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Salvia fruticosa, Salvia officinalis and rosmarinic acid induce apoptosis and inhibit proliferation of Human Colorectal cell lines: the role in MAPK/ERK pathway

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ABSTRACT

Epidemiologic studies have shown that nutrition is a key factor in modulating sporadic colorectal carcinoma (CRC) risk. Aromatic plants of the genus *Salvia* (sage) have been attributed many medicinal properties, which include anticancer activity. In the present study, the antiproliferative and pro-apoptotic effects of water extracts of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO) and of their main phenolic compound rosmarinic acid (RA) were evaluated in two human colon carcinoma-derived cell lines, HCT15 and CO115, which have different mutations in the MAPK/ERK and PI3K/Akt signalling pathways. These pathways are commonly altered in CRC leading to increased proliferation and inhibition of apoptosis. Our results show that SF, SO and RA induce apoptosis in both cell lines, whereas cell proliferation was inhibited by the two sage extracts only in HCT15. SO, SF and RA inhibited ERK phosphorylation in HCT15 and had no effects on Akt phosphorylation in CO115 cells. The activity of sage extracts seems to be due, at least in part, to the inhibition of MAPK/ERK pathway.

Introduction

Cancer is an important health problem and one of the most common forms is colorectal carcinoma (CRC). Phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathways play critical roles in cell proliferation and survival and are frequently activated in CRC (1-3). Deregulation of these pathways is also thought to determine response to treatment (4). Mutations of KRAS and BRAF in sporadic CRC [70-80% of total cases (5)] are alternative, where the former constitutively activates both MAPK/ERK and PI3K/Akt pathways and the latter activates MAPK/ERK pathway (3, 4, 6-8). As presented by Schubbert *et al.* (9), mutations in CRC of either KRAS or
BRAF genes occur in 32% and 14% of cases, respectively. Studies have also shown that CRC is frequently associated with mutations in genes that encode for PI3K, PI3KCA, and PTEN (an endogenous inhibitor of PI3K activity), resulting in an overexpression of Akt (10-13). Considering the high incidence of CRC, inhibitors of these pathways are actively being searched for use in the control of cancer progression (14-16).

Epidemiologic studies have shown that western type diets, poor in vegetables and fruits, are risk factors known associated with CRC, suggesting that nutritional factors may also be preventive and also helpful in the control of cancer (17-19). In fact, green and black tea consumption has been shown to be effective in the initiation, promotion and progression stages of carcinogenesis, although effects on colon cancer are inconclusive (20). Plants of the genus Salvia (sage) such as Salvia miltiorrhiza and Salvia menthaefolia have also been suggested to have anticancer properties based on antiproliferative activity on tumor cells (21, 22). In addition, reactive oxygen species (ROS) have been reported to play a role in signalling transduction enhancing proliferation and survival of cancer cells. Antioxidant phytochemicals through their ROS scavenging activity, may suppress altered redox-sensitive signalling events in cancer (23, 24).

Salvia fruticosa (SF) and Salvia officinalis (SO), poorly studied with regard to their anticancer activity, are mediterranean medicinal and aromatic plants which contain rosmarinic acid (RA; Fig. 1) as major phenolic compound in their water extracts. RA constitutes about 58% of all phenolic compounds present in SF water extract and 70% in SO water extract (25, 26). This phenolic compound has high antioxidant and anti-inflammatory activities (22, 27), but little is known about its effects on cancer cells and especially on CRC.
In the present study, we report on the antiproliferative and pro-apoptotic effects of two *Salvia* water extracts, SF and SO, and their major phenolic compound, RA, in two human colon cancer-derived cell lines, HCT15 and CO115, through effects on the MAPK/ERK and PI3K/Akt pathways and caspase mediated apoptosis. These two cell lines possess different activating mutations in these two pathways: HCT15 has a KRAS (G13D) mutation (28) whereas CO115 has a BRAF (V599E) mutation (29).

In view of these genetic differences we further speculate on the mechanisms behind the antiproliferative and pro-apoptotic effects of sage extracts and RA and the involvement of PI3K/Akt and MAPK/ERK signalling pathways in these effects.

