Apigenin impairs oral squamous cell carcinoma growth 

*in vitro* inducing cell cycle arrest and apoptosis

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**Abstract.** In the present study, we investigated the effect of apigenin, a flavonoid widely present in fruits and vegetables, on a tongue oral cancer-derived cell line (SCC-25) and on a keratinocyte cell line (HaCaT), with the aim of unveiling its anti-proliferative mechanisms. The effect of apigenin on cell growth was evaluated by MTT assay, while apoptosis was investigated by phosphatidyl serine membrane translocation and cell cycle distribution by propidium iodide DNA staining through flow cytometry. In addition the expression of cyclins and cyclin-dependent kinases was evaluated by western blotting. A reduction of apigenin-induced cell growth was found in both cell lines, although SCC-25 cells were significantly more sensitive than the immortalized keratinocytes, HaCaT. Moreover, apigenin induced apoptosis and modulated the cell cycle in SCC-25 cells. Apigenin treatment resulted in cell cycle arrest at both G₀/G₁ and G₂/M checkpoints, while western blot analysis revealed the decreased expression of cyclin D₁ and E, and inactivation of CDK1 upon apigenin treatment. These results demonstrate the anticancer potential of apigenin in an oral squamous cell carcinoma cell line, suggesting that it may be a very promising chemopreventive agent due to its cancer cell cytotoxic activity and its ability to act as a cell cycle modulating agent at multiple levels.

**Introduction**

Oral squamous cell carcinoma (OSCC) is the sixth most widespread cancer with an annual estimated incidence of 263,000 worldwide, two-thirds of these cases occurring in developing countries (1,2). Although its incidence rate is not increasing in the general population of Western countries, an increase in the number of OSCC cases has been reported in young adults in the United States and in some countries of Europe (3-5). The 5-year survival rate remains <60% of diagnosed cases, depending on the stage of the tumor at presentation, in addition, OSCC has devastating consequences negatively affecting the patients quality of life (5). Therefore, developments of new approaches to prevent OSCC occurrence and growth are highly desirable.

Epidemiological studies have identified several nutrients derived from plants with anticancer properties and, among these, flavonoids, a family of polyphenolic compounds, have proved to be effective in cancer prevention (6). Apigenin (Api) is a flavonoid widely found in fruits such as oranges and grapefruits, vegetables and in plant-derived beverages such as chamomile, tea and wine (7). Due to its low toxicity and its biological activities, i.e. anti-inflammatory and anti-oxidant effects, there has been increasing interest in the potential use of Api as an anticancer agent (8). Several studies have proved Api to be effective in inhibiting tumor growth *in vitro* in several types of cancer cells including breast, cervical, prostate and colorectal ones (9-12). The *in vivo* effectiveness of Api oral administration has been demonstrated against prostate cancer (13,14). Moreover, the topical application of Api was found to counteract skin carcinoma growth in mice (15). Although Api has been widely studied the cellular mechanisms responsible of its beneficial effects are still far from being completely understood and scarce information is available regarding the use of Api in oral cancer (16,17). Api anticancer action depends on various mechanisms that can vary according to cell type and it involves apoptosis, modulation of the cell cycle and alteration of kinase pathways (8). Therefore, the aim of the present study is to investigate the anti-proliferative effect of Api on OSCC cells, elucidating the underlying molecular mechanisms. To this end we evaluated Api’s effects on tongue derived OSCC cell lines, SCC-25 and on the spontaneously immortalized keratinocytes, HaCaT.

**Materials and methods**

**Cell lines and materials.** SCC-25, derived from a human oral squamous cell carcinoma of the tongue (obtained from ATCC,
USA) were maintained in DMEM/F12 (Euroclone, Pero, Italy) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 400 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Euroclone). HaCaT cells (obtained from German Cancer Research Center, Heidelberg, Germany) were cultured in high glucose DMEM (Euroclone) supplemented with 10% FBS and 1% penicillin/ streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO₂. Apigenin (Sigma-Aldrich) was dissolved in pure DMSO and subsequently diluted in culture medium. DMSO concentration did not exceed 0.1% during treatments.

