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Naringenin as an opener of mitochondrial potassium channels in dermal fibroblasts

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Abstract

Flavonoids belong to a large group of polyphenolic compounds that are widely present in plants. Certain flavonoids, including naringenin, have cytoprotective properties. Although the antioxidant effect has long been thought to be a crucial factor accounting for the cellular effects of flavonoids, mitochondrial channels have emerged recently as targets for cytoprotective strategies. In the present study, we characterized interactions between naringenin and the mitochondrial potassium (mitoBK$_{Ca}$ and mitoK$_{ATP}$) channels recently described in dermal fibroblasts. With the use of the patch-clamp technique and mitoplasts isolated from primary human dermal fibroblast cells, our study shows that naringenin in micromolar concentrations leads to an increase in mitoBK$_{Ca}$ channel activity. The opening probability of the channel decreased from 0.97 in the control conditions (200 μmol/L Ca^{2+}) to 0.06 at a low Ca^{2+} level (1 μmol/L) and increased to 0.85 after the application of 10 μmol/L naringenin. Additionally, the activity of the mitoK$_{ATP}$ channel increased following the application of 10 μmol/L naringenin. To investigate the effects of naringenin on mitochondrial function, the oxygen consumption of dermal fibroblast cells was measured in potassium-containing media. The addition of naringenin significantly and dose-dependently increased the respiratory rate from 5.8 ± 0.2 to 14.0 ± 0.6 nmol O_2 × min^{-1} × mg $protein^{-1}$. Additionally, a Raman spectroscopy analysis of skin penetration indicated that the naringenin was distributed in all skin layers, including the epidermis and dermis. In this study, we demonstrated that a flavonoid, naringenin, can activate two potassium channels present in the inner mitochondrial membrane of dermal fibroblasts.

KEYWORDS
dermal fibroblast, mitochondria, naringenin, potassium channel

Abbreviations: mitoBK$_{Ca}$ channel, mitochondrial large-conductance Ca^{2+}-regulated potassium channel; mitoK$_{ATP}$ channel, mitochondrial ATP-regulated potassium channel; P_o, open probability of the channel; ROS, reactive oxygen species.

1 | INTRODUCTION

The following potassium channels, similar to plasma membrane potassium channels, have been identified in the inner mitochondrial membrane: mitochondrial (a) ATP-regulated potassium channels (mitoK$_{ATP}$ channels),[1–4] (b) small-, intermediate- and large-conductance calcium-activated potassium channels,[5–8] (c) voltage-dependent potassium Kv1.3/Kv7.1 channels (mitoKv channels)[9–11] and (d) twin-pore TASK-3 potassium channels (mitoTASK).[12,13] Potassium ion transport through the inner mitochondrial membrane regulates mitochondrial metabolism, primarily via the regulation of mitochondrial membrane potential, respiration and mitochondrial reactive oxygen species (ROS) synthesis.[14]

Potassium transport through the mitochondrial inner membrane via mitochondrial potassium channels has also been described as an important player in cell life and death phenomena.[15] For example, activation of the mitochondrial ATP-regulated potassium channel (mitoK$_{ATP}$) or the mitochondrial large-conductance Ca^{2+}-regulated potassium channel (mitoBK$_{Ca}$) with potassium channel openers may induce cytoprotective effects in various cell types, such as cardiac or neuronal tissue.[16,17] In contrast, mitochondrial channel blockade has the opposite effect. It has been shown that inhibition of the mitochondrial Kv1.3 channel may lead to cell death in cancer cells.[18]

Recently, we have demonstrated the presence of various mitochondrial potassium channels in the following cells present in skin tissue: keratinocytes,[13] fibroblasts[1,19] and endothelial cells.[20] In the human keratinocyte HaCaT cells, a patch-clamp technique has demonstrated a potassium-selective channel activity with a conductance of 83 pS.[13] Using reverse transcriptase-PCR, an mRNA transcript for the TASK-3 (tandem pore domain acid-sensitive K channels) channels has been detected. In the HaCaT cells, a co-localization of the TASK-3 protein and a mitochondrial marker is observed.[13] We have also analysed the activity of the mitoBK$_{Ca}$ channel in mitoplasts isolated from a primary human dermal fibroblast cells.[19] A potassium-selective current has been recorded with a mean conductance of 280 ± 2 pS in a symmetrical 150 mmol/L KCl solution. The mitoBK$_{Ca}$ channel of dermal fibroblast cells is activated by the Ca^{2+} and by potassium channel opener NS1619. The channel activity is irreversibly inhibited by paxilline, a selective inhibitor of the BK$_{Ca}$ channels. Additionally, the α- and β-subunits (predominantly the β3-form) of the BK$_{Ca}$ channels have been identified in fibroblast mitochondria. Interestingly, the presence of the BK$_{Ca}$ channel in the mitochondria of the human endothelial cell has been also observed.[20] Recently, for the first time, we have also shown the presence of mitochondrial ATP-regulated potassium channels in human dermal fibroblast cells.[1] Using the patch-clamp technique on the inner mitochondrial membrane of fibroblasts, a potassium channel with a mean conductance equal to 100 pS in symmetric 150 mmol/L KCl has been detected. Using reverse transcriptase-PCR, also an mRNA transcript for the KCNJ1 (ROMK) channel has been identified in fibroblasts. Moreover, the presence of the ROMK protein has been shown in the inner mitochondrial membrane fraction from fibroblasts.[1]

Naringenin is a natural substance, belonging to the flavonoid group, which has a beneficial influence on organisms and interacts with potassium channels.[21,22] This compound naturally occurs in grapefruits (*Citrus paradisi*) and is responsible for bitter taste of those fruits. Naringenin is known for its pharmacological effects, such as antiatherogenic, antithrombotic, anti-inflammatory, hypolipidemic and antioxidative properties.[23] Furthermore, naringenin and its structural analogues have been shown to activate BK$_{Ca}$ and K$_{ATP}$ channels present in the plasma membrane of smooth muscle cells and motor neuron-like cells.[24,25] A stimulatory effect of naringenin on the cardiac mitoBK$_{Ca}$ channel has been recently observed.[22]

In this study, with the use of patch-clamp and biochemical techniques, we provide direct evidence that naringenin has a stimulatory effect on the activity of the large-conductance, calcium-regulated (mitoBK$_{Ca}$) and ATP-regulated potassium channels (mitoK$_{ATP}$) present in the mitochondria of human dermal fibroblasts.