**Material and Methods**

**Reagents and Plant Extracts**

All reagents and chemicals used were of analytical grade. Wortmannin (W), rosmarinic acid (RA) and staurosporine were purchased from Sigma-Aldrich (St. Louis, MO, USA) and PD-98059 (PD) was from Calbiochem (San Diego, CA, USA). The primary antibodies anti-phospho-Akt (Ser473), anti-Akt total, anti-phospho-PTEN (Ser380/Thr382/383), anti-PTEN total, anti-p44/42 MAPK total and anti-cleaved caspase-9 (Asp315) were purchased from Cell Signaling (Danvers, MA, USA), the anti-phospho-ERK and caspase-3 (H-277) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the anti-β-actin from Sigma-Aldrich. The secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were from GE Healthcare (Bucks, UK).

The water extracts of *Salvia fruticosa* and *Salvia officinalis* were prepared as previously described by Lima et al. (30), by pouring boiling water onto the dried plant material (at ratio of 150ml of water to each 2g of plant) and allowing to steep for 5min.
After filtering, the water extract was lyophilized to dryness. The extracts of both sages were made using batches of the plants which composition, in terms of phenolics compounds, have already been published (25, 26). In brief, SF water extract contain as major phenolic compound rosmarinic acid (RA; 71.5 µg/ml), 6-hydroxyluteolin-7-glucoside (22.7 µg/ml), a not identified flavone heteroside (28.6 µg/ml) and the remaining phenolic compounds representing 0.8 µg/ml. SO water extract contain as major phenolic compounds RA (52.0 µg/ml), luteolin-7-glucoside (19.7 µg/ml) and the remaining phenolic compounds representing 2.7 µg/ml.

Stocks solutions of PD and W were made in dimethyl sulfoxide (DMSO) and aliquots were kept at -20°C. Therefore, DMSO (0.5%) was included in cell culture for the other conditions (controls and extracts/RA) to exclude any possible DMSO effect.

**Cell culture**

HCT15 and CO115 human colon carcinoma-derived cell lines were a gift from Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained in culture at 37°C in a humidified 5% CO₂ atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 0.1 mM pyruvate, 1% antibiotic-antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard, Cambrex, Verviers, Belgium). Cells were seeded onto six well plates at a density of 0.75 x 10⁵ (HCT15) and 1.0 x 10⁵ (CO115) cells/well. Incubations with different concentrations of sage extracts and RA were performed in serum free medium for 48h to analyze BrdU incorporation and TUNEL positive cells, and for 24h for western blot analysis.

**Assessment of proliferation by BrdU incorporation**
Preliminary experiments using the MTT assay were performed in order to choose concentrations of SF and SO extracts that inhibited around 50% cell proliferation without cytotoxic effects. RA was tested in similar concentrations to the ones found in the extracts at the concentrations used and also did not induce cytotoxic effect. After 45h of treatment with sage extracts or RA at different concentrations, bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the culture medium in order to give a final concentration of 10µM, and then incubated for another 3h. Both adherent and non-adherent cells were collected from each sample, fixed with 4% paraformaldehyde for 15min at room temperature and then attached into a polylysine treated slide using a Shandon Cytospin (Thermo Fisher Scientific Inc, Waltham MA, USA). Cells were incubated with HCl 2M for 20min, washed in PBS containing 0.5% Tween-20 and 0.05% BSA (TPBS-B) and then incubated with monoclonal mouse anti-BrdU antibody (DakoCytomation, Glostrup, Denmark) for 1h at room temperature. After washing in TPBS-B, cells were incubated with anti-mouse IgG FITC-conjugated secondary antibody (Sigma-Aldrich) for 1h at room temperature, washed again and then incubated with Hoechst for nuclei staining. The percentage of proliferating cells was calculated as the ratio between BrdU positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Assessment of apoptosis by TUNEL assay

Cells treated as above for 48 h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for 15min at room temperature and then attached into a polylysine treated slide using a Shandon Cytospin. Cells were washed in PBS and
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permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed using a kit from Roche (Mannheim, Germany), following the manufacture’s instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope.

Results are presented as mean ± SEM of at least three independent experiments.

Protein extraction and western blotting

After 24h of treatment with sage extracts or RA at the highest concentration used in the BrdU and TUNEL assay, cells were washed with PBS and lysed for 15min at 4ºC with ice cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM Na$_2$V$_3$O$_4$ and protease inhibitor cocktail (Roche). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA as a protein standard. Twenty micrograms of protein for each sample were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA, incubated with the primary antibody and followed by the secondary antibody conjugated with IgG horseradish peroxidase. Membranes were washed 3 times with TPBS between the different incubations. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as a
loading control. Results are presented as mean ± SEM of at least three independent experiments.