**MTT assay.** The effect of Api on cell vitality was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay. Cells were transferred to 96-well plates (5,000 cells/cm²) and were incubated with Api for 24 and 48 hours (h). Subsequently, the medium was removed and the cells were incubated with MTT (Sigma-Aldrich). Four hours later, formazan crystals were dissolved with DMSO and the absorbance was recorded at 560 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). The effect of Api on cell survival was calculated by normalizing the data to the absorbance of vehicle-treated cells (considered as 100%). The IC₅₀ value was estimated.

**Annexin V-propidium iodide assay.** In order to evaluate Api-induced apoptosis, the FITC conjugated Annexin V-propidium iodide (PI) apoptosis detection kit (BD Biosciences, Bucinacaso, Italy) was used according to the manufacturer's instructions. Briefly, the cells were plated in 6-well plates (5,000 cells/cm²) and treated for 24 h with Api 100 µM. Both floating and attached cells were harvested, washed with PBS and, finally, suspended in binding buffer containing Annexin V and PI. After 15-min incubation in the dark, the presence of apoptotic cells was analysed by the FACSCanto flow cytometer (BD Biosciences).

**Analysis of cell cycle distribution.** After Api treatment, both floating and attached cells were harvested, washed twice with cold PBS, spun in a refrigerated centrifuge at 200 g for 10 min and resuspended in cold 70% ethanol overnight (ON) at 4°C. The following day the cells were washed in cold PBS and then incubated in 5 µg/ml PI and 12.5 µg/ml RNase (both from Sigma-Aldrich) for 48 h. When the cell synchronization was required, SCC-25 and HaCaT cells were maintained in serum-free medium for 30 and 48 h, respectively, and subsequently treated with Api in complete medium. DNA content analysis was performed using an enhanced chemiluminescence (ECL) system (Genespin Srl., Milan, Italy). After blot scanning, the densitometric analysis of the bands was performed using the Gel Logic 100 Imaging System (Eastman Kodak, Rochester, NY, USA). Results were expressed as a relative optical density (OD) considering the ratio between the optical density of the protein band of interest and that of the corresponding actin band.

**Statistical analysis.** Data are reported as means ± standard deviation from at least three independent experiments. The statistical evaluation of the results was performed using GraphPad Prism 3 Software (GraphPad Software Inc., La Jolla, CA, USA). The differences between controls and treated cell MTT results were analyzed by the one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test. Differences in the percentages of apoptotic cells in the Annexin V-PI assay and differences in the cell cycle distribution between controls and treated cells were analyzed by Student's t-test. Statistical significance was set at p<0.05.

**Results**

**Apigenin treatment inhibits cell proliferation.** The effect of different Api concentrations on cell survival was evaluated using MTT assay. The data obtained showed a dose- and time-dependent decrease in cell vitality in both HaCaT and SCC-25 cells (Fig. 1).

As can be observed in the graph of Fig. 1A, the highest doses of Api used induced a marked, statistically significant, decrease in SCC-25 cells survival after 24-h treatment, in particular Api 50 and 100 µM reduced cell viability to values <50% with respect to untreated controls. The Api-induced decrease in cell viability was observed also in HaCaT cells; however, the effect of Api in these cells was milder than in SCC-25 cells, in fact, only Api 100 µM significantly affected HaCaT cell viability.

After 48-h treatment all the tested doses statistically significantly reduced SCC-25 cells viability, in particular a dramatic drop in cell vitality to 18.1±7.5% and 7.9±2.0%
was observed after 48-h treatment with 50 and 100 µM Api, respectively. The Api-induced decrease in cell viability with respect to vehicle-treated cells was observed also in HaCaT cells; however, the effect of Api in these cells was milder than in SCC-25 cells, in fact, only Api 100 µM reduced HaCat cell viability below 50% (Fig. 1B).

