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Quercetin enhances 5-fluorouracil-induced apoptosis in MSI colorectal cancer cells through p53 modulation

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Abstract

Purpose: Colorectal tumors (CRC) with microsatellite instability (MSI) show resistance to chemotherapy with 5-fluorouracil (5-FU), the most widely used pharmacological drug for CRC treatment. The aims of this study were to test the ability of quercetin (Q) and luteolin (L) to increase sensitivity of MSI CRC cells to 5-FU and characterize the dependence of the effects on cells´ p53 status.

Methods: Two MSI human CRC derived cell lines were used, CO115 wild-type (wt) for p53 and HCT15 that harbors a p53 mutation. Apoptosis induction in these cells by 5-FU, Q and L alone and in combinations were evaluated by TUNEL and western. The dependence on p53 of the effects was confirmed by small interference RNA (siRNA) in CO115 cells and in MSI HCT116 wt and p53 knockout cells.

Results: CO115 p53-wt cells are more sensitive to 5-FU than the p53 mutated HCT15. The combination treatment of 5-FU with L and Q increased apoptosis with a significant effect for Q in CO115. Both flavonoids increased p53 expression in both cell lines, an effect particularly remarkable for Q. The significant apoptotic enhancement in CO115 incubated with Q plus 5-FU involved the activation of the apoptotic mitochondrial pathway. Importantly, knockdown of p53 by siRNA in CO115 cells and p53 knockout in HCT116 cells totally abrogated apoptosis induction, demonstrating the dependence of the effect on p53 modulation by Q.

Conclusion: This study suggests the potential applicability of these phytochemicals for enhancement 5-FU efficiency in MSI CRC therapy, especially Q in p53 wt tumors.

Keywords: Apoptosis; Colorectal carcinoma; 5-fluorouracil; p53; Quercetin

Introduction

Chemotherapy with 5-fluorouracil (5-FU) is the basis for colorectal carcinoma (CRC) treatment, one important cause of cancer related death in western societies [1]. However, significant resistance to this drug has been reported [2-4]. To overcome resistance, drugs such as irinotecan and oxaliplatin are used in combination with 5-FU and have provided increased efficacy although not in all patients [3,5,6]. Particularly, tumors with microsatellite instability (MSI) do not generally respond satisfactorily to 5-FU [7-10]. MSI tumors, which occur in approximately 15% of sporadic CRC cases and in 90% of hereditary non-polyposis colorectal cancer (HNPCC), have mutations in the mismatch repair (MMR) genes, resulting in an inability to correct DNA replication errors and in the accumulation of mutations [8,9,11]. *In vitro* studies have also shown that DNA MMR deficiency may be responsible for tumor resistance to 5-FU [7,8,10].

Previous studies have shown that, independently of MSI status, mutations in the gene *P53* contribute to 5-FU resistance in CRC and have profound effects on drug responses [12,13] with reduced induction of apoptosis and inhibition of cell cycle [14,15]. The prognosis in patients presenting MSI tumors with p53 mutations have been shown to be poorer compared to those having MSI tumors with p53 wild-type [16].

The induction of apoptosis by 5-FU may occur through p53 activation and both the intrinsic and extrinsic pathways with activation of caspases [17]. In the intrinsic pathway, Bcl-2 family proteins modulate mitochondrial membrane permeabilization, which leads to the release of cytochrome c and activation of caspase-9 that in turn activates the effector caspase-3. Activation of death receptors on the cell membrane (extrinsic pathway), which subsequently activates caspase-8 and caspase-3, may also be induced by 5-FU [17].

Dietary phytochemicals have been shown to induce apoptosis through modulation of different pathways contributing to decrease tumor malignance and chemoresistance [18-20].

In addition to effects on mitochondrial and death receptors pathways, some proapoptoitc effects of natural compounds have also been attributed to c-Jun N-terminal kinase (JNK) and p38 stress-activated protein kinases [19,21-23].

In a previous study, we showed that two structurally related flavonoids quercetin (Q; a flavonol) and luteolin (L; a flavone), commonly found in fruits and vegetables, have antiproliferative effects in HCT15 (mutant KRAS) and CO115 (mutant BRAF) human CRC cells through regulation of KRAS and both the MAPK/ERK and the PI3K pathways [24]. The anticarcinogenic effects of these flavonoids suggest the suitability of diet rich in Q and/or L for CRC patients undergoing treatment with 5-FU. In the present study, we tested the possible therapeutic efficacy of Q and L in combination with the pharmaceutical drug 5-FU in a MSI p53 wild-type (wt) and a p53 mutant CRC cell lines, CO115 and HCT15, respectively [25,26]. In addition, the mechanism of Q and L in inducing apoptosis in these MSI CRC cell lines was investigated and the dependence on p53 confirmed by siRNA. Our data shows the potential applicability of these flavonoids for use in combination with 5-FU to induce apoptosis in CRC, particularly for Q in a p53 wt background.

Material and methods

Reagents and antibodies

Quercetin (Q), z-VAD-fmk (zVAD), staurosporine (STS), 5-fluorouracil (5-FU) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin (L) was purchased from Extrasynthese (Genay, France). Stock solutions of test compounds were made in dimethyl sulfoxide (DMSO) and aliquots were kept at -20ºC. All other reagents and chemicals used were of analytical grade.