2 | METHODS

2.1 | Cell culture

As an experimental model, we used primary human dermal fibroblasts from breast skin (Cat. No. ATCC® PCS-201-012™). The cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The cells were reseeded every fourth day and cultivated until 12 passages.

2.2 | Mitochondria and mitoplast preparation

For electrophysiological measurements, mitochondria and subsequent mitoplasts were prepared by differential centrifugation and hypotonic swelling, respectively, as previously described.[19] Mitoplasts were prepared from the fibroblast mitochondria by incubation in a hypotonic solution (5 mmol/L HEPES, 200 μmol/L $CaCl_2$, pH 7.2) for approximately 1 minute, and then a hypertonic solution (750 mmol/L KCl, 30 mmol/L HEPES, and 200 μmol/L $CaCl_2$, pH 7.2) was subsequently added to restore the isotonicity of the medium. For each patch-clamp experiment, mitoplasts were prepared based on hypotonic swelling assay.

2.3 | Patch-clamp experiments

Patch-clamp experiments using mitoplasts were performed as previously described.[19,26] In brief, a patch-clamp pipette was filled with an isotonic solution containing 150 mmol/L KCl, 10 mmol/L HEPES and 200 μmol/L $CaCl_2$ at pH 7.2. All of the modulators of the mitoBK$_{Ca}$ and mitoK$_{ATP}$ channel were added as dilutions in the isotonic solution. To apply these substances, a perfusion system was used. The mitoplasts at the tip of the measuring pipette were transferred into the openings of a multibarrel "sewer pipe" system

in which their outer faces were rinsed with the test solutions (Figure 1A). The current-time traces of the experiments were recorded in single-channel mode. The illustrated channel recordings are representative of the most frequently observed conductance for the given condition. The open probability (P_o) of the channels was determined using the single-channel search mode of the Clampfit 10.7 software.

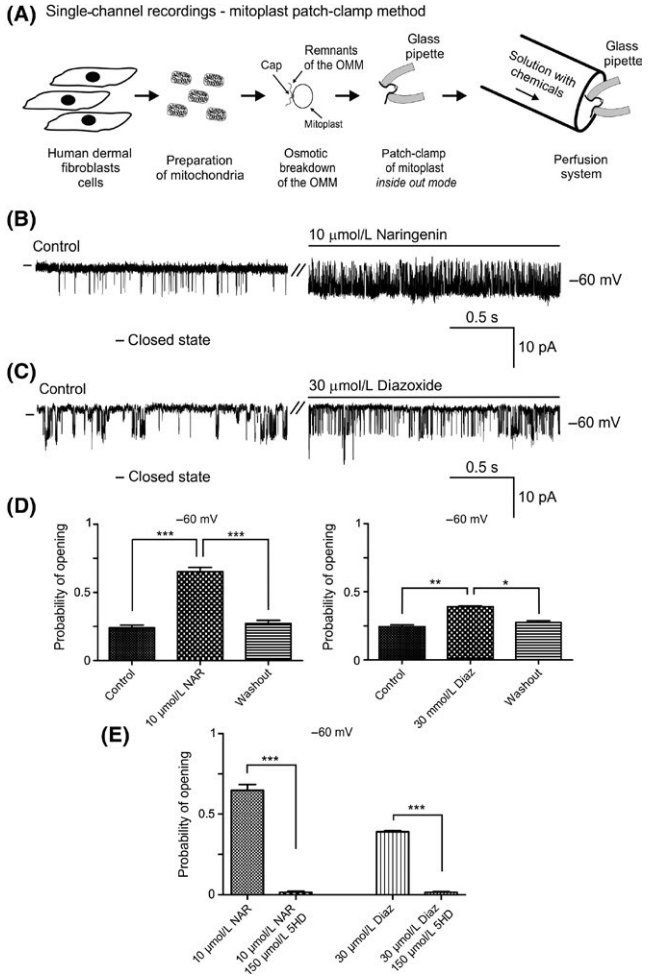

FIGURE 1 Influence of naringenin and diazoxide on the activity of the mitoK$_{ATP}$ channel from the mitochondria of primary human dermal fibroblasts. A, A schematic representation of the mitochondria and mitoplast preparation from primary human dermal fibroblasts, mitoplast patching and the patch-clamp experiment in the inside-out mode using the perfusion system. The matrix side of the mitochondrial membrane was exposed to externally added substances. Representative single-channel current-time recordings of the mitoK$_{ATP}$ channel in a symmetric 150/150 mmol/L KCl isotonic solution at −60 mV after (B) 10 µmol/L naringenin (C) and 30 µmol/L diazoxide treatment. D, Analysis of the open probability of the mitoK$_{ATP}$ channel at −60 mV under control solution, 10 µmol/L naringenin (NAR) (left panel) and 30 µmol/L diazoxide (Diaz) (right panel) application and after washout to control solution. E, Open probability analysis of the 5-hydroxydecanoic acid (5HD) inhibitory effects on the mitoK$_{ATP}$ channel activated by 10 µmol/L naringenin and 30 µmol/L diazoxide. Data (D, E) are presented as the mean ± SD (n = 5)

2.4 | Measurements of cell respiration

Human fibroblast cells were harvested as described previously.[19] The final cell pellet was resuspended in PBS (1 g of cells per 2 mL of medium) and maintained on ice. Protein content was determined using the Bradford method (Bio-Rad). Cells were resuspended in cold DMEJ medium containing 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, 110 mmol/L NaCl, 44 mmol/L $NaHCO_3$, 1.1 mmol/L NaH_2PO_4 and 10 mmol/L Na/Na buffer (pH 7.2). The oxygen consumption rate was measured at 37°C using a Clark-type electrode (Hansatech) in 0.7 mL of DMEJ medium with 4 mg of protein, as previously described.[27] Pyruvate (5 mmol/L) was used as a respiratory substrate. Oligomycin (1 µg/mg of protein) was added to inhibit ATP synthesis.

