New therapies targeting aging cells in the skin

Senescence is accompanied by numerous processes that lead to alterations in cell metabolism, cell cycle arrest, and increased production and secretion of senescence-associated secretory phenotype (SASP). Consequently, signaling pathways cascades are activated, leading to inflammation that can trigger multiple disorders, including cancer. Recently, a novel therapeutic approach was proposed based on targeting senescent cells using senolytics. This group of biologically active compounds includes fisetin, quercetin, dasatinib, and others. These compounds were shown to affect laboratory animals (rodents) by improving the quality of life and significantly increasing the length of life by reducing senescent cells pool in different organs. Based on these findings, we decided to evaluate the potential of these compounds in targeting senescent cells in human skin using in vitro model based on human-derived keratinocytes (HEKa) and...
Introduction

The accumulation of senescent cells within the skin contributes to age-related skin damage, which can manifest in the depletion of skin functions, especially disturbed epidermal barrier, skin neoplasms and autoimmune skin diseases development [1, 2]. Senescence-associated secretory phenotype (SASP), being a set of proinflammatory chemokines, cytokines, growth factors, lipids, and proteases produced and secreted by senescent cells, induces chronic inflammation and tissue changes in all skin layers, including epidermis (keratinocytes) and dermis (fibroblasts). The use of a strategy that eliminates senescent cells (senolytics) or neutralizes SASP components (senostatics) represents promising option for delaying skin aging and treatment of age-related skin diseases [1, 2]. There is convincing evidence that flavonoids, initially found in fruits and vegetables, can target cellular pathways crucial for clearing senescent cells and reducing the SASP [3]. Several biologically active compounds, including fisetin, quercetin, and curcumin, have been reported to trigger positive effects against skin cellular senescence in vivo and in vitro [1, 3]. Notably, these natural plant-derived compounds were reported safe. Fisetin was shown to inhibit PI3K/AKT/mTOR pathway, topoisomerase, and TNF-α-induced inflammation and oxidative damage in human keratinocytes. Applied topically in hairless mice prevented UVB-induced skin damage and restored epidermal function by increased expression of filaggrin and aquaporins [1, 4]. Another flavanol, quercetin, was shown to decrease the number of stress-induced senescent cells, while curcumin led to the selective elimination of senescent cells by inducing apoptosis [3]. Combination of two senolytics was also evaluated, showing promising results [5]. In this study we aimed to assess the biological activity of fisetin and quercetin with dasatinib on skin cell metabolism, including survival and senescent cells elimination.

Materials and methods

Permeability of fisetin by Raman spectroscopy in an in vitro model

Skin penetration of fisetin (3,7,3’,4’-tetrahydroxy-flavone) was examined using Confocal Raman spectroscopy. The commercially available fisetin (Indofine, Hillsborough, NJ, USA) was used to prepare the 3% fisetin cream in multi-component medium with a pH 5.5 Lekobaza Pharma Cosmetic base (Fagron, Kraków, Poland).

Raman spectroscopy was performed using a WITec Alpha 300 spectrometer equipped with a confocal microscope (WITec alpha300 R, Ulm, Germany), TrueSurface attachment, and an electron-multiplying CCD (EMCCD) camera using previously described protocol [6]. Skin layer permeability analysis was performed on cross-sections through layers of the skin after incubation with the samples. Skin samples were obtained from excess skin during abdominoplasty from healthy middle-aged females. Samples (three repeats) prepared in a 6-well plate containing PBS to maintain hydration were incubated for 6 hours in 3% fisetin cream and then mounted on slides for Raman spectroscopy. The biophysical skin parameters (e.g. TEWL) must be evaluated in further detailed studies.

Cell culture

Adult human keratinocytes cell line (HEKa) and adult human fibroblasts cell line (HDFa) were purchased from ATCC (American Type Culture Collection) and cultured according to ATCC guidelines. Cells were studied in varied intervals including passages 2 to 6 to verify potential association with senescence status.
Viability test
MTT test was used to assess changes in cells viability as previously described (7). HEKa or HDFa cells were plated into each well of 96-well plate at a density of 3 x 10^3 cells per well for 24h incubation (for cell adhesion) and then cells were treated for 24 or 72h with: fisetin solution (0.1, 1, 5, 10, 20, 100 or 200 μM) or Quercetin/Dasatinib combination (0.1 μM/1 nM, 1 μM/10 nM, 2.5 μM/25 nM, 5 μM/50 nM, 10 μM/100 nM, 25 μM/250 nM or 50 μM/500 nM). Results were presented relative to control cells (DMSO).