**Statistical analysis**

One-way ANOVA followed by the Student-Newman-Keuls test was used to perform statistical analysis for BrdU, TUNEL and western blot data. GraphPad Prism 4.0 software (San Diego, CA, USA) was used and $P$-values ≤0.05 were considered statistically significant.

**Results**

**Effects on cell proliferation**

To test the effects of SF, SO and RA on cell proliferation of human colon cancer cells, two different colon carcinoma-derived cell lines, HCT15 and CO115, were used. Based on preliminary experiments using the MTT assay (data not shown), where cells were incubated with several concentrations of sage extracts for 48h, concentrations of each extract that were not cytotoxic and inhibited cell proliferation around 50% were chosen for the subsequent studies. Since RA is the main phenolic compound of these extracts, we also tested RA in similar concentrations to the ones found in the extracts under our experimental conditions.

The effects of sage extracts and RA on cell proliferation of both cell lines were tested using the BrdU incorporation assay. As shown in Fig. 2A, a significant inhibition of HCT15 cell proliferation by both SF and SO was observed at all concentrations tested. Levels of BrdU incorporation significantly decreased from 26.2% in the control to 4.7% in HCT15 cells treated with 50µg/ml of SF and SO extracts. In CO115 cells, SF
and SO did not inhibit significantly cell proliferation (Fig. 2B). No significant inhibition of cell proliferation was observed in both cell lines treated with RA (Fig. 2). Comparing the effects of sage extracts in the two cell lines, we observed that SF extract was somewhat more active than SO and HCT15 cells were more sensitive to the sage extracts.

**Effects on apoptosis**

The ability of SF, SO and RA to induce apoptosis in human colon carcinoma-derived cells were studied using the TUNEL assay. As shown in Fig. 3, both *Salvia* extracts and RA significantly induced apoptosis in a concentration dependent manner in both HCT15 and CO115 cells. Apoptotic cells in HCT15 increased from 0.4% in the control to 6.6%, 5.8% and 2.5% in SF, SO and RA treatments, respectively, at the higher concentrations tested (Fig. 3A). In CO115 cells, apoptotic cells increased from 1.8% in the control to 6.8%, 3.8% and 3.6% in the conditions treated with the higher concentrations tested of SF, SO and RA, respectively (Fig. 3B). Since the basal levels of apoptosis were higher in the CO115 cell line, overall it seems that the HCT15 cells were more sensitive to the extracts and RA. Again, SF extract showed to be more active than SO extract and RA alone.

The involvement of caspases 3 and 9 in the apoptosis induction by sage extracts and RA was also studied by western blot. After 24h of treatment with the highest concentrations used of SF, SO and RA, we did not observe cleaved caspase-9 and caspase-3 in either of cell lines, in contrast with the reference compound, staurosporine (data not shown).

**Effects on MAPK/ERK pathway**
The effects of sage extracts and RA for 24h were studied on the MAPK/ERK pathway by western blot. Salvia extracts and RA significantly decreased phospho-ERK protein levels in HCT15 cells (Fig. 4A) while no effects were observed in CO115 cells (Fig. 4B). The reference inhibitor of phospho-ERK, PD-98059 (PD) was effective in both cell lines (Fig. 4), in a similar extension than SF, SO and RA in HCT15 cells.

Effects on PI3K/Akt pathway

The effects of sage extracts and RA on the expression of phospho-Akt and phospho-PTEN (a negative regulator of PI3K/Akt pathway) were also tested. Phospho-Akt was observed in CO115, however was not detected in HCT15, in medium with and without serum (data not shown). Neither of the Salvia extracts nor RA inhibited significantly the expression of phospho-Akt in CO115 cells (Fig. 5A). A significant inhibition of Akt phosphorylation was observed for the reference PI3K inhibitor, wortmannin (W). HCT15 cells expressed phospho-PTEN and this expression was not significantly changed by Salvia extracts, RA or W (Fig. 5B). CO115 cells did not express phospho-PTEN or total PTEN, in medium with and without serum (data not shown).