2.5 | Carriers for naringenin

The following two formulation carriers were developed for testing naringenin penetration into the skin: carrier no. 1: naringenin 0.1%, dimethicone/vinyl dimethicone 0.93%, silica 0.075%, cyclopentasiloxane 0.63%, dimethicone 1.33%, sodium polyacrylate 1.82%, trideceth-6 0.42%, ethylhexylglycerin 0.05%, glycerin 23.4%, butylene glycol 0.05%, methylparaben, fenoksyetanol 0.47%, aqua (water) and carrier no. 2: naringenin 0.1%, hydrogen dimethicone 0.07%, stearic acid 0.09%, palmitic acid 0.11%, laureth-7 0.11%, xanthan gum 0.41%, hydroxyacetophenone 0.5%, 1,2-hexanediol 1.5%, titanium dioxide (nano) 1.51%, potassium cetyl phosphate 2%, cetyl alcohol 2.5%, dicaprylyl carbonate 4%, octocrylene 5%, aqua (water).

2.6 | Raman spectroscopy analysis: skin penetration tests of naringenin

Materials used for the study were fragments of the skin obtained during planned surgical procedures. The studied skin was obtained during the facial plastic surgery of a 34-year-old woman. The collected skin was then transported in culture medium (RPMI-1640) at room temperature. Within 16 hours of collection, the skin was divided into pieces of approximately 25 mm^2, which were placed on cell culture inserts within the wells of tissue culture plates in the culture medium. 30 µL of naringenin in two different cosmetic formulation carriers (described above carrier no. 1 and carrier no. 2) was applied on the skin surface, which was subsequently incubated at 37°C for 72 hours. After this time, each piece of skin was cut along the transverse axis to obtain a cross-sectional profile of each layer of the sample and then placed on gold-coated glass slides (BION 300 nm, Gold-Coated Glass Slides, Phasis). Raman microscopy (RamanMicro 300, Perkin-Elmer) was used to examine the substance penetration depth, according to Ref. [28] The spectra were taken using an infrared laser (785 nm). The size of the scanning area was approx. 1.4 × 3.0 mm, and the area contained 7 lines (98 points for carrier no. 1 and 105 points for carrier no. 2, which were located at a distance of 2200 µm from one another). The points were irradiated with 70 mW laser power (785 nm) with 50 seconds total acquisition time for each point (10 exposures). To analyse the distribution

of naringenin in the inner layers of the skin, the resulting spectrum of tissues was compared with the reference spectra of the tested substances. An analysis of the distribution of samples resulted from a comparison of the test and reference spectra and from expressing their similarities with the correlation of the two images.

The study design was accepted by the Ethics Committee of the Pomeranian Medical University in Szczecin.

2.7 | Statistical analysis

The results are expressed as the mean ± SD obtained from at least three independent experiments, and each determination was performed at least in triplicate. An unpaired two-tailed Student's *t* test was used to identify any significant differences. For patch-clamp experiments, one-way ANOVA was used to compare the means of three or more treatment conditions. For all tests, a *P*-value was considered to be significant if $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

3 | RESULTS

3.1 | Patch-clamp studies of the mitoBK$_{Ca}$ and mitoK$_{ATP}$ channel pharmacology

The patch-clamp technique was used to investigate the biophysical and pharmacological properties of the large-conductance Ca^{2+}-regulated (mitoBK$_{Ca}$) and ATP-regulated (mitoK$_{ATP}$) potassium channels present in the inner mitochondrial membranes of primary human dermal fibroblast cells. A flow chart of the experiments using the patch-clamp technique is shown in Figure 1A. Selection of fibroblast mitoplasts was performed based on the vesicle size, transparency and the presence of a characteristic "cap," that is a region containing the remnants of the outer mitochondrial membrane. Additionally, to exclude false positives and to confirm the mitochondrial identity of the applied vesicles, we performed an assay called single-mitoplast PCR, as previously described[19] (data not shown). In the patch-clamp experiments with fibroblast mitoplasts, currents characteristic of the mitoBK$_{Ca}$ (n = 28) and mitoK$_{ATP}$ (n = 31) channels were observed. We detected channels with mean single-channel conductances of 280 ± 2 pS and 100 ± 3 pS for mitoBK$_{Ca}$ and mitoK$_{ATP}$ channels, respectively. The conductances were calculated from the current-voltage relationship. Rectification of the current was not observed (data not shown). The effect of mitoK$_{ATP}$ channel activation by naringenin was observed (Figure 1B) (n = 8), and an analysis of the open probability (P_o) was performed (Figure 1D, left panel). P_o increased from 0.24 to 0.65 (in the presence of 10 μmol/L naringenin). This activation was immediately reversed by the washout to control solution. Substances known to regulate mitoK$_{ATP}$ channel activity were also used to confirm the properties of the ion channels observed in our experiments. Figure 1C illustrates the activity of the mitoK$_{ATP}$ channel under control conditions and upon the application of 30 μmol/L diazoxide, a known activator of mitoK$_{ATP}$ channel activity (n = 6). The presence of the diazoxide increased the P_o from 0.24 to 0.39 (n = 3) (Figure 1D, right panel). This effect was reversed by the washout. The open probability of the channel decreased from 0.39 (in the presence of the diazoxide) to 0.27 (upon washout). The effect of a mitoK$_{ATP}$ channel inhibitor on single-channel activity was then examined. 5-Hydroxydecanoic acid inhibited the channel activity in the presence of naringenin and diazoxide (Figure 1E). The probability of the channel opening diminished significantly from 0.65 to 0.02 in the presence of 10 μmol/L naringenin and from 0.39 to 0.01 in the presence of 30 μmol/L diazoxide (Figure 1E).

