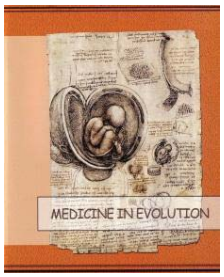
Evaluation of the cytotoxic and anti-angiogenic potential of flavone apigenin using the B164A5 mouse melanoma cell line



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Abstract

Aim and objectives: The present study was designed in order to evaluate the cytotoxic and anti-angiogenic potential of apigenin using the B164A5 mouse melanoma cell line. Material and methods: The effect of apigenin against B164A5 mouse melanoma cell line was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Lactate dehydrogenase (LDH) assays. For the determination of the anti-angiogenic potential CAM assay was performed. Results: At 72h post-stimulation, Api provoked a dose-dependent decrease of melanoma cells viability. Significant statistic results were found at the concentrations of 10 µM 30µM and 60 µM for reduction of proliferation, respectively 30µM and 60 µM for cytotoxicity. Data from this study showed that at both tested doses Api reduced the migration potential of melanoma cells. Conclusions: Apigenin induced, dose-dependent antiproliferative, cytotoxic and anti-angiogenic effects on B164A5 cells. The compound represents a promising candidate for further studies of experimental animal models of melanoma.

Keywords: apigenin, B164A5 mouse melanoma cell line, antiproliferative, cytotoxic, antiangiogenic effects.

INTRODUCTION

Although used since ancient times, in recent years medicinal plants and natural compounds have been in the spot light of the medical research due to the continued development of physico-chemical and analytical methods that allowed a comprehensive qualitative and quantitative characterization of various plant extracts. Along with this aspect, countless studies attest the therapeutic value of natural compounds for the treatment and/or prevention of a wide range of both acute and/or chronic pathologies [1, 2].

Nowadays, over 50% of current medication derives from natural compounds [3] , and globally more than 80% of the substances used in various pathologies such as cardiovascular, immune, bacterial or viral diseases have a vegetal source. For neoplastic diseases, substances of plant origin used as such or in the form of chemical derivatives account for more than 60% [1].

Carcinogenesis is an accumulation of genetic and epigenetic anomalies leading to cell alteration. As a treatable pathology, but with very rapid progress most of the time, the stage of the disease and the type of cancer are very important [4]. According to the latest WHO statistics, cancer is one of the most common causes of death in recent years [5]. The latest statistics show that there are around 9.6 million deaths and 18 million new diagnoses [6].

Skin cancer is one of the most common types of cancer. It is frequently classified into four categories: basocellular cancer, squamous cell cancer, actinic keratosis and melanoma. Due to its fast and high capacity of metastasis, melanoma represents one of the most aggressive type of cancer [7].

Among years an increased number of phytochemicals have represented scaffolds for the development of anti-cancer drugs. Apigenin (4,5,7– trihydroxyflavone) belongs to the class of flavonoids, a very broad therapeutic family, widely studied, which includes numerous therapeutic substances, currently used in medicine for various ailments (8).

Apigenin has a multitude of pharmacological properties, of which: vasoprotective, hypotensitive, antibacterial, antiviral, immunosuppressive, anti-angiogenic, hemostatic effects and antitumor potential [8, 9]. Apigenin has also anti-inflammatory, antimutagenic, antioxidant effects, regulates the cell cycle, activates the apoptotic process and has an antiproliferative role in case of some cancer cell lines including: cervical and ovarian cancer, breast, neck and head cancer, colorectal, lung, skin and prostate cancer [10].

Matricaria chamomilla L. is one of the most important sources of apigenin [11] [5010-5320 mg / 100 g dry product] [12]. This phytocompound can be also found in important amounts in Apium graveolens L., Rosmarinus officinalis L., Petroselinum crispum L., Camelia sinensis L. Piper nigrum L. [8], Carum carvi L. [13], Mentha longifolia L. [14], Scutellaria barbata L. [15], Lycopodium clavatum L. [16] and Euterpe oleracea L. [17].

So far, multiple specialized studies have proved the antiproliferative, proapoptotic and cytotoxic action of apigenin. Experimental tests with apigenin have been shown to be effective in vitro in case of several cancer cell lines including: breast cancer [MCF-7] [18], [HER2] [19], gastric carcinoma [SGC-790] [4], thyroid carcinoma [BCPAP], prostate cancer [DU145] [20], lung carcinoma [A549], human colon cancer [HCT116], ovarian cancer [A2780] [21], human melanoma [A375 and C8161] [22], murine melanoma [B16F10] [23].

