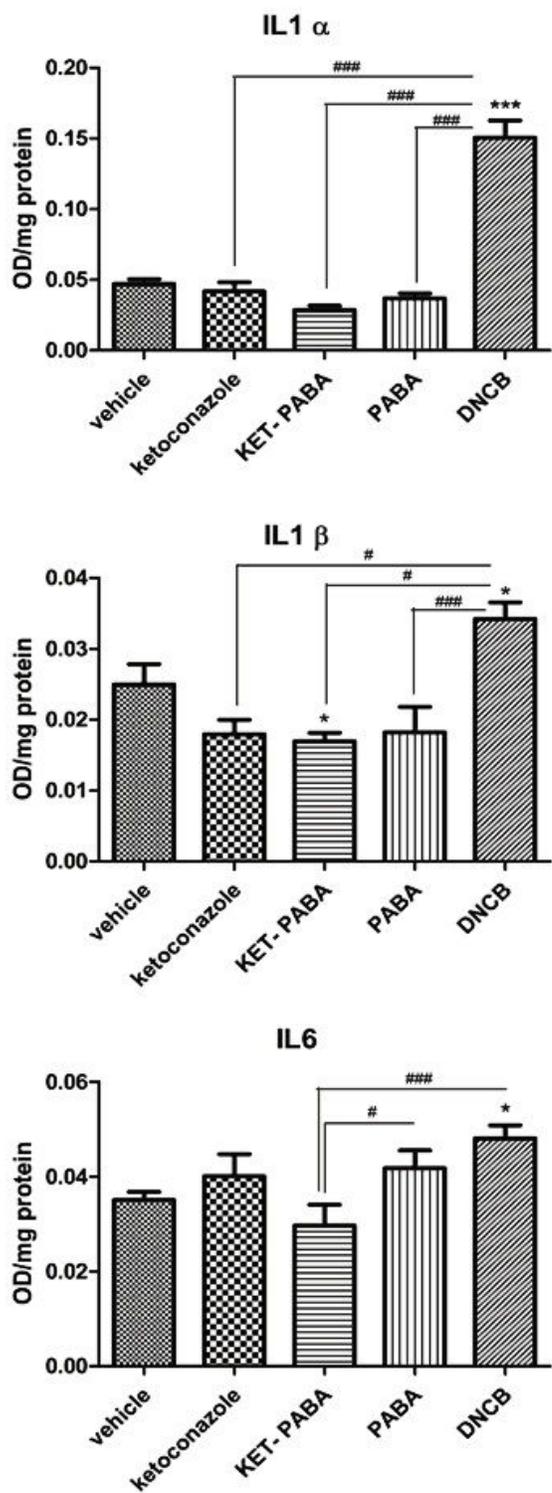


Figure 3

ELISA measurement of pro-inflammatory interleukins IL1 α , IL1 β and IL6. Data are presented as mean \pm SD, n=3, * = p< 0.05, ***= p< 0.0001, compared to vehicle; #=p<0.05, ###= p<0.0001, compared to other groups.

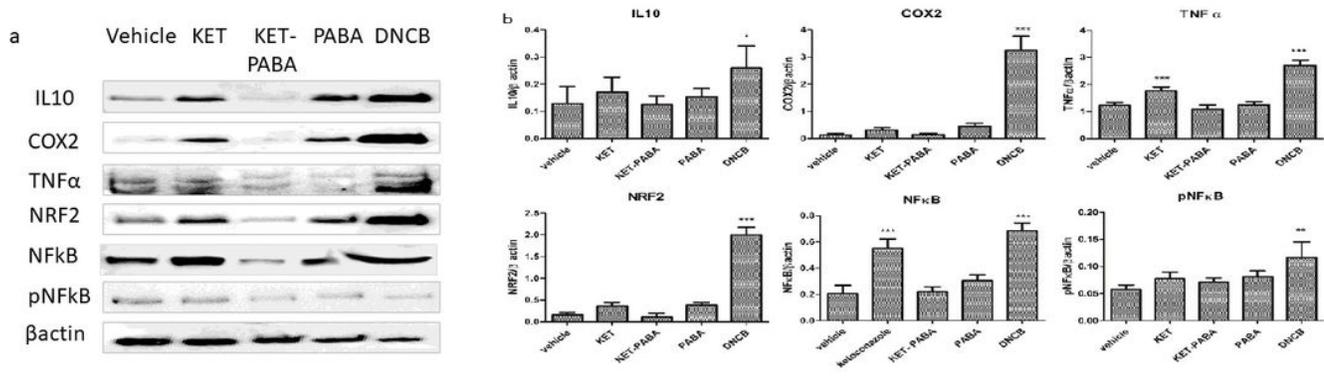


Figure 4

Western Blot assessment of inflammatory markers: IL10, COX2, TNF α , NRF2, NF κ B and pNF κ B in the ear tissue: a. images of the WB bands; b. image analysis of WB bands was done by densitometry, results were normalized to β actin. Each bar represents mean \pm SD (n = 3); * = p<0.05, *** = p<0.0001, compared to vehicle.