The mitoBK$_{Ca}$ channel activation by naringenin was also studied. Figure 2A illustrates typical activity of the mitoBK$_{Ca}$ channel under control conditions in the presence of 200 μmol/L Ca^{2+} (left panel), a low-calcium solution (1 μmol/L Ca^{2+}) (middle panel), and a low-calcium solution with 10 μmol/L naringenin (right panel). Activation of the mitoBK$_{Ca}$ channel by naringenin was immediate at all applied voltages. Slight changes in conductance were observed from 280 pS under control conditions to 255 pS after 10 μmol/L naringenin application (Figure 2B), but the changes were not significant. The distribution of the mitoBK$_{Ca}$ open probability at different voltages is shown in Figure 2C. The activation effects of 10 μmol/L naringenin were completely reversible. After washout of naringenin, P_o was 0.05. The open probability of the mitoBK$_{Ca}$ channel at a holding potential of +40 mV and −40 mV demonstrated an immediate strong inactivation effect of the low Ca^{2+} concentration on the single-channel activity. The probability of the channel opening diminished significantly ($P < 0.001$) from 0.97 to 0.06 at +40 mV and from 0.54 to 0.02 at −40 mV (Figure 2D). The addition of 10 μmol/L naringenin increased the mitoBK$_{Ca}$ channel activity at low Ca^{2+} concentration. The probability of the channel opening increased significantly from 0.06 to 0.85 at +40 mV and from 0.02 to 0.44 at −40 mV (Figure 2D). The activation effects of 10 μmol/L naringenin were completely blocked upon the addition of 1 μmol/L paxilline, a known inhibitor of mitoBK$_{Ca}$ channel activity. Paxilline inhibited the channel activity; P_o dropped from 0.85 at +40 mV and 0.44 at −40 mV to 0.02 (Figure 2D).

3.2 | Effects of potassium channel modulators on the respiratory rate in human dermal fibroblasts

To investigate the effects of naringenin on potassium permeability in fibroblast mitochondria, the oxygen consumption rate of fibroblast cells was measured in potassium-containing media. As shown in Figure 3A, the addition of 5–30 μmol/L naringenin significantly and dose-dependently increased the respiratory rate from 5.8 ± 0.2 up to 14.0 ± 0.6 nmol $O_2 \times min^{-1} \times$ mg $protein^{-1}$ (~120% increase). The saturation of the effect was observed at 20 μmol/L naringenin, and thus, this concentration was used in subsequent experiments. The stimulatory effect of naringenin was partially reversed by the mitochondrial BK$_{Ca}$ potassium channel inhibitor paxilline, as shown in Figure 3B. The addition of 20 μmol/L paxilline decreased the effect of naringenin by ~50%. As naringenin was shown in electrophysiological experiments to activate both mitochondrial potassium channel types, BK$_{Ca}$ and K$_{ATP}$ (Figures 1 and 2), the effect of mitoK$_{ATP}$ channel inhibitors on naringenin-stimulated oxygen consumption

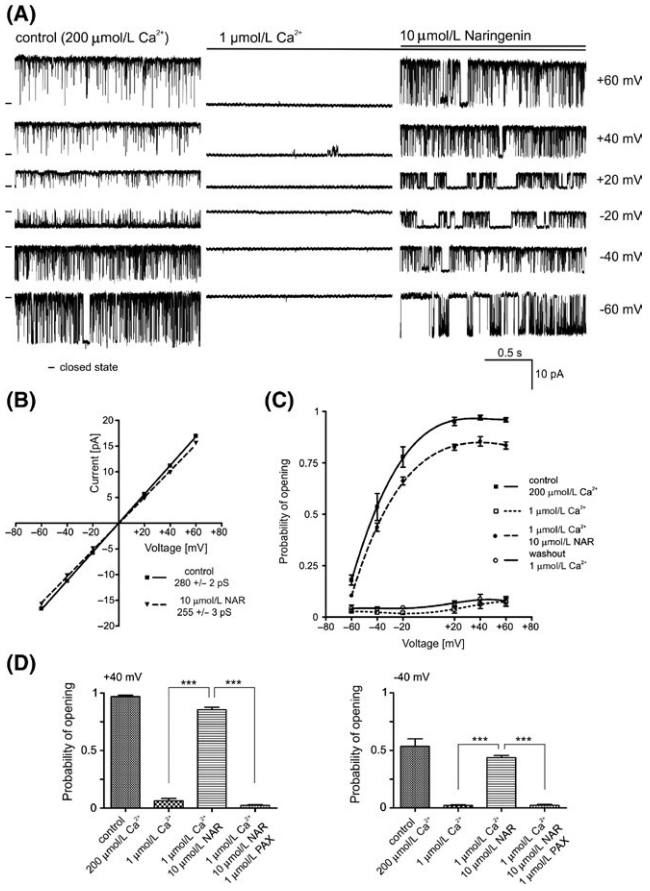

FIGURE 2 Effect of naringenin on the activity of the mitoBK$_{Ca}$ channel present in the inner mitochondrial membrane of primary human dermal fibroblasts. A, Representative traces of single-channel recordings of the mitoBK$_{Ca}$ channel at different voltages in the rage of −60 to +60 mV performed in a symmetric 150/150 mmol/L KCl control solution (200 µmol/L Ca^{2+}), low-calcium solution (1 µmol/L Ca^{2+}) or low-calcium solution containing 10 µmol/L naringenin. B, The current-voltage relationship based on a single-channel recording in a symmetric 150/150 mmol/L KCl isotonic solution (200 µmol/L Ca^{2+}) and low-calcium solution containing 10 µmol/L naringenin (NAR). C, The open probability of the mitoBK$_{Ca}$ channel at different voltages in the order of experimental conditions: symmetric 150/150 mmol/L KCl control solution (200 µmol/L Ca^{2+}, ■), low-calcium solution (1 µmol/L Ca^{2+}, □), low-calcium solution containing 10 µmol/L naringenin (1 µmol/L Ca^{2+} plus 10 µmol/L NAR, ●) and after washout to low-calcium solution (1 µmol/L Ca^{2+}, ○). D, Analysis of the open probability at +40 (left panel) and −40 mV (right panel) under control solution (200 µmol/L Ca^{2+}), low-calcium solution (1 µmol/L Ca^{2+}), low-calcium solution containing 10 µmol/L naringenin (1 µmol/L Ca^{2+} plus 10 µmol/L NAR) and after the application of 1 µmol/L paxilline (PAX) in a low-calcium solution containing 10 µmol/L naringenin (1 µmol/L Ca^{2+} plus 10 µmol/L NAR plus 1 µmol/L PAX). All data are presented as the mean ± SD (n = 5)