Assessment of senescence marker, p16
For the senescence marker assessment 3 x 10^5 HEKa or HDFa cells were plated into 60mm Petri dish for 24h (for cells adhesion) as previously described (8). After 72h treatment cells morphology was assessed using microscope (photos at 400x magnification) and then cells were lysed (lysates were stored in -80°C). ELISA test was performed according to manufacturer guidelines (Abcam, as previously described [9]). For absorbance measurement multimode plate reader EnSpire (Perkin Elmer) was used.

Statistical Analysis
Results were expressed as mean ± SD. All statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were assessed for statistical significance using repeated-measures ANOVA, followed by post-hoc the Dunnett’s test method. All experiments were performed in triplicates unless specified otherwise. The threshold for significance was defined as p < 0.05 and are indicated by the (*) symbol.

Results

Permeability assessment
Skin penetration assessment was performed with fisetin (3,7,3’,4’-tetrahydroxyflavone) using Confocal Raman spectroscopy showed significant penetration of the compound through the skin up to at least 610μm up to 1330μm (Table 1).

Biological potential of senolytics
Evaluation of HDFa cells viability after 2 passages showed that fisetin alone provoked significant decrease of cell viability starting from 10 or 20% reduction (for 24 and 72h, respectively) at 0.1 μM up to 50 and 70% reduction (for 24 and 72h, respectively) at 200 μM (Figure 1A). Cells subjected to further passaging (i.e. four passages) also showed significant reduction in metabolic activity and with almost 20 and 30% up to 30 and 50% reduction (for 24 and 72h, respectively) in cell survival at 0.1 μM and 200 μM, respectively (Figure 1B). HDFa cells evaluated after 6 passages demonstrated reduced cell viability by 10 and 30% at 0.1 μM (24 and 72 h respectively) up to 40 and 50% at 200 μM (24 and 72h respectively) (Figure 1C).

Assessment of the biological activity of fisetin in HEKa cells showed cytotoxic effect of the compound after both time intervals but with a different pattern. Treatment of cells after 2 passages showed decreased viability of HEKa cells by 20%
at 10 μM for 24h treatment while 72h-treatment revealed reduced cell viability by 15% at 0.1 μM. Further increase of the fisetin concentration led to more efficient inhibition of metabolic activity of studied cells, up to 80% decrease of viability at 200 μM when cells were treated for 24h and 90% decrease of viability at the same concentration when cells were treated for 72h (Figure 1D). In turn, treatment of cells after 4 passages led to a significant decrease of their survival by 5% when 1μM fisetin was applied (24h) and by 10% when 0.1 μM fisetin was used (72h) (Figure 1E). Further increase of the fisetin concentration led to more efficient inhibition of metabolic activity of studied cells up to almost 80% decrease at 200 μM for 24h and 95% when 72h treatment with 200 μM fisetin was applied (Figure 1E). When experiments were performed with the use

Figure 1. Cytotoxicity assessment of fisetin in human skin fibroblasts and keratinocytes.

A  
HDFa cells passage 2  

B  
HDFa cells passage 4  

C  
HDFa cells passage 6  

D  
HEKa cells passage 2  

E  
HEKa cells passage 4  

F  
HEKa cells passage 6
of HEKa cells after 6 passages, significant viability decrease was observed at the concentration of 10 μM for both time intervals. Further concentration increases provoked significant reduction of cell survival up to 70 and 95% for 24 and 72h treatment with 200 μM fisetin, respectively (Figure 1F).

**Biological potential of quercetin and dasatinib**

HDFa cells were subjected to the quercetin/dasatinib (Q+D) combination (0.1 μM/1 nM, 1 μM/10 nM, 2.5 μM/25 nM, 5 μM/50 nM, 10 μM/100 nM, 25 μM/250 nM or 50 μM/500 nM). As demonstrated, the youngest cells (passage 2, Figure 2A) showed relatively low sensitivity to Q+D combination showing significant viability decrease at 2.5 μM/25 nM for both time intervals (10 and 15% survival reduction for 24 and 72h treatment, respectively). Increased concentration of Q+D provoked further reduction of HDFa cells viability up to 20 and 40% reduction at 50 μM/500 nM for 24 and 72h treatment time, respectively. When experiments were performed for cells after 4 passages, a more significant reduction of cell survival was observed, i.e. irrespectively to the treatment time, viability of cells was reduced by 10% at the lowest applied concentration (0.1 μM/1 nM) up to almost 80% reduction at 50 μM/500 nM (Figure 2B). Very similar results were observed when cells after 6 passages were studied with no relation to treatment time (Figure 2C).