Aim and objectives

Since data from the literature present informations regarding B16F10, the high metastatic melanoma cell line, the aim of this study was to evaluate the antiproliferative, cytotoxic and anti-angiogenic potential of flavone apigenin using the B164A5 mouse melanoma cell line. B164A5 is one of the most widely used cell line for the murine melanoma model, being well accepted by the C57BL6J mouse strain [24].

MATERIAL AND METHOD

Cell Culture

The murine melanoma cell line B164A5 (code no. 94042254) was purchased from Sigma-Aldrich (Germany). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Germany), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Germany) and 1% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/mL; Sigma-Aldrich, Germany). The cells were maintained in standard conditions (humidified atmosphere with 5% CO2 and 37°C).

MTT Assay

The effect of Api on B164A5 murine melanoma cells viability was evaluated by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The method was conducted as previously described [27]. Briefly, 1 × 104 cells/well were seeded in 96-well culture plates and allowed to adhere overnight. The second day, the cells were stimulated with different concentrations of Api (1, 3, 10, 30 and 60 µM) and incubated for 72h. The Control group is represented by cells treated with the solvent dimethyl sulfoxide (DMSO). After the 72h incubation period, the cells were treated with 10 µL of 5 mg/mL MTT solution from the MTT kit (Sigma-Aldrich) and incubated for an additional 3h. The obtained formazan crystals were dissolved in 100 µL of lysis solution provided in the MTT kit. The absorbance was determined at 570 nm with a microplate reader (BioRad, xMark Microplate Spectrophotometer).

LDH Assay

The cytotoxic effect of Api on B164A5 murine melanoma cells was determined by means of Lactate dehydrogenase (LDH) assay (Thermo Fisher Scientific). The protocol was applied as previously described [27]. Briefly, 1 × 104 cells/well were seeded in 96-well culture plates and allowed to adhere overnight. The second day, the cells were stimulated with two concentrations of Api (30 and 60 µM) and incubated for 72h. After the incubation period, 50 µL from each well was transferred into a 96-well culture plate and mixed with 50 µL of reaction mixture. The plate was incubated for 30 minutes at room temperature and then 50 µL of the stop solution was added into each well. The level of LDH release in the medium was measured at 490 nm and 680 nm using a microplate reader (BioRad, xMark Microplate Spectrophotometer).

Tumor angiogenesis evaluation on the chorioallantoic membrane (CAM) using melanoma B164A5 cells

The CAM assay makes use of fertilized chicken (Gallus gallus domesticus) eggs, according to an adapted technique [25, 26], that involves incubation at controlled 37°C and 50% humidity. On the third, and fourth day of incubation, 4-5 ml of albumen were removed and a window was cut on the upper side of the eggs.

Subsequently, the B164A5 mouse melanoma cells, cultured according to the above described protocol, were inoculated using concentrations of 105 cells/5µl, on top of the developing membrane on day 10 of incubation (0H) [25]. Apigenin in concentration of 30 µM, 60 µM and solvent control DMSO 1% were applied in volumes of 5 µl and was repeated for 48h. The treated CAMs were daily monitored, and in ovo photographs were registered.

The evaluation was performed by means of a stereomicroscope (Discovery 8 Stereomicroscope, Zeiss) and relevant images were registered using the Axio CAM 105 colour, Zeiss digital camera and processed by Zeiss ZEN software, ImageJ and GIMP.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). The statistical analysis was performed by means of GraphPad Prism 5. Comparison among groups was performed using One-way ANOVA followed by Dunnett's post-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

RESULTS

MTT Assay

The effect of Api was assessed on a murine melanoma cell line and compared to Control group (cells that were stimulated with the solvent DMSO). In Figure 1 is represented the effect of Api after a stimulation period of 72h. Api produced a dose-dependent decrease of tumor cell viability, the most significant effects were obtained at 30 and 60 µM (cells viability was for 30 µM 76 ± 1.7 % vs. Control and for 60 µM it was 57.8 ± 1.8 % vs. Control)