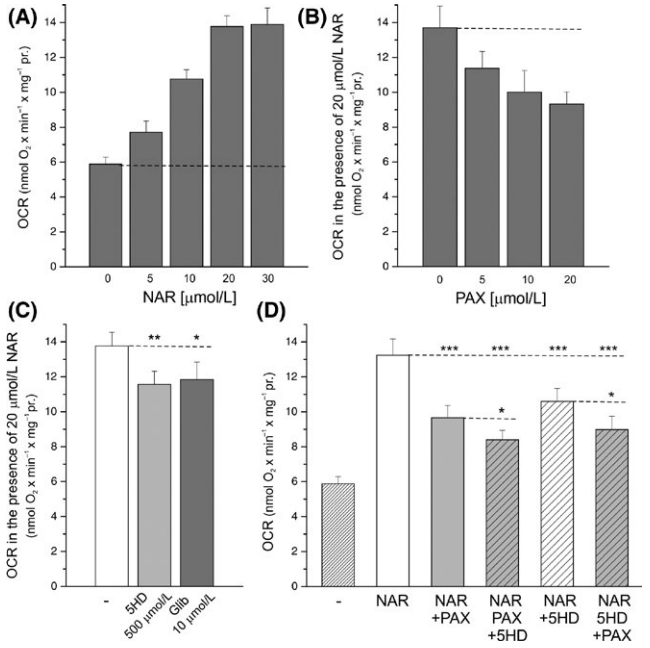

FIGURE 3 Influence of naringenin on the oxygen consumption rate of fibroblasts in the presence of inhibitors of potassium channels. A, The effect of increasing concentrations of naringenin (NAR) on the oxygen consumption rate (OCR). B, The effect of increasing concentrations of the BK_{Ca} channel inhibitor, paxilline (PAX), on naringenin-induced changes in OCR. C, The effect of mitoK_{ATP} channel inhibitors, 500 µmol/L 5-hydroxydecanoic acid (5HD) and 10 µmol/L glibenclamide (Glib) on OCR in the presence of naringenin. D, The cumulative effect of the mitoBK$_{Ca}$ and mitoK_{ATP} inhibitors on the naringenin-stimulated OCR. Experiments were performed in the presence of pyruvate (5 mmol/L) as a respiratory substrate, oligomycin (1 µg/mg of protein), and in the absence or presence of 20 µmol/L naringenin (B, C). OCR is expressed in nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$

rates was also investigated (Figure 3C,D). Both 500 µmol/L 5-hydroxydecanoic acid and 10 µmol/L glibenclamide (Figure 3C) significantly (by 28% and 24%, respectively) diminished the naringenin-induced oxygen consumption rates. 5-Hydroxydecanoic acid, a slightly more potent inhibitor (Figure 3C), was used in later experiments (Figure 3D). The cumulative effect of inhibitors specific for both types of mitochondrial potassium channels is illustrated in Figure 3D. These experiments demonstrate that the naringenin-induced increase in oxygen consumption was sensitive to inhibitors of both types mitochondrial potassium channels, and the inhibitory effect was additive. The addition of 5-hydroxydecanoic acid to cells treated with naringenin and paxilline further reduced the effect by 17%. The same was true when inhibitors were added in reverse order. 5-Hydroxydecanoic acid alone diminished the effect of naringenin by 36%, while the addition of paxilline increased the inhibition up to 58%.

3.3 | Skin penetration tests of naringenin

Substantial information regarding the chemical composition of the biological samples, which enabled the identification of characteristic bands of such molecules as nucleic acids, proteins, lipids or flavonoids, can be obtained from the Raman spectra.[28,29] In biological and medical applications, Raman spectroscopy has the advantage that a spectrum containing a large amount of information can be

obtained from intact tissue, thus without interfering with the structure. Therefore, it is possible to obtain a detailed chemical analysis of biological material, despite its high complexity. Another advantageous feature is the high sensitivity of Raman spectroscopy, even for small structural changes.

In our study, an analysis of the distribution of samples (naringenin in carrier no. 1 and naringenin in carrier no. 2) resulted from a comparison of the test and reference spectra and from expressing their similarities with the correlation of the two images (Figure 4). The greatest intensity of the characteristic peaks of the spectrum of the test formulation, and thus most of its contents, is marked in Figure 4. The colour scale reflects the level of correlation between the spectrum of the tested conjugates and the spectrum of the skin after exposure. Pink-coloured spots represent the highest content of the tested naringenin in carriers in the skin. The results of the present study revealed that within 72 hours of incubation, carrier no. 1 was distributed in all layers of the epidermis and dermis (Figure 4A), gradually penetrating to the lower part of dermis. Carrier no. 2 was distributed unevenly, and the highest content was present in the epidermis and the inner layer of the dermis (Figure 4B). The results indicate that naringenin in tested carriers penetrates human skin layers.

4 | DISCUSSION

Mitochondrial potassium channels are distributed in a variety of tissues and cell types.[14,30] These channels were identified both in tissue containing a high number of mitochondria, such as cardiac muscle[31,32] and brain,[33,34] and in tissue less dependent on oxidative phosphorylation, such as endothelium.[20] It is believed that potassium flux through the inner mitochondrial membrane plays a significant role in the cell life/death cycle.[15]

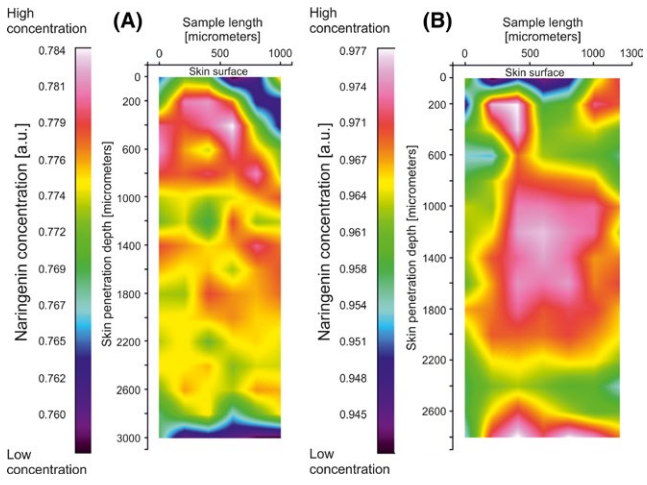

FIGURE 4 Raman spectroscopy analysis of the distribution of naringenin in the skin. Distribution of naringenin using carrier no. 1 (A) and naringenin using carrier no. 2 (B) in the inner layers of the skin. Pink regions represent the highest content of the tested naringenin in the skin