Discussion

In order to assess the potential of sage in the control of CRC progression, the antiproliferative and pro-apoptotic effects of Salvia fruticosa (SF) and Salvia officinalis (SO) water extracts and their main phenolic compound, rosmarinic acid (RA), were studied in two human colon carcinoma-derived cell lines, HCT15 and CO115. Both sage water extracts (SF and SO) were effective in inhibiting proliferation in a concentration-dependent manner in HCT15 but not in CO115 cells. SF, SO and RA
induced apoptosis. SF was more effective than SO with regard to both antiproliferative and proapoptotic effects. To identify the bioactive compound behind these effects, sage’s major phenolic compound (RA), was tested individually at concentration similar to those present in the extracts. However, RA was found not to have antiproliferative activity but to be proapoptotic in both cell lines, although at less extent than sage extracts. In view of these results, it seems that other active compounds present in the extracts may be responsible for the antiproliferative and proapoptotic effects of SF and SO.

The two cell lines used harbor different activating mutations: HCT15 has a KRAS (G13D) activating mutation (28) with potential to constitutively activate both PI3K/Akt and MAPK/ERK pathways, whereas CO115 harbors a BRAF (V599E) mutation (29) which affects the MAPK/ERK pathway. The highest sensitivity of HCT15 could be a result of these genetic differences. HCT15 cells, even though presenting an activating mutation of the RAS oncogene, did not express phospho-Akt possibly as a consequence of the high levels of the strong negative regulator of this pathway, phospho-PTEN, found in this cell line. In these cells, the antiproliferative effects of SF and SO correlate with an inhibition of phospho-ERK. However, RA showed a significant inhibition of phospho-ERK without inhibiting HCT15 cell proliferation. Inhibition of phospho-ERK seems, therefore, not to be the only factor involved in inhibition of cell proliferation in this cell line. Our findings are in agreement with previous studies (6, 31), which have shown that an inhibition of MAPK/ERK pathway in KRAS mutated cell lines is not sufficient to inhibit cell proliferation. Therefore, the KRAS mutated HCT15 cells do not depend exclusively on MAPK/ERK pathway to proliferate and, as a result, SF and SO seem also to be inhibiting other proliferation pathways, which in these cells do not include Akt phosphorylation (Fig. 6).
In CO115 cells, where SF and SO did not have antiproliferative effect, there was no inhibition of phospho-ERK or phospho-Akt. RA also did not inhibit proliferation of CO115 cells. However, in contrast to the effects on the other cell line, RA was without effect on phospho-ERK. Inhibition of MAPK/ERK pathway by sage extracts and RA in HCT15 and not CO115 indicates that the effect may be upstream of BRAF and could be on KRAS (Fig. 6). In CO115 cells, a potential inhibition of RAS by sage extracts would not result in antiproliferative effects due to the downstream activating mutation of BRAF (Fig. 6). An inhibition of RAS oncogene has also been recently shown for quercetin, a common natural-occurring phenolic compound (32, 33). It seems that the effects of RA depend on cell type and/or genetic background, since also others have shown that RA decreases ERK phosphorylation in cardiac muscle cells but it is without effect on Akt and ERK in melanoma cells (34, 35).

SF, SO and RA induced apoptosis in both cell lines. It seems, however, that under these conditions apoptosis is not dependent on the cleavage of neither caspase-9 nor caspase-3 in both cell lines. Nevertheless, some authors have shown that RA promotes apoptosis in human Jurkat cells and HepG2 cells via the mitochondrial pathway and Bcl-2 suppression in which caspases are involved (36-38). Also the mitochondrial pathway was induced by RA in activated T cells from rheumatoid arthritis patients (39). It seems, therefore, that the induction of caspase pathways by RA is cell type specific and/or dependent on concentration and time of exposure which may explain the discrepancy between these and our results. The inhibition of MAPK/ERK pathway may contribute, at least in part, to the effects on apoptosis in HCT15 cells.

Besides a possible interaction with KRAS, sage extracts may act as antiproliferative and proapoptotic in these cancer cell lines through their antioxidant activity. It is known that cancer cells produce increased amounts of ROS, in particularly
hydrogen peroxide (H$_2$O$_2$), which could inhibit protein fosfatases and also be associated with signalling events in MAPK pathways that lead to activation of redox-sensitive transcription factors, mediating cancer cell proliferation and survival (23, 24).

Therefore, the radical scavenging activity of the phenolic compounds present in the sage extracts may be reducing the ROS levels in these cancer cells contributing also to a decreased activity of redox-sensitive proliferating pathways, through RAS signalling. Based on RA results, the effects describe in the present study seem, however, not to be totally explained by the antioxidant properties of the sage extracts.