Primary antibodies were purchased to the following sources: anti-cleaved caspase-9 and anti-phospho-p38 MAPK (Thr180/Tyr182) to Cell Signaling (Danvers, MA, USA); anti-caspase-3 to Calbiochem (San Diego, CA); anti-Bcl-2, anti-Bax, anti-PARP-1, anti-phospho-JNK, anti-JNK, anti-p38 total and anti-p53 to Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-β-actin to Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased to GE Healthcare (Bucks, UK).

Cell lines

HCT15 and CO115 human colon carcinoma-derived cell lines were kindly provided by Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal. The two isogenic HCT116 colon carcinoma, p53-wild type (p53-wt) and p53 complete knockout for p53 (p53-null) cells were kindly provided by Vogelstein [27]. The cell lines were maintained at 37ºC in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic/antimycotic solution (Sigma-Aldrich) and 6% heat-inactivated fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium).

Cell viability/proliferation assay

To investigate the effects of 5-FU on cell viability/proliferation in HCT15 and CO115 cells, as well as, the effect of Q and 5-FU in the two isogenic HCT116 cell lines, the MTT reduction assay were used as described previously [24]. Cells were treated with different concentrations of 5-FU and Q for 46h and then two more hours in the presence of MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04M in isopropanol was then used to dissolve the formazan crystals. The number of viable cells in each well was estimated by the cell capacity to reduce MTT, using a spectrophotometer. The results were expressed as percentage relative to the control (cells without any test compound), and MTT reduction at the beginning

of incubation (t= 0h) was subtracted from all experimental conditions, including the control. Since the effects of the compounds were studied in 48h incubations and cells grow significantly during this period, this treatment of the results allows to distinguish between significant cell death (negative values) and inhibition of proliferation (values between 0 and 100%). The IC50 corresponds to the concentration that inhibits cell viability/proliferation by 50%. Results are presented as mean ± SEM of at least three independent experiments.

TUNEL assay

TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed to estimate the percentage of apoptotic cells treated for 48h with different concentrations of 5-FU alone and in combination with Q and L. In HCT15 and CO115 cell lines, the concentrations of Q and L used induce significant inhibition of cell proliferation and cell viability without substantial severe and acute cell death, as determined by BrdU assay and MTT test in a previous study using the same cells and conditions [24]. Both cell lines were also treated with Q and L in combination with 20µM z-VAD-fmk (zVAD), a general caspase inhibitor, for 48h, to assess the involvement of caspases activation in the apoptotic process induced by the test compounds. Staurosporine (STS) 0.25µM, an apoptosis inducer, was also used as a positive control. In HCT116 isogenic cell lines (p53 wt and p53-null) and CO115 cells depleted for p53 by small interference RNA (siRNA), the concentrations of 5-FU and Q used significantly inhibited cell proliferation without substantial severe and acute cell death.

After treatments, cells were collected (both floating and attached cells), fixed with 4% paraformaldehyde for 15min at room temperature and attached onto a polylysine treated slide using a Shandon Cytospin. Cells were then washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Hoechst

was used for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells, 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.

Western blot analysis

Cells were treated with Q, L, 5-FU and STS alone and co-incubated with Q and 5-FU for 48h and total cell lysates were prepared to measure the expression of different proteins. The 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 $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as a protein standard. To perform western blot analysis, 20µg of protein were resolved by SDS-polyacrylamide gel and then electroblotted onto 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 (bovine serum albumin), washed in TPBS and then incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and 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 loading control.

p53 knokdown in CO115 cells

CO115 human colon cancer cells were transiently transfected with Oligofectamine (Invitrogen, Carlsbad, CA) and 100nM of p53 siRNA in OPTI-MEN (Invitrogen) according to the manufacturer's instructions. The p53 siRNA target sequence was guggaguauuuggaugaca, which was purchased from Invitrogen. Control of siRNA experiments were included by using a siRNA against GFP. Confirmation of p53 knockdown was done by western blot analysis. Twenty four hours after transfection, cells were incubated with 5-FU or Q alone and with both in combination, for 48h, and apoptosis measured by the TUNEL assay.

Statistical analysis

Statistical analyses were done using *t*-test, GraphPad Prism 4.0 software (San Diego, CA, USA). *P*-values ≤ 0.05 were considered statistically significant.

Results

Colon cancer cells` sensitivity to 5-FU

The effect of 5-FU on cell viability/proliferation and apoptosis in HCT15 and CO115 cells were established by the MTT and TUNEL assays, respectively. As shown in Fig. 1a, 5-FU was more effective in decreasing cell viability/proliferation in CO115 than HCT15 after 48h treatment. The 5-FU concentrations that inhibit cell viability/proliferation by 50% (IC50) are around 100µM in HCT15 and 1µM in CO115. The differences in susceptibility of the two cell lines to 5-FU were also observed for apoptosis, with HCT15 being more resistant to apoptosis compared to CO115 (Fig. 1b). IC50 concentrations of 5-FU were selected for the next experiments (100µM for HCT15 and 1µM for CO115).