Mitochondrial potassium channel activation with potassium channel openers induces cytoprotection.[35] For example, ischaemic preconditioning in heart and brain tissue can be mimicked with the use of diazoxide acting on mitochondria.[36] In contrast, mitochondrial potassium channel inhibition may lead to cell death. Recently, it has been shown that inhibition of the mitochondrial Kv1.3 channel in primary chronic B-lymphocytic leukaemia tumor cells, melanoma cells and pancreatic cancer cells causes cell death.[37] The explanation for these phenomena is likely based on the modulation of mitochondrial ROS generation due to inhibition of potassium ion flux via the inner mitochondrial membrane.[38]

Naringenin, the predominant flavonoid in such citrus fruits as lemon, orange, tangerine and grapefruit, has pleiotropic actions on cellular function.[23] This flavonoid has antioxidant, antifibrogenic, anti-inflammatory and anticancer properties.[39] Moreover, naringenin inhibits UVB irradiation-induced skin inflammatory oedema, cytokine production, myeloperoxidase activity, matrix metalloproteinase-9 activity and oxidative stress.[40] Naringenin also interacts with various plasma membrane potassium channels, both in a stimulatory[21] or inhibitory[41] manner. Additionally, it has been shown that naringenin could be an efficient therapeutic agent reducing superoxide anion-driven inflammatory pain. The anti-inflammatory and antioxidant effects are mediated via activation of the nitric oxide-cGMP-dependent protein kinase-K_{ATP} channel signalling and involve the induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/heme oxidase pathway.[42] Recently, cardioprotective effects of naringenin have been observed in rat heart due to the activation of the mitoBK$_{Ca}$ channel.[16,22]

In this study, we have analysed the interaction of naringenin with the following two potassium channels identified in the mitochondria of human skin fibroblasts: the ATP-regulated potassium channel, mitoK$_{ATP}$ channel,[1] and the large-conductance calcium-regulated potassium channel, mitoBK$_{Ca}$ channel.[19] Our results indicate that these channels have similar properties, both biophysical and pharmacological, to mitochondrial potassium channels described in other cell types (for review see [14]). The application of a patch-clamp technique and single-channel recordings of potassium channels present in the inner mitochondrial membrane is a key approach to identify and study the properties of these proteins. Using this methodology, we demonstrated the direct stimulation of the mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels of human dermal fibroblasts by micromolar concentrations of naringenin. Stimulation of the channels was observed by diazoxide (mitoK$_{ATP}$ potassium channel opener[43]) and Ca^{2+} (activator of mitoBK$_{Ca}$ channels[44]), confirming the identity of these channels in the mitochondria. Naringenin-induced stimulation of mitoK$_{ATP}$ channel was inhibited by 5-hydroxydecanoic acid, a classical inhibitor of mitoK$_{ATP}$ channels.[45] Similarly, paxilline (a blocker of BK$_{Ca}$ channels[46]) inhibited naringenin-induced stimulation. After application of naringenin, an increase in open probability of the mitoBK$_{Ca}$ channel is twice as high at positive potentials than that at negative voltages. This observation is consistent with endogenous voltage dependence of the mitoBK$_{Ca}$ channel.[19]

Naringenin is considered to be an antioxidant compound with substantial activity against oxidative damage in cells.[23] This property raises the following questions: Is there any chemical reaction between mitochondrial potassium channels and naringenin? Is the stimulatory effect towards channel proteins based on a redox reaction? The reversibility of the stimulatory effect upon washout of naringenin suggests, rather, a ligand-protein interaction, similarly for the potassium channel opener diazoxide.[43] Additionally, the activation of mitoBK$_{Ca}$ channel in fibroblasts is similar to the stimulation observed in cardiomyocytes.[16] The cardiac mitoBK$_{Ca}$ channel is likely the DEC isoform,[32] but the molecular identity of the fibroblast mitoBK$_{Ca}$ needs further investigations.

The stimulatory effect of naringenin on mitochondrial potassium channels was further confirmed by respiration measurements of fibroblast cells. It was previously observed that activation of the channel followed by an influx of K^+ into the mitochondrial matrix results in the stimulation of mitochondrial respiration measured in cells.[47] In our study, in fibroblast cells, naringenin-stimulated respiration (mitochondrial respiratory activity) and its effect was lowered by the application of channel inhibitors such as 5-hydroxydecanoic acid, glibenclamide and paxilline. These findings confirm the stimulatory action of naringenin on mitochondrial potassium channels. Similar effects were observed in other cellular systems.[16] Additionally, this observation confirms that naringenin can penetrate the membranes of skin fibroblasts. Naringenin accumulation into other cells was also observed.[48]

Naringenin is a poor water-soluble compound and has a minimal oral bioavailability due to its largely hydrophobic ring structure.[49] In our study, the results of the skin penetration tests indicated that after treatment, the naringenin is distributed in all skin layers, the epidermis and dermis. The presence of naringenin in the dermis, in both carriers, suggests the possibility of activating the mitochondrial potassium channels of fibroblasts after topical treatment with naringenin. Moreover, the results of this study indicate that a cytoprotective bioactive substances in an appropriate delivery systems may play a crucial role in skin topical treatment.[50] Recently, topical formulations containing naringenin have been studied regarding their physicochemical characteristics and antioxidant activity. It has been shown that application of naringenin formulation in the dorsal skin of hairless mice protects from UVB irradiation. Additionally, formulations containing naringenin inhibit UVB irradiation-induced lipid hydroperoxide formation, oedema and cytokine production.[51] In other studies, naringenin inhibited fibroblast activation and local inflammation in mechanical stretch-induced hypertrophic scar mouse model.[52] All these data open the possibility for future studies of action of the naringenin as well as novel applications for other inflammatory and oxidative skin diseases.

Previously, it has been shown that naringenin promotes cardioprotective effects against ischaemia/reperfusion injury through the activation of mitoBK$_{Ca}$[22] and mitoK$_{ATP}$[53] channels. Our study confirms that naringenin is an opener of mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels in dermal fibroblasts. Further studies are needed to understand why naringenin is able to activate two channels belonging to different molecular classes. Currently, understanding of the role of mitochondrial potassium channels in skin fibroblast physiological function is limited. Investigating the role of mitochondrial channels in impaired dermal fibroblast function, such as wound healing or ageing-related skin diseases, represents future challenges.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHORS' CONTRIBUTIONS

PB, RPK, AK, WJ, MPP, BD, RD, AS conceptualized and designed experiments. PB, RPK, AK, BD conducted experiments. PB, RPK, AK, WJ, MPP, BD, RD analysed the data. PB, RPK, AK, WJ, MPP, BD, RD, AS prepared the manuscript. All authors reviewed the manuscript.