In conclusion, our results show that SF and SO water extracts inhibit proliferation and induce apoptosis in colon carcinoma-derived cell lines whereas RA was only effective on the induction of apoptosis. Sage extracts and RA did not affect the PI3K/Akt pathway but inhibited the MAPK/ERK pathway in the KRAS mutated HCT15 cell line. The inhibitory effects of sage extracts on phospho-ERK seem to result from an inhibition of KRAS, upstream to BRAF, since it was not observed in CO115 cells. The inhibition of MAPK/ERK by sage extracts seems, however, not to completely explain the inhibition of cell proliferation in HCT15, since RA inhibits phospho-ERK without affecting cell proliferation. These data add *S. fruticosa* and *S. officinalis* to the list of potential sources of new active anticancer compounds useful in particular in tumors with a mutagenic KRAS activation and also suggest their possible use in dietary strategies for the control of CRC progression.

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References


Appendixes

Figure 1. Chemical structure of rosmarinic acid (RA).

Figure 2. Effect of different concentrations of *Salvia fruticosa* (SF), *Salvia officinalis* (SO) and rosmarinic acid (RA), for 48h, on BrdU incorporation in HCT15 (A) and CO115 (B) cells. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.

Figure 3. Effect of different concentrations *Salvia fruticosa* (SF), *Salvia officinalis* (SO) and rosmarinic acid (RA) on apoptosis, for 48h, as assessed by the TUNEL assay, of HCT15 (A) and CO115 (B) cell lines. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

Figure 4. Effects of *Salvia fruticosa* 50µg/ml (SF50), *Salvia officinalis* 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-ERK in HCT15 cells (A) and CO115 cells (B). PD-98059 50µM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.

Figure 5. Effects of *Salvia fruticosa* 50µg/ml (SF50), *Salvia officinalis* 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-Akt in CO115 cells (A) and phospho-PTEN in HCT15 cells (B). Wortmannin 1 µM (W1) was used as a reference inhibitor of PI3K. No phospho-Akt expression was observed in...
HCT15 cells and no PTEN expression was observed in CO115 cells. Values are mean ± SEM of at least 3 independent experiments. **P≤ 0.01 when compared to control.

**Figure 6.** Model for the inhibition of ERK phosphorylation by *Salvia fruticosa* (SF), *Salvia officinalis* (SO) and rosmarinic acid (RA) in HCT15 but not in CO115 cells. SF, SO and RA inhibit mutant KRAS leading to a decrease on the levels of phospho-ERK in HCT15 cell line. In CO115 cells, SF, SO and RA do not change ERK phosphorylation levels due to a BRAF activating mutation downstream RAS oncogene. The missing PTEN in CO115 cells and phospho-Akt in HCT15 cells were also observed in this study.
Chemical structure of rosmarinic acid (RA).

80x44mm (600 x 600 DPI)
Effect of different concentrations of Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid (RA), for 48h, on BrdU incorporation in HCT15 (A) and CO115 (B) cells. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.
Effect of different concentrations Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid (RA) on apoptosis, for 48h, as assessed by the TUNEL assay, of HCT15 (A) and CO115 (B) cell lines. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

80x122mm (600 x 600 DPI)
Effects of Salvia fruticosa 50µg/ml (SF50), Salvia officinalis 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-ERK in HCT15 cells (A) and CO115 cells (B). PD-98059 50µM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. *P ≤ 0.05 and ***P ≤ 0.001 when compared to control.

160x94mm (600 x 600 DPI)
Effects of *Salvia fruticosa* 50µg/ml (SF50), *Salvia officinalis* 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-Akt in CO115 cells (A) and phospho-PTEN in HCT15 cells (B). Wortmannin 1 µM (W1) was used as a reference inhibitor of PI3K. No phospho-Akt expression was observed in HCT15 cells and no PTEN expression was observed in CO115 cells. Values are mean ± SEM of at least 3 independent experiments. **P≤ 0.01 when compared to control.

160x94mm (600 x 600 DPI)
Model for the inhibition of ERK phosphorylation by Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid (RA) in HCT15 but not in CO115 cells. SF, SO and RA inhibit mutant KRAS leading to a decrease on the levels of phospho-ERK in HCT15 cell line. In CO115 cells, SF, SO and RA do not change ERK phosphorylation levels due to a BRAF activating mutation downstream RAS oncogene. The missing PTEN in CO115 cells and phospho-Akt in HCT15 cells were also observed in this study.