Combined effect of 5-FU and test compounds on apoptosis

The induction of apoptosis in both cell lines treated with Q or L, at IC50 concentrations, as tested previously [24], or 5-FU (IC50), was monitored by the TUNEL assay in both cell lines. As shown in Fig.2, the flavonoids induced a higher percentage of apoptotic cells in CO115 when compared with HCT15. In HCT15 cells, L or Q in combination with 5-FU demonstrated an additive effect on the induction of apoptosis, i.e., the effects of the combination was similar to the sum of the effects of Q and 5-FU when used alone (Fig. 2a). In CO115 cells, L in combination with 5-FU showed an additive effect in apoptosis induction whereas Q demonstrated to significantly potentiate the induction of apoptotic cell death when combined with 5-FU (Fig. 2b). In all cases, the effects on apoptosis of co-incubations were higher than 5-FU alone or test compound alone.

Effects of Q, L and 5-FU on markers of pathways related with apoptosis

In order to determine the role of caspase activation on the apoptotic effects of the test compounds, the caspase inhibitor zVAD was used and apoptosis measured by TUNEL assay. Apoptosis induced by the test compounds, Q and L, and STS, the apoptosis inducer, were inhibited by zVAD in CO115 (Fig. 3b) but not in HCT15 (Fig. 3a). In addition, as shown in Fig. 3c, none of the compounds induced cleaved (active) caspase-9 in HCT15 cells, and only STS induced cleavage of caspase-3 (active form) and of PARP (inactive form). On the other hand, in CO115 cleaved caspase-9 and caspase-3 were observed with all compounds as well as cleavage of PARP and/or a remarkable decreased of uncleaved PARP (active form).

To further elucidate the apoptotic effects of the test compounds and 5-FU, the expression of the positive mediators of apoptosis, p53 and Bax, as well as the negative regulator, Bcl-2, were analysed by western blot (Fig. 4a). It was observed that Q, L and 5-FU induced p53 levels in both cell lines. Levels of Bcl-2 were notably decreased by all the compounds in both cell lines. On the other hand, Bax levels were increased by all the

compounds in HCT15, although only slightly by Q. Bax was not detected in CO115, which is in accordance to a previous report [28].

In addition, the possible involvement of the JNK and p38 pathways on the induction of apoptosis by the test compounds and 5-FU were evaluated. The results show no effect on phospho-JNK levels by Q, L and 5-FU in neither of the cell lines (Fig. 4b). In HCT15 cells no effect on phospho-p38 levels was observed by the flavonoids and 5-FU while in CO115 cells Q and L slightly increased the levels of phospho-p38 (Fig. 4c). STS significantly induced phospho-JNK levels and decreased the expression of phospho-p38 in both cell lines.

Combination of Q and 5-FU: dependence on p53

Since a significant enhancement of 5-FU-induced apoptosis was observed in CO115 p53-wt cells with Q, an effect concomitant with a robust p53 induction, we further investigated this effect. First, as shown in Fig. 5, combination of Q with 5-FU in CO115 cells also remarkably increased the cleavage of caspase-3, caspase-9 and PARP and decreased Bcl-2 expression, but no further induction of p53 was observed. These results indicate that the apoptosis enhancement of Q with 5-FU is at the mitochondrial caspase pathway.

To explore the involvement of p53 in the induction of apoptosis by Q and 5-FU, CO115 cells were depleted for p53 (around 80%) by siRNA. As shown in Fig. 6a, p53-depleted CO115 cells were significantly resistant to apoptosis when incubated with Q or 5-FU. In addition, the synergy between Q and 5-FU on the induction of apoptosis was absent in the p53 knockdown cells.

The role of p53 in the induction of apoptosis by Q was further confirmed by using two isogenic KRAS activated HCT116 CRC cell lines, one p53-wt and the other with a complete knockout of p53 (p53-null). These cells were incubated with 5-FU or Q alone or with both in combination, at concentrations that induce significant inhibition of cell proliferation without

substantial necrotic death (as observed by the MTT assay; data not shown). HCT116 p53-wt cells were much more susceptible than HCT116 p53-null cells to apoptosis induced by Q and 5-FU (Fig. 6b). As in CO115 (KRAS wt), a synergy between Q and 5-FU in the induction of apoptosis was observed in the KRAS mutated HCT116 p53-wt cells.

Discussion

5-Fluorouracil (5-FU) is the pharmaceutical drug most commonly used in CRC chemotherapy, however, tumor cell resistance to this drug remains a significant concern. Failure to induce apoptosis has been reported to reduce the efficacy of 5-FU, particularly in tumors presenting MSI and/or mutant p53 [7,13,15]. Thus, new compounds are needed to use in combination with 5-FU in order to increase treatment efficacy. In a previous paper, we reported that Q and L, two structurally related dietary flavonoids, possess potential anticarcinogenic effects in two MSI resistant CRC cell lines, HCT15 and CO115, through inhibition of PI3K/Akt and MAPK/ERK pathways [24]. Inhibitory effects on these two pathways have showed to contribute to an induction of apoptosis and to sensitize to chemotherapeutic drugs [29-33]. Here, we tested these flavonoids in combination with 5-FU and an enhancement of apoptosis was found. HCT15 and CO115 cell lines showed different susceptibilities to 5-FU. As expected, HCT15 cells, harboring a p53 mutation, were more resistant to 5-FU than CO115 cells (wt for p53). Q (in HCT15 cells) and L (in both cell lines) in combination with 5-FU showed to increase apoptosis additively. A significant potentiation of apoptosis induction was detected when treating CO115 p53-wt cells with Q and 5-FU, which indicates a synergy between these two compounds in CO115. The effect on apoptosis of this combination was even more pronounced than that of a 100 times higher concentration of 5-FU when tested alone.