Apigenin induces apoptosis in SCC-25 cells. To assess if the Api-induced decrease in cell survival was related to apoptosis, we performed a flow cytometric analysis of the membrane translocation of phosphatidyl serine with FITC-conjugated Annexin V (A), combined with PI DNA staining (PI). As shown in Fig. 2A, 24 h exposure to 100 µM Api significantly increased the percentage of apoptotic SCC-25 cells (A+/PI-). In particular, the population of cells showing A+/PI- increased from <1.47±0.78% in the controls to 19.8±4.1% in Api-treated cells (p<0.01), whereas the percentage of cells in late apoptosis, being positive for both Annexin V and PI staining (A+/PI+), increased from 4.7±0.74 in the controls to 14.6±2.05% in Api-treated cells (p<0.01), whereas the percentage of cells in late apoptosis, being positive for both Annexin V and PI staining (A+/PI+), increased from 4.7±0.74 in the controls to 14.6±2.05% in Api-treated cells (p<0.01). On the contrary, 100 µM Api treatment induced only a slight, statistically insignificant, increase in the percentage of early and late apoptotic cell populations in HaCaT cells (Fig. 2B).

Cell cycle alteration induced by apigenin. We aimed at correlating the Api-induced inhibition of cell growth with an alteration of the cell cycle progression. Therefore, we performed cell cycle analysis in cells treated with the most effective dose of Api (100 µM) for 24 and 48 h.

An increase in the percentage of cells in the G2/M phase in Api-treated cells was observed in both HaCaT and SCC-25 cells compared with untreated controls. This increase was associated with a slight, but statistically significant, decrease in the percentage of cells in both G0/G1 and S phase after 48 h exposure. The modulation of SCC-25 cell cycle distribution due to Api, although already notable after 24 h Api exposure, became statistically significant after 48 h (Fig. 3A), so that Api 100 µM treatment resulted in 48.2±4% of cells in G2/M, versus the 9.3±0.8% observed in controls (Fig. 3A). In HaCaT cells, 48 h Api treatment resulted in an increase in the G2/M population from 7.1±1.7 in controls to 26.1±1.2% in treated cells (p<0.001) (Fig. 3B).

Despite the increase in the G2/M population, there was a considerable number of cells still in the G0/G1 phase, thus suggesting that another checkpoint might be involved in Api-induced modulation of cell cycle progression. In an attempt to better clarify the effect of Api on cell cycle distribution, we analyzed the effect of 100 µM Api on synchronized cells. For this purpose, SCC-25 and HaCaT cells were maintained in serum-free medium for 30 and 48 h, respectively, then cells were allowed to re-enter the cell cycle by culturing them in complete medium. As shown in Fig. 4, we obtained
satisfactory synchronization, with almost 90% of cells being in the G0/G1 phase following serum starvation. Api treatment on synchronized HaCaT and SCC-25 cells resulted in the maintenance of cells into the G0/G1 phase, in fact, while in untreated controls the majority of cells after 24 h were in the S phase as a result of cycle re-entering after serum starvation, Api-treated cells maintained the same distribution of serum starvation synchronized cells both after 24 and 48 h of treatment (Fig. 4). In synchronized SCC-25 cells, the exposure to 100 µM Api for 24 h resulted in 85.7±11.5% of cells being in G0/G1 (p<0.01 versus controls) and a very similar effect was observed in HaCaT cells. The blockage of cells in G0/G1 persisted also after 48 h treatment. Despite this, the blockage at G2/M transition was still evident in HaCaT cells since a slight, but statistically significant (p<0.01), increase in the percentage of cells in G2/M was found in 24 h Api-treated cells when compared to controls.