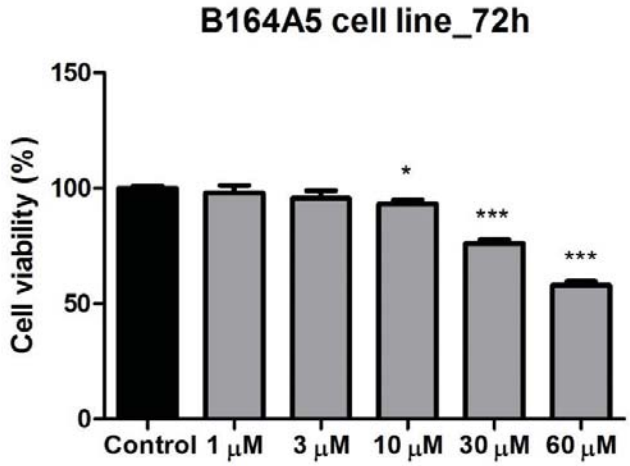

Figure 1. B164A5 murine melanoma cells viability after 72h stimulation with Apigenin (1, 3, 10, 30 and 60 µM)

Determination of the Cytotoxic Potential by LDH Release

The cytotoxic potential of Api was evaluated using two concentrations that proved to be the most efficient in decreasing tumour cells viability. At 72h post-stimulation, at both tested doses of Api there was a significand release of lactate dehydrogenase compared to DMSO groups. At 30 µM Api produced a cytotoxicity rate of 13.3 ± 1.7% and at 60 µM the cytotoxicity rate was slightly higher 15.1 ± 1.8% (Figure 2).

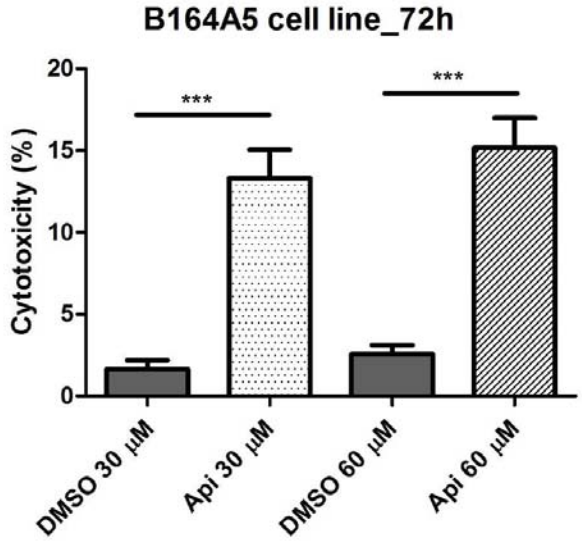

Figure 2. The cytotoxic effect of Apigenin and DMSO (30 and 60 µM) on B164A5 murine melanoma cell line at 72h post-stimulation

Chorioallantoic Membrane Assay (CAM)

The evaluation of the specimens was performed 48 hours post inoculation of the mouse melanoma cells on top of the developing CAMs and after the administration of two doses of test solutions. The specimens exposed to only DMSO showed a more compact tumor area, adherent to the inner surface of the application ring, next to several areas of migrated cells outside the ring, displaying an intense angiogenic reaction.

In contrast, when treated with Api, the compact tumour areas were reduced and a more scattered display of the cells was observed. For both concentrations, Api reduced the migration potential of melanoma cells, and only a few dispersed cells were observed in the outer proximity of the application ring. The number of pigmented cells was also reduced, with a slightly higher degree of pigmentation induced by Api at the higher tested concentration (60 µM).

When analysing the impact induced by the samples on the tumor angiogenesis process, there were some differences between the tested samples. Api in concentration of 30 µM caused a spokes wheel angiogenic reaction converging toward the application ring, while inside the ring the capillaries had a fine aspect and an ununiform distribution. Meanwhile, the higher concentration of Api, induced a higher vascular reaction inside the ring, but still vessels were displayed in an irregular pattern (Figure 3).