In CO115 cells, we observed that the caspase inhibitor zVAD totally abrogated apoptosis induction by Q and L, which was in agreement with the expression of apoptotic-associated molecular markers, such as cleaved (activated) caspase-9 and caspase-3 as well as a decrease in Bcl-2 expression. These results indicated that Q and L induce apoptosis via caspase dependent pathway in CO115 cells with a contribution of the mitochondrial pathway. Even though CO115 cells do not express Bax, apoptosis induction mediated by mitochondria occurs possibly through interaction with the pro-apoptotic protein Bak. The induction of apoptosis by 5-FU in these cells was low, but also caspase dependent. A remarkable synergy was found for the co-incubation of Q with 5-FU in CO115. In these cells, Q showed a more dramatic induction of p53 compared to L, indicating a possible implication of p53 in the synergy observed between Q and 5-FU in CO115. The co-incubation of Q with 5-FU also remarkably enhanced cleavage of caspase 9, caspase 3 and PARP as well as decreased Bcl-2 levels, compared with each of the compounds alone, suggesting the involvement of the apoptotic mitochondrial pathway in the synergy observed for Q and 5-FU in CO115. This synergy was not observed for HCT15 (p53 mutated) cells which corroborates the dependence of Q on p53 for the observed effect.

In order to elucidate the dependence on p53 of the apoptosis induced by Q and 5-FU, p53 expression was decreased by siRNA in CO115 cells. A phenotype more resistant to apoptosis was observed, and interestingly, the synergy between Q and 5-FU observed in control cells (transfected cells with no p53 silencing) was lost in CO115 cells after p53 knockdown. This dependence on p53 was further confirmed using two isogenic MSI (KRAS mutated) HCT116 cell lines. HCT116 p53-null cells showed a smaller apoptotic response to Q and 5-FU as compared to HCT116 p53-wt cells. Furthermore, apoptosis was significantly enhanced when Q was combined with 5-FU only in HCT116 p53-wt cells, with the effect being lost in the HCT116 p53-null cells. These results clearly indicate that Q induces

apoptosis through modulation of p53, and that this contributes to the synergy found for the combination with 5-FU in p53-wt CRC cells. Moreover, the dependence on p53 in induction of apoptosis by Q seems to be independent of KRAS status, since this effect was observed in CO115 KRAS-wt cell line as well as in the KRAS mutant HCT116 cell line.

The resistance to 5-FU of *TP53*-deficient CRC cells has previously been reported [15]. In some studies, this chemotherapeutic drug has been combined with natural compounds in different genetic backgrounds, such as triptolide and rosiglitazone in microsatellite stable (MSS) CRC cell lines [34,35] and notoginseng and its ginsenosides in MSI HCT116 p53-wt cells [36], with favourable outcome. Moreover, luteolin was also demonstrated by others [37] to increase the apoptotic effect of the chemotherapeutic drug, cisplatin, in a p53 dependent manner in different cell types including in HCT116 p53-wt cells.

In HCT15 cells zVAD did not inhibit apoptosis induced by any of the test compounds or the reference inducer STS. The lack of caspase-dependent apoptosis was corroborated by the absence of cleaved caspase-9 and caspase-3 when these cells were incubated with Q, L or 5-FU. Although all compounds induced p53 expression in HCT15 cells, alteration in the expression of p53 protein is not expected to be of functional significance for apoptosis in this p53 mutated cell line. Also, Bax expression levels increased and Bcl-2 decreased in response to test compounds which, however, did not activate apoptosis through mitochondrial caspase pathway in HCT15 cells. JNK and p38 pathways seem not to be involved in the induction of apoptosis in these cells, contrarily to what was observed with CO115 where the p38 pathway may contribute to the induction of apoptosis. An induction of JNK phosphorylation and a decrease on p38 expression was observed for STS in both cell lines. The activation of JNK by STS has been reported in breast cancer cells [38] but the effect of this compound on these two stress activated kinases in CRC is not well established.

CRC MSI patients require treatment alternatives that enhance 5-FU responsiveness and would gain from customized treatment modalities based on p53 status. The present study shows the potential applicability of Q and L in the enhancement of the apoptotic effects of 5-FU in MSI CRC cells. Of particular relevance, Q shows the ability to cooperate with 5-FU to potentiate the induction of apoptosis in p53 wt colorectal cancer cells through p53 signaling.

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Conflict of interest

None.