**Apigenin inactivates CDK1 in SCC-25 cells.** In order to address in more detail the effect of Api on the cell cycle, we evaluated the expression of some cyclins and the associated CDKs that regulate both G0/G1 and G2/M phase transition. Therefore, we considered the protein level of cyclin B1, which is involved in G2/M transition, in unsynchronized cells. However, no significant modulations of its expression by Api were evidenced either in SCC-25 or in HaCaT cell lines (data not shown). We subsequently evaluated the level of the CDK1, also known as p34cdc2, which is functionally associated with cyclin B1. Even in this case we did not observe any variation/modulation between treated and control cells. Nevertheless
the regulation of CDK1 is quite complex; in fact, it is strictly regulated by phosphorylation so that its enzymatic activity is inhibited by phosphorylation on the tyrosine in position 15. Therefore, we evaluated the level of the tyrosine 15 phosphorylated form of CDK1. As shown in Fig. 5, a marked, although not statistically significant, increase in tyrosine 15 phosphorylated CDK1 was observed in SCC-25 cells following 100 µM Api treatment for 24 h (Fig. 5C), whereas Api treatment did not modulate the level of tyrosine 15 phosphorylation in HaCaT cells (Fig. 5D).

Apigenin modulates cyclin D1 and E expression. In view of the different Api action on the cell cycle in synchronized versus asynchronous cells, we analyzed also the cyclins and CDKs involved in the regulation of G1/S transition in synchronized cells. First, we evaluated the expression of cyclin D1, which drives the progression through the G1 phase of cell cycle, by exposing synchronized cells to 100 µM Api for 24 h. As can be seen in Fig. 6, Api treatment modulated cyclin D1 expression in both cell lines, with a statistically significant decrease being observed in SCC-25 cells (Fig. 6C), while the decrease in cyclin D1 was slight in HaCaT cells (Fig. 6B and D). Secondly, we evaluated the Api effect on the expression of cyclin E, whose levels are generally high during the mid G1 phase and which is involved in the regulation of G1/S transition. We did not find any cyclin E expression in SCC-25 cells (data not shown). On the contrary, a statistically significant decrease in HaCaT cells after Api treatment was found. The level of cyclin E expression significantly dropped after 24 h Api treatment in HaCaT cells (Fig. 7).

Discussion

Apigenin, a flavonoid belonging to the class of flavones has proved its effectiveness against various cancer types (8-13), but up to now scarce data are available regarding the Api effects and molecular mechanisms on OSCC.

In the present study, we aimed at evaluating Api's action on OSCC using the cell line SCC-25, derived from tongue OSCC. In addition, Api's effect was analyzed also on a spontaneously immortalized, non-tumorigenic, keratinocyte cell line: HaCaT cells. These cell lines have been widely used and they represent a valid model for studying the action of potential chemotherapeutic agents (18,19). In addition, HaCaT cells may be considered a good model of the initial transformation of squamous cell carcinoma. In fact, although they can be referred to as normal keratinocytes, they show some molecular alterations, found in the first steps of malignant transformation of epithelial cells, such as the mutations in both p53 alleles (20).

We demonstrated that Api inhibits cancer cell growth in vitro; it was able to significantly decrease cell survival in SCC-25 cells. In our paradigm, the SCC-25 cells were much more sensitive to Api than immortalized keratinocytes (HaCaT). In agreement with our findings a selective growth inhibitory effect of Api in prostate cancer cells when compared with non-transformed cells was reported (9). Masuelli and colleagues (17) also reported a comparable proliferation inhibition in SCC-15, OSCC derived cells.