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REFERENCES

[1] P. Bednarczyk, A. Kicinska, M. Laskowski, B. Kulawiak, R. Kampa, A. Walewska, M. Krajewska, W. Jarmuszkiewicz, A. Szewczyk, *Biochim. Biophys. Acta* **2018**, *1859*, 309.
[2] R. Bajgar, S. Seetharaman, A. J. Kowaltowski, K. D. Garlid, P. Paucek, *J. Biol. Chem.* **2001**, *276*, 33369.
[3] Y. A. Dahlem, T. F. Horn, L. Buntinas, T. Gonoi, G. Wolf, D. Siemen, *Biochim. Biophys. Acta* **2004**, *1656*, 46.
[4] I. Inoue, H. Nagase, K. Kishi, T. Higuti, *Nature* **1991**, *352*, 244.
[5] B. Honrath, I. E. Krabbendam, C. Culmsee, A. M. Dolga, *Neurochem. Int.* **2017**, *109*, 13.
[6] I. E. Krabbendam, B. Honrath, C. Culmsee, A. M. Dolga, *Cell Calcium* **2018**, *69*, 101.
[7] S. Frankenreiter, P. Bednarczyk, A. Kniess, N. I. Bork, J. Straubinger, P. Koprowski, A. Wrzosek, E. Mohr, A. Logan, M. P. Murphy, M. Gawaz, T. Krieg, A. Szewczyk, V. O. Nikolaev, P. Ruth, R. Lukowski, *Circulation* **2017**, *136*, 2337.
[8] U. De Marchi, N. Sassi, B. Fioretti, L. Catacuzzeno, G. M. Cereghetti, I. Szabò, M. Zoratti*Cell Calcium*. **2009**;*45*:509.
[9] L. Testai, V. Barrese, M. V. Soldovieri, P. Ambrosino, A. Martelli, I. Vinciguerra, F. Miceli, I. A. Greenwood, M. J. Curtis, M. C. Breschi, M. J. Sisalli, A. Scorziello, M. J. Canduela, P. Grandes, V. Calderone, M. Taglialatela, *Cardiovasc. Res.* **2016**, *110*, 40.
[10] I. Szabo, J. Bock, A. Jekle, M. Soddemann, C. Adams, F. Lang, M. Zoratti, E. Gulbins, *J. Biol. Chem.* **2005**, *280*, 12790.