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Figure legends

Figure 1 - Effect on cell viability/proliferation (**a**) and apoptosis (**b**) of different concentrations of 5-fluorouracil (5-FU), for 48h, in HCT15 and CO115 colon cancer cells, using MTT and TUNEL assay, respectively. The MTT value in the beginning of the assay (t= 0h) was subtracted from all experimental conditions at 48h. Negative values in MTT assay indicate induction of severe and acute cell toxicity after 48h incubation with test compound. Results are presented as mean ± SEM of at least 3 independent experiments. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001.

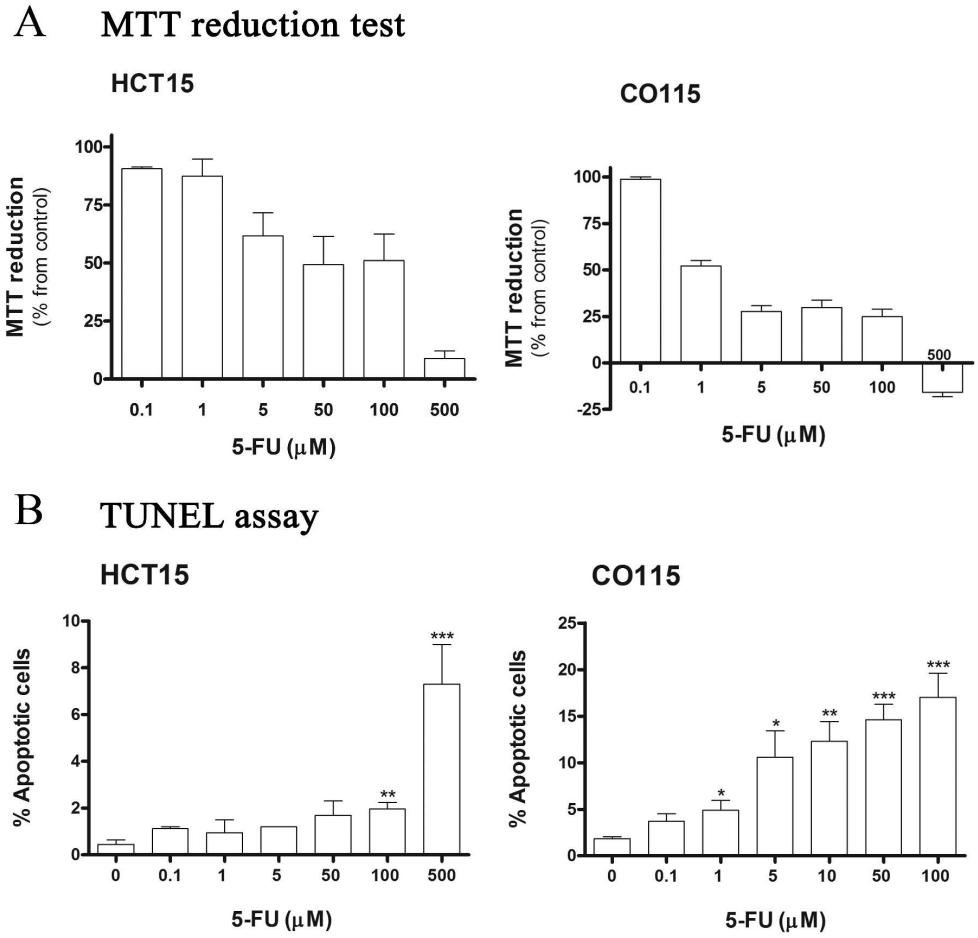
Figure 2 - Effect on apoptosis by 5-fluorouracil (FU) 500, 100 and 1µM, quercetin 12µM (Q12) and luteolin 12µM (L12) alone, as well as the natural compounds co-incubated with FU for 48h, in HCT15 (**a**) and CO115 (**b**) cells, using TUNEL assay. Results are presented as mean ± SEM of at least 3 independent experiments. *** P≤ 0.001, when compared with control; ++ P≤ 0.01, when compared with the respective natural compound alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with FU alone; NS, not significant differences observed between each other.

Figure 3 - Effect of a caspase inhibitor zVAD-FMK (zVAD) 20µM on the apoptosis induction by quercetin 12µM (Q12), luteolin 12µM (L12) and staurosporine (STS) 0.25µM, for 48h, in HCT15 (**a**) and CO115 (**b**) cells, using TUNEL assay. Results are presented as mean ± SEM of at least 3 independent experiments. * P≤ 0.05, *** P≤ 0.001, when compared to control; ++ P≤ 0.01 and +++ P≤ 0.001, when compared with the respective compound alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with zVAD alone; NS, not significant differences observed between each other. Effects on caspase-9, caspase-3 and PARP-1 expressions, for 48h, of Q, L, 5-FU (FU) and STS alone, in HCT15 and CO115 cells (**c**), by western blot. Images are representative of at least 3 independent experiments.