Consistent with data reported in breast (10) and in lung cancer (21), the analysis of the phosphatidyl serine membrane
translocation, an early apoptotic marker, showed that Api induced apoptosis in SCC-25 cells. Apoptosis was not detected in HaCaT cells, thus confirming a selective cytotoxic action of Api towards tumoral SCC-25 cells. The absence of apoptosis in HaCaT cells is not in agreement with previously reported data from Abu-Yousif et al (22), where the exposure of HaCaT cells to Api led to apoptosis in almost 15% of cells. One possible reason for this difference might be that in the study of Abu-Yousif et al Api treatment was conducted in serum-free medium, while in the present study HaCaT cells were maintained in complete medium during evaluation of Api cytotoxicity. However, the ratio of apoptotic cells alone was not enough to explain the decrease in cell viability observed in SCC-25 cells following Api treatment. Furthermore, the absence of apoptosis in HaCaT cells, whose proliferation was inhibited by Api, led us to hypothesize that mechanisms other
than apoptosis had a role in the cell number decrease observed after Api treatment. We therefore investigated whether the impairment of cell growth was associated with alterations in the cell cycle progression. The analysis of cell cycle distribution showed a marked increase in the percentage of cells in the G1/M phase after Api treatment in both cell lines. These results are in agreement with data reported by Ujiki et al. (23) and Choi and Kim (10) that demonstrated an Api-induced G2/M arrest in pancreatic and breast cancer cells, respectively. In addition, an increase in the G1/M population after Api exposure was observed also in immortalized keratinocytes (24,25) and in primary cultures of OSCC (16). On the contrary, our results differ from the data reported by Masuelli and co-workers (17). Although they reported an Api-induced decrease in cell survival similar to our findings, they found a significant decrease in cells in both G1 and G2/M phases after Api exposure in SCC-15. The main regulator of G2/M progression is the CDK1/cyclin B complex whose activity is required for cells to enter mitosis. In our paradigm, Api induced only a slight modulation of the level of cyclin B and CDK1 (data not shown). However, CDK1 activity is also regulated by phosphorylation and dephosphorylation mediated by wee1 and cdc25 and, in particular dephosphorylation of CDK1 at the tyrosine 15 site is required for its activity (26). Api treatment induced a significant increase in the level of tyrosine 15 phosphorylated CDK1 in SCC-25 cells, thus indicating that Api can inactivate this CDK. Similar results were obtained in human melanoma cells (27). Api's ability to inactivate CDK1 through phosphorylation on tyrosine 15 sites was demonstrated also by McVean et al. (25) in epidermal keratinocytes. On the contrary, HaCaT cells did not show any modulation of CDK1 phosphorylation after Api treatment, thus indicating that Api induces G2/M arrest through different mechanisms in different cell types.

Even after 48 h Api exposure, a relatively large percentage of cells still remained in the G1/G0 phase leading us to hypothesize that Api could also impair G1 progression. Api treatment in synchronized cells resulted in an almost complete G1/G0 arrest in both the cell lines. These data are consistent with results by Lepley and Pelling (28) who reported a G1 accumulation in synchronized skin fibroblasts exposed to Api, while unsynchronized cells were arrested in G1/M. Western blot analysis demonstrated that Api-induced G1/G0 arrest depended on different pathways in SCC-25 and HaCaT cells. A statistically significant decrease in the expression of the cyclin E cell was found in HaCaT, whereas we found no expression of this cyclin in SCC-25 cells, although cyclin E overexpression is often found in oral cancer (29). However, the loss of cyclin E expression has been reported in two other tongue-derived SCC cell lines (30). It is, therefore, conceivable that cell growth deregulation in SCC-25 cells might depend on the loss of cyclin E. These observations suggest that Api has a double action on the cell cycle, inducing both a G1/G0 and a G2/M arrest, accompanied by a reduction in cyclin D1 and cyclin E expression and inactivation of CDK1.

In conclusion, we demonstrated an inhibitory effect on cell survival and apoptotic effect of Api in oral cancer cells. The model of action we hypothesize implies the induction of apoptosis in SCC-25 cells by Api, which also determines the cell cycle arrest acting as a CDK1 inhibitor and inducing a decrease in cyclin D1 expression.

Therefore, it appears that Api deserves to be further studied as a potential agent for oral cancer prevention and treatment since it is able to induce cell cycle arrest and apoptosis in OSCC cells. In addition, Api seems to be a very promising cell cycle regulating agent since it is able to induce a double cell cycle arrest in different phases. It could, therefore, be capable of arresting cell cycle progression also in cells, such as SCC-25 where the cell cycle regulation machinery is deregulated but not completely compromised.

References