[11] P. Bednarczyk, J. E. Kowalczyk, M. Beresewicz, K. Dolowy, A. Szewczyk, B. Zablocka, *Biochem. Biophys. Res. Comm.* **2010**, *397*, 614.
[12] Z. Rusznak, G. Bakondi, L. Kosztka, K. Pocsai, B. Dienes, J. Fodor, A. Telek, M. Gönczi, G. Szucs, L. Csernoch, *Virchows Arch.* **2008**, *452*, 415.
[13] R. Toczylowska-Maminska, A. Olszewska, M. Laskowski, P. Bednarczyk, K. Skowronek, A. Szewczyk, *J. Invest. Dermatol.* **2014**, *134*, 764.
[14] I. Szabo, M. Zoratti, *Physiol. Rev.* **2014**, *94*, 519.
[15] B. O'Rourke, S. Cortassa, M. A. Aon, *Physiology (Bethesda)* **2005**, *20*, 303.
[16] L. Testai, A. Martelli, A. Marino, V. D'Antongiovanni, F. Ciregia, L. Giusti, A. Lucacchini, S. Chericoni, M. C. Breschi, V. Calderone, *Biochem. Pharmacol.* **2013**, *85*, 1634.
[17] O. G. Deryagin, S. A. Gavrilova, K. L. Gainutdinov, A. V. Golubeva, V. V. Andrianov, G. G. Yafarova, S. V. Buravkov, V. B. Koshelev, *Front Neurosci.* **2017**, *11*, 427.
[18] L. Leanza, P. O'Reilly, A. Doyle, E. Venturini, M. Zoratti, E. Szegezdi, I. Szabo, *Curr. Pharm. Des.* **2014**, *20*, 189.
[19] A. Kicinska, B. Augustynek, B. Kulawiak, W. Jarmuszkiewicz, A. Szewczyk, P. Bednarczyk, *Biochem. J.* **2016**, *473*, 4457.
[20] K. P. Bednarczy, A. Koziel, W. Jarmuszkiewicz, A. Szewczyk, *Am. J. Physiol. Heart Circ. Physiol.* **2013**, *304*, H1415.
[21] S. Saponara, L. Testai, D. Iozzi, E. Martinotti, A. Martelli, S. Chericoni, G. Sgaragli, F. Fusi, V. Calderone, *Br. J. Pharmacol.* **2006**, *149*, 1013.
[22] L. Testai, E. Da Pozzo, I. Piano, L. Pistelli, C. Gargini, M. C. Breschi, A. Braca, C. Martini, A. Martelli, *Front Pharmacol.* **2017**, *8*, 71.
[23] D. Barreca, G. Gattuso, E. Bellocco, A. Calderaro, D. Trombetta, A. Smeriglio, G. Laganà, M. Daglia, S. Meneghini, S. M. Nabavi, *BioFactors* **2017**, *43*, 495.
[24] Z. Yang, A. Pan, W. Zuo, J. Guo, W. Zhou, *J. Ethnopharmacol.* **2014**, *155*, 1177.
[25] H. T. Hsu, Y. T. Tseng, Y. C. Lo, S. N. Wu, *BMC Neurosci.* **2014**, *15*, 135.
[26] P. Bednarczyk, M. R. Wieckowski, M. Broszkiewicz, K. Skowronek, D. Siemen, A. Szewczyk, *PLoS ONE* **2013**, *8*, e68125.
[27] A. Koziel, A. Woyda-Ploszczyca, A. Kicinska, W. Jarmuszkiewicz, *Pflugers Arch.* **2012**, *464*, 657.
[28] M. Maksymiak, R. Debowska, K. Bazela, A. Dzwigalowska, A. Orchel, K. Jelonek, B. Dolegowska, M. Kowalczuk, G. Adamus, *Biomacromol* **2015**, *16*, 3603.
[29] J. Zeranska, M. Pasikowska, B. Szczepanik, K. Mlosek, S. Malinowska, R. M. Dębowska, I. Eris, *Postepy Dermatol. Alergol.* **2016**, *33*, 28.
[30] A. Szewczyk, W. Jarmuszkiewicz, W. S. Kunz, *IUBMB Life* **2009**, *61*, 134.
[31] E. Soltysinska, B. H. Bentzen, M. Barthmes, H. Hattel, A. B. Thrush, M. E. Harper, K. Qvortrup, F. J. Larsen, T. A. Schiffer, J. Losa-Reyna, J. Straubinger, A. Kniess, M. B. Thomsen, A. Brüggemann, S. Fenske, M. Biel, P. Ruth, C. Wahl-Schott, R. C. Boushel, S. P. Olesen, R. Lukowski, *PLoS ONE* **2014**, *9*, e103402.
[32] H. Singh, R. Lu, J. C. Bopassa, A. L. Meredith, E. Stefani, L. Toro, *Proc. Natl Acad. Sci. USA* **2013**, *110*, 10836.
[33] A. Jafari, E. Noursadeghi, F. Khodagholi, R. Saghiri, R. Sauve, A. Aliaghaei, A. Eliassi, *Exp. Neurol.* **2015**, *269*, 8.
[34] M. Richter, C. Nickel, L. Apel, A. Kaas, R. Dodel, C. Culmsee, A. M. Dolga, *Neurochem. Int.* **2015**, *81*, 63.
[35] H. T. Facundo, M. Fornazari, A. J. Kowaltowski, *Biochim. Biophys. Acta* **2006**, *1762*, 202.
[36] L. Wang, Q. L. Zhu, G. Z. Wang, T. Z. Deng, R. Chen, M. H. Liu, S. W. Wang, *Neurosci. Lett.* **2011**, *491*, 63.
[37] L. Leanza, M. Romio, K. A. Becker, M. Azzolini, L. Trentin, A. Managò, E. Venturini, A. Zaccagnino, A. Mattarei, L. Carraretto, A. Urbani, S. Kadow, L. Biasutto, V. Martini, F. Severin, R. Peruzzo, V. Trimarco, J. H. Egberts, C. Hauser, A. Visentin, G. Semenzato, H. Kalthoff, M. Zoratti, E. Gulbins, C. Paradisi, I. Szabo, *Cancer Cell* **2017**, *31*, 516.
[38] R. Peruzzo, A. Mattarei, M. Romio, C. Paradisi, M. Zoratti, I. Szabò, L. Leanza, *Front Oncol.* **2017**, *7*, 239.
[39] E. Hernandez-Aquino, P. Muriel, *World J. Gastroenterol.* **2018**, *24*, 1679.
[40] R. M. Martinez, F. A. Pinho-Ribeiro, V. S. Steffen, C. V. Caviglione, J. A. Vignoli, D. S. Barbosa, M. M. Baracat, S. R. Georgetti, W. A. Verri Jr, R. Casagrande, *J. Nat. Prod.* **2015**, *78*, 1647.
[41] E. P. Scholz, E. Zitron, C. Kiesecker, S. Lück, D. Thomas, S. Kathöfer, V. A. Kreye, H. A. Katus, J. Kiehn, W. Schoels, C. A. Karle, *Naunyn Schmiedebergs Arch. Pharmacol.* **2005**, *371*, 516.
[42] M. F. Manchope, C. Calixto-Campos, L. Coelho-Silva, A. C. Zarpelon, F. A. Pinho-Ribeiro, S. R. Georgetti, M. M. Baracat, R. Casagrande, W. A. Verri Jr, *PLoS ONE* **2016**, *11*, e0153015.
[43] K. D. Garlid, P. Paucek, V. Yarov-Yarovoy, H. N. Murray, R. B. Darbenzio, A. J. D'Alonzo, N. J. Lodge, M. A. Smith, G. J. Grover, *Circ. Res.* **1997**, *81*, 1072.
[44] D. Siemen, C. Loupatatzis, J. Borecky, E. Gulbins, F. Lang, *Biochem. Biophys. Res. Commun.* **1999**, *257*, 549.
[45] M. Jaburek, V. Yarov-Yarovoy, P. Paucek, K. D. Garlid, *J. Biol. Chem.* **1998**, *273*, 13578.
[46] G. J. Kaczorowski, M. L. Garcia, *Int. Rev. Neurobiol.* **2016**, *128*, 439.
[47] B. Honrath, L. Matschke, T. Meyer, L. Magerhans, F. Perocchi, G. K. Ganjam, H. Zischka, C. Krasel, A. Gerding, B. M. Bakker, M. Bünemann, S. Strack, N. Decher, C. Culmsee, A. M. Dolga, *Cell Death Differ.* **2017**, *24*, 761.
[48] L. Testai, *Life Sci.* **2015**, *135*, 68.
[49] A. W. Khan, S. Kotta, S. H. Ansari, R. K. Sharma, J. Ali, *Drug Deliv.* **2015**, *22*, 552.
[50] S. Davinelli, J. C. Bertoglio, A. Polimeni, G. Scapagnini, *Curr. Pharm. Des.* **2018**, *24*, 99.
[51] R. M. Martinez, F. A. Pinho-Ribeiro, V. S. Steffen, T. C. Silva, C. V. Caviglione, C. Bottura, M. J. Fonseca, F. T. Vicentini, J. A. Vignoli, M. M. Baracat, S. R. Georgetti, W. A. Verri Jr, R. Casagrande, *PLoS ONE* **2016**, *11*, e0146296.
[52] S. Shan, Y. Zhang, M. Wu, B. Yi, J. Wang, Q. Li, *Mol. Med. Rep.* **2017**, *16*, 4643.
[53] L. M. Meng, H. J. Ma, H. Guo, Q. Q. Kong, Y. Zhang, *Can. J. Physiol. Pharmacol.* **2016**, *94*, 973.

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