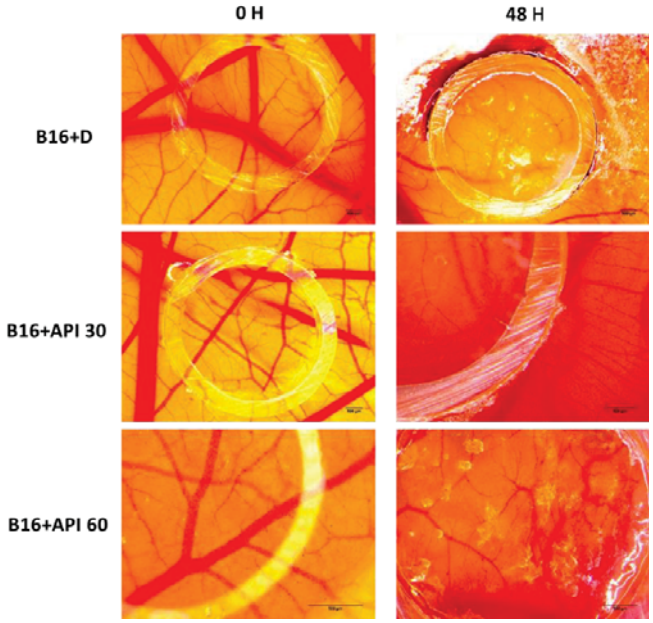

Figure 3. Apigenin treated B164A5 melanoma cells on CAM: apigenin 30, 60 µM and DMSO 1% as solvent control, 0 H and 48 H post inoculation; in ovo, stereomicroscopy photographs, scale bar 500 µm

DISCUSSIONS

In a previous published paper by our group [27] it was shown that Api had an antiproliferative and proapoptotic effect against a human melanoma cell line (A375 cells). Comparing the data obtained in the present study with the data obtained on A375 cells, we can indicate that Api has a more potent effect on the human melanoma cell line than on the murine one.

Cao et al. indicated that the flavonoid apigenin reduced A375 human and B16F10 murine melanoma cells migration and invasion in a dose dependent manner [23]. In another paper Caltagirone et al. showed that Api inhibited B16-BL6 murine melanoma growth. Furthermore, the authors indicated that Api inhibited lung colonization of B16-BL6 cells in an in vivo model [28].

As previously showed by our group [27] Api at both concentration of 30 and 60 μM reduced the development of A375 human melanoma cells, while reducing the angiogenesis reaction. Here we explored the behaviour of a murine melanoma cell line, namley B164A5, when exposed to Api in vivo using the CAM assay. We noticed that in the case of these cells too, the tumor formation process was impaired, by means of reducing cell adherence and by inducing a dysregulated vessel plexus. The migration potential of the cells was reduced in this in vivo model. A number of recent publications explore the antimetastatic potential of apigenin by inhibiting both upstream and downstream aberrations of the STAT-3 pathway, including the decrease of VEGF, HIF-1, MMP-2, MMP-9 and oncogenes Src and JAK 2 [23, 29, 30]. Also a possible melanogenic effect was observed here, more intensively at the higher tested concentration, confirming the tyrosinase inhibition shown by others [31].

Another group conducted by Xu et al. performed a series of assays to examine the influence of apigenin on the cell proliferation and apoptosis of A375, A2058 and RPMI-7951 melanoma cells. It was obtained that apigenin suppressed the propagation of all three melanoma cell lines, in a concentration-dependent manner, maximum effect being at the concentration of 60 μM and that apigenin treatment resulted in significant increases in the percentages of apoptotic populations in A375, A2058 and RPMI-7951 cells. Also, the group investigated the influence of apigenin on the tumorigenesis of melanoma in vivo, generating melanoma xenograft mouse models using the B16-F10 melanoma cell line derived from the C57BL/6 strain. It turned out that apigenin treatment significantly suppressed the growth of B16-F10 xenograft tumours [32].

In a similar approach Ye et al. demonstrated that apigenin presents potent melanogenic activity in murine melanoma B16 cells without affecting proliferation [33]. In the study designed by Nasr Bouzaiene et al., it was observed that incubation of B16F10 mouse melanoma cells with apigenin-7-glucoside in the dose range [10-50 μM] inhibited cell proliferation in a dose-dependent manner [34].

CONCLUSIONS

The in vitro evaluation of Api effects against B164A5 murine melanoma cells indicated that the compound decreased tumour cells viability and had a cytotoxic effect especially at the highest doses tested (30 and 60 μM).

It was observed a reducing effect of the angiogenesis process at the concentration of 30 μM at a higher extent then at 60 μM. Both concentrations reduced the migration potential of the cells, while at 60 μM a stronger melanogenic effect was observed. As far as we know no other study was performed on the CAM assay using the highly metastatic B164A5 cells for the evaluation of apigenin.

The study concludes that apigenin induced, dose-dependent antiproliferative, cytotoxic and anti-angiogenic effects on B164A5 cells. The compound represents a promising candidate for further studies of experimental animal models of melanoma.

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