Figure 4 - Effects on p53, Bax and Bcl-2 (**a**), phospho-JNK and total JNK (**b**) and phospho-p38 and total P38 (**c**) expressions, for 48h, of quercetin 12µM (Q12), luteolin 12µM (L12), staurosporine (STS) 0.25µM and 5-fluorouracil (FU) 1µM and 100µM, in HCT15 and CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

Figure 5 – Effects on caspase-9, caspase-3, PARP-1, p53 and Bcl-2 expressions, for 48h, of co-incubation of quercetin 12µM (Q12) and 5-fluorouracil 1µM (FU1) in CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

Figure 6 – Dependence on p53 for apoptosis induction by quercetin (Q) and 5-fluorouracil (FU). (**a**) CO115 cells knockdown for p53 by siRNA (100nM) were incubated with FU 1µM (FU1) or Q 12µM (Q12) alone, and with both in combination for 48h and apoptosis assessed by TUNEL assay. Control cells were transfected with control siRNA. Compounds were added 24h after transfection. p53 knockdown efficiency was monitored by western blot (inset). (**b**) HCT116 p53-wt (p53 +/+) and HCT116 p53-null (p53 -/-) cells were used to observe effects of FU, Q and both in combination on apoptosis after 48h of incubation, as assessed by TUNEL assay. FU10: FU 10µM; FU100: FU 100µM; Q15: Q 15µM. (a; b) Results are presented as mean ± SEM of at least 3 independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, when compared with control; + $P \leq 0.05$, when compared with each other; ### $P \leq 0.001$, when compared with respective FU alone; Ø $P \leq 0.05$, when compared with respective Q alone; NS, not significant differences observed between each other.



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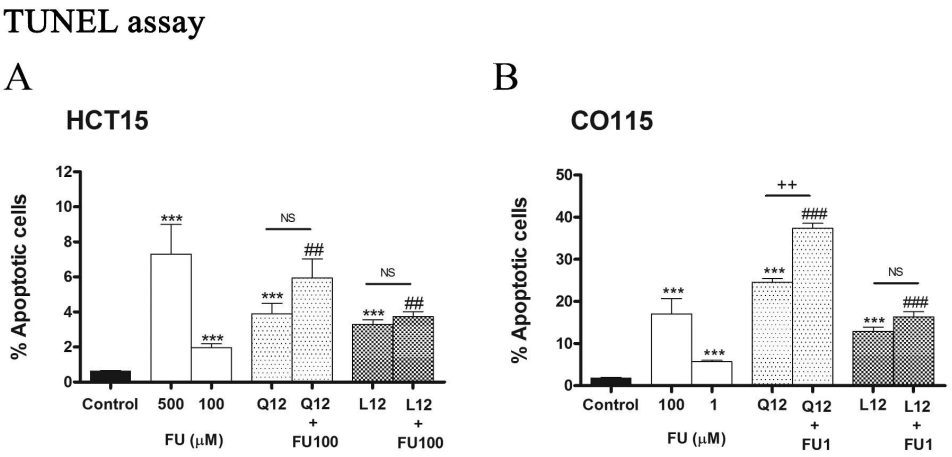

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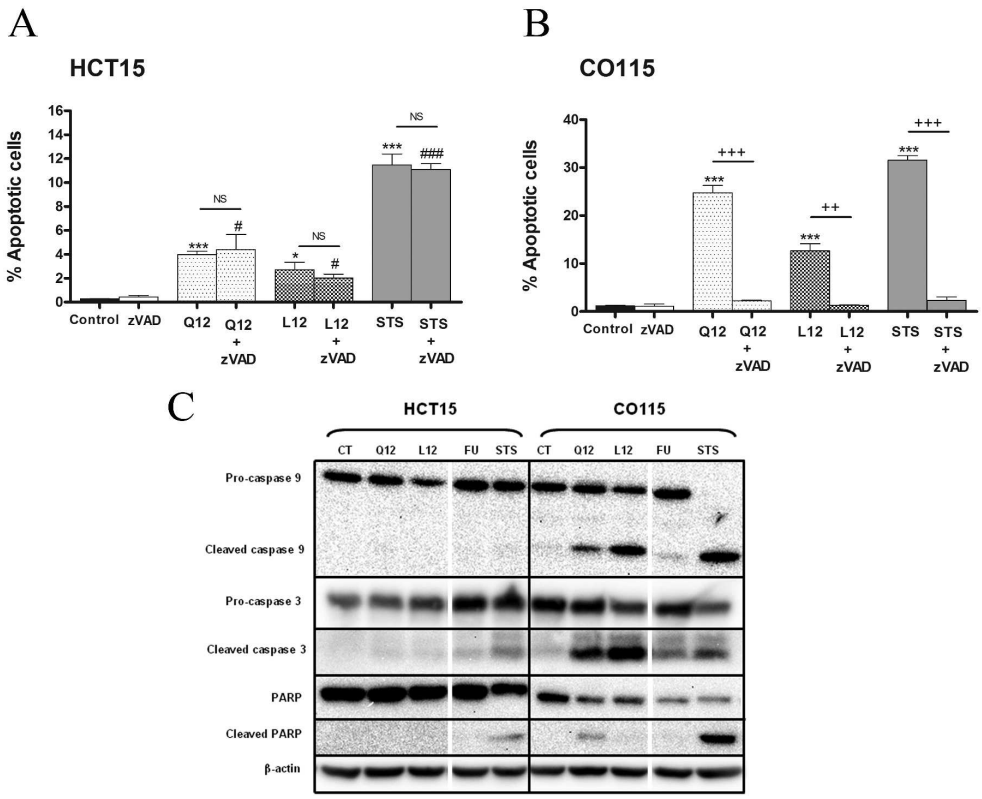