Simultaneous assessment of p16 showed that its relative level was significantly reduced in HDFa cells after 72h treatment with studied compounds but only in cells treated with Q+D at 5 μM/50 after 4 (10%, p = 0.05) and 6 passages (30%, p = 0.05) with no significant alterations when lower concentration was applied (1 μM/10 nM) or in younger cells (i.e. after 2 passages) irrelevant to the concentration applied (Figure 2D). Interestingly, treatment of HDFa cells with fisetin alone did not show any significant alterations in p16 accumulation (Figure 2D).

Similarly, HEKa cells were subjected to the same concentrations of studied compounds. When cells after 2 passages were subjected to

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**Figure 2.** Cytotoxicity assessment of fisetin, dasatinib and quercetin in human skin fibroblasts.
(Q+D) combination a significant viability decrease was observed at concentration 5 μM/50 nM (20% at 24h) and 0.1 μM/1 nM (15% at 72h). Further concentration increase led to more efficient metabolism inhibition i.e. up to 20% for 24h and 90% for 72h treatment, respectively (both at 50 μM/500 nM, Figure 3A). Evaluation of cells after 4 passages showed more than 10% cell viability inhibition at 2.5 μM/25 nM for 24h treatment and also 10% viability reduction but at 0.1 μM/1 nM for 72h treatment (Figure 3B). Further concentration increase led to higher cytotoxicity up to 50 and 70% at 50 μM/500 nM for both time intervals, respectively. Assessment of cell survival after 6 passages and treatment with Q+D combination showed higher cytotoxicity of the compounds starting from 1 μM/10 nM for 24h (5% decrease) and 0.1 μM/1 nM for 72h (15% decrease) (Figure 3C). Higher concentrations led to more efficient metabolic activity inhibition up to 70 and ca 80% decrease at 50 μM/500 nM for 24 and 72h, respectively.

Verification of p16 accumulation showed significant decrease of the protein accumulation after 2 passages when cells were treated with 5 μM/50 (Figure 3D), while in cells after 4 passages, a significant reduction of this protein was observed in cells treated with 5 μM fisetin, and 1 μM/10 nM or 5 μM/50 nM Q+D. Interestingly, older cells (6 passages) did not show any significant alterations in p16 levels when Q+D were involved but fisetin alone (1 μM) led to a significant reduction of p16 level (Figure 3D).

Discussion

Cellular senescence represents the major cellular mechanism associated with aging and age-related dermal diseases [1, 2]. Better understanding of the molecular mechanism and impact of these cells on skin conditions during aging including inflammation, metabolic alterations, carcinogenesis induction etc. will help to develop better therapeutic approaches by prevention and/or blocking the senescence pathway. Such approach can be provided by different senolytics including fisetin or dasatinib with quercetin.
The latter two compounds were shown by Kirkland lab to target senescent cells in vitro and in vivo in different organs in mice and humans by oral treatment [10]. Since the activity of dasatinib and quercetin (DQ) was already demonstrated we used them as a reference sample and focused on fisetin. First, we evaluated the ability of fisetin to penetrate skin tissue. As demonstrated, the skin was permeated for more than one millimeter (1330 μm) in depth indicating that not only keratinocytes but also skin fibroblasts can be affected. Following our observation we completed cytotoxicity assessment of selected senolytics and their effect on senescence marker accumulation, p16 gene using in vitro human skin model based on human-derived keratinocytes (HEKa) and fibroblasts (HDFa). In both cell types, DQ showed gradually higher cytotoxic potential in older cells. Interestingly, in fibroblasts treated with DQ we observed lowered accumulation of p16 relative to control, untreated cells suggesting high efficiency in targeting and eliminating senescent cells. Additionally, cytotoxicity assay revealed that the activity of fisetin was time- and dose-dependent as well as cell-type dependent. As demonstrated, it was more efficient in attenuation of the metabolic activity of keratinocytes than fibroblasts. Additionally, fisetin was more efficient in the elimination of younger keratinocytes (passage 2 and 4), especially after a long time exposure (72 h) at 0.1–20 μM while higher concentrations (100–200 μM) was even more potent (up to 90% reduction). In turn, fisetin was more efficient in reducing the metabolism of fibroblasts when elder cells were studied (passages 4 and 6) compared to younger cells indicating more selective targeting of senescent rather than healthy, non-senescent cells. Most of current clinical trials focus on oral administration of fisetin so it is difficult to compare to any other studies [11,12]. Thus our initial study shows the need to better understand the potential of topical treatment since the mechanism and metabolic effects of fisetin in skin cells is not known yet.

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Conflict of interest statement
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