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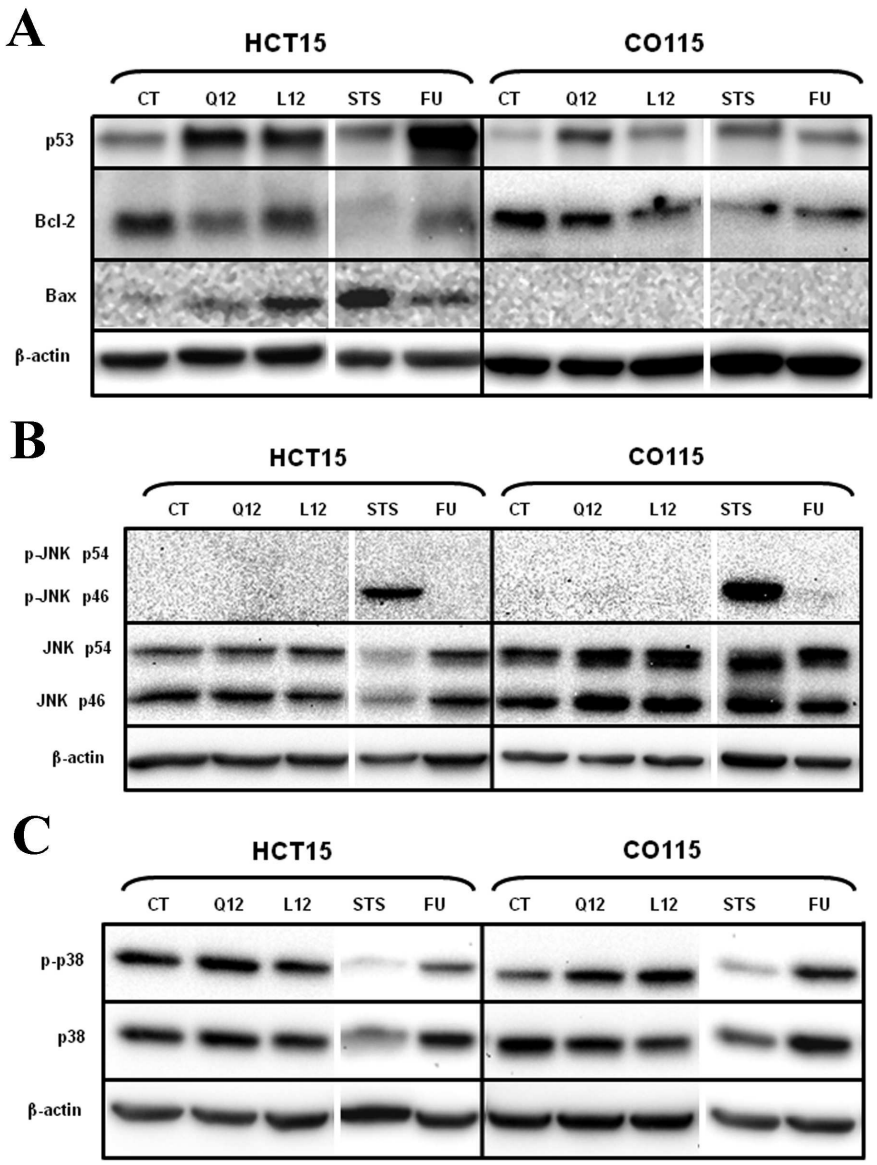

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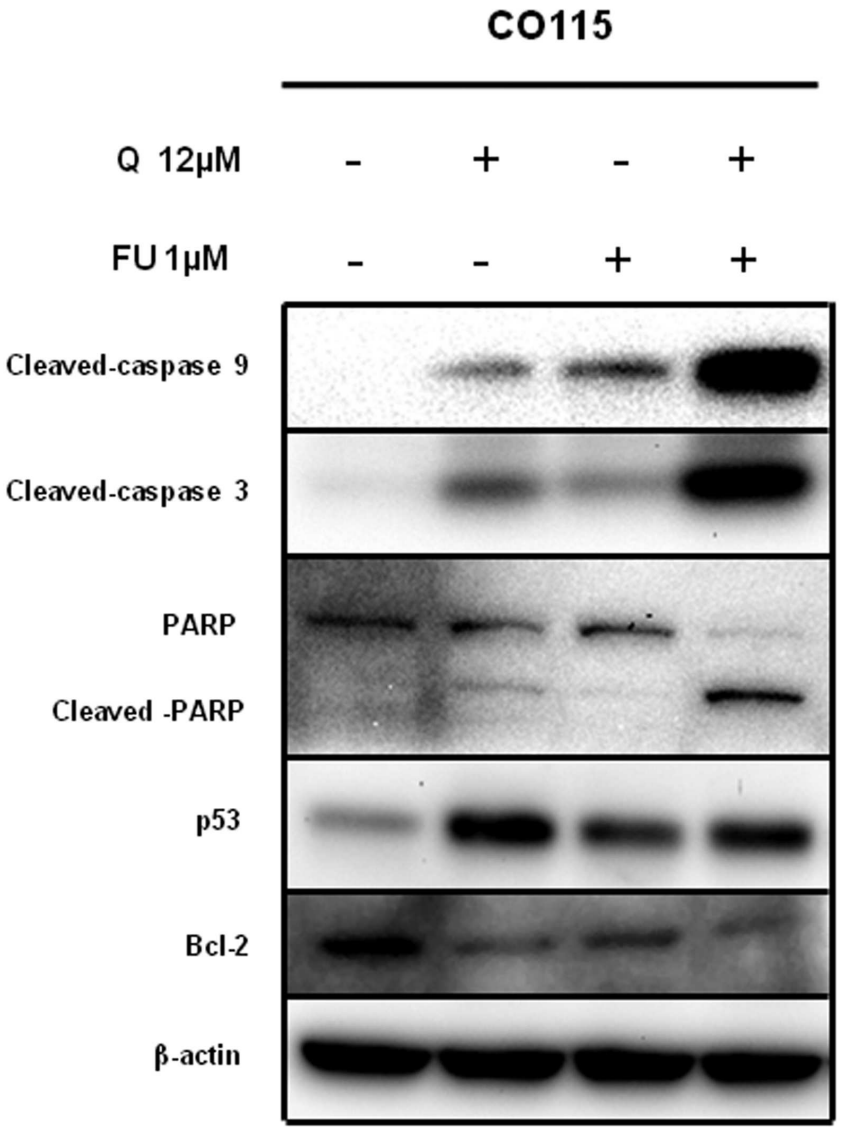

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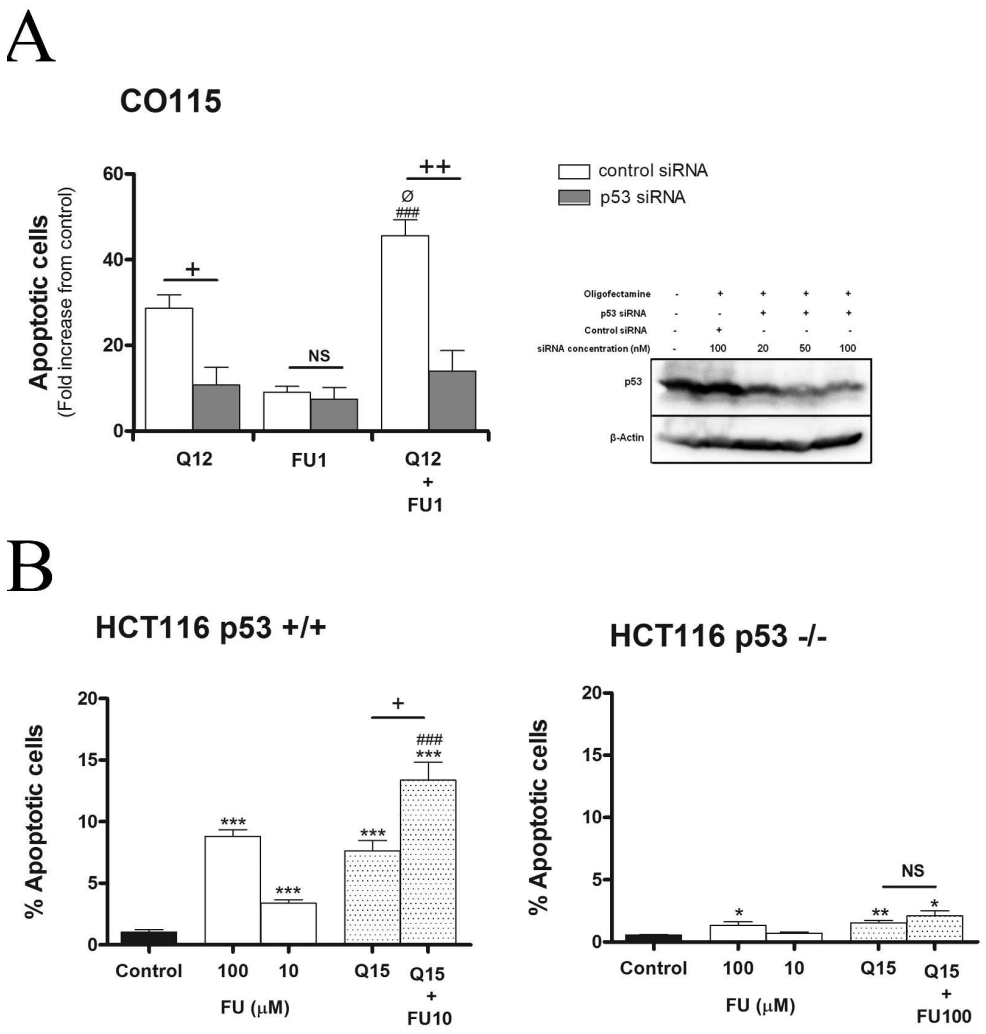

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