Title

Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells

Cristina P.R. Xavier[1,2], Cristovao F. Lima[1,3], Ana Preto[1,2,4], Raquel Seruca[4], Manuel Fernandes-Ferreira[1,3] and Cristina Pereira-Wilson[1,2,*]

[1]*Department of Biology, University of Minho, 4710-057 Braga, Portugal*
[2]*CBMA – Molecular and Environmental Biology Centre*
[3]*CITAB – Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas*
[4]*Institute of Molecular Pathology and Immunology, University of Porto (IPATIMUP), 4200-465 Porto, Portugal*

[*] Corresponding author: Tel.: +351 253604318; fax: +351 253678980 (C. Pereira-Wilson).
E-mail address: cpereira@bio.uminho.pt (C. Pereira-Wilson).

Abstract

KRAS and BRAF mutations are frequent in colorectal carcinoma (CRC) and have the potential to activate proliferation and survival through MAPK/ERK and/or PI3K signalling pathways. Because diet is one of the most important determinants of CRC incidence and progression, we studied the effects of the dietary phytochemicals quercetin (Q), luteolin (L) and ursolic acid (UA) on cell proliferation and apoptosis in two human CRC derived cell lines, HCT15 and CO115, harboring KRAS and BRAF activating mutations, respectively. In KRAS mutated HCT15 cells, Q and L significantly decreased ERK phosphorylation, whereas in BRAF mutated CO115 cells the three compounds decreased Akt phosphorylation but had no effect on phospho-ERK. Our findings show that these natural compounds have antiproliferative and proapoptotic effects and simultaneously seem to act on KRAS and PI3K but not on BRAF. These results shed light on the molecular mechanisms of action of Q, L and UA and emphasize the potential of dietary choices for the control of CRC progression.

Keywords: Flavonoids, Ursolic acid, colorectal carcinoma, MAPK/ERK and PI3K pathways, molecular nutrition

Introduction

Colorectal carcinoma (CRC) is the third most common cancer worldwide. In particular sporadic CRC corresponding to 70-80% of total cases [1] is influenced by environmental factors, many of which diet related [2]. The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt are signalling pathways that have been implicated in oncogenic transformation in CRC. They confer a proliferative phenotype and resistance to therapy which is reflected in low patient survival [3-7]. Components of MAPK/ERK and PI3K/Akt pathways constitute, therefore, molecular targets for anticancer strategies [8-10]. Mutations of either KRAS (32%) or BRAF (14%) genes occur alternatively [11] in CRC, causing activation of either MAPK/ERK and PI3K pathways or MAPK/ERK pathway, respectively. Activation of MAPK/ERK pathway regulates the expression of a large number of proteins involved in the control of cell proliferation, differentiation and apoptosis [4, 12]. Activation of PI3K gene and inactivation of PTEN, common in CRC, result in overexpression of downstream targets, including Akt and PKC, which promote cell growth and rescue from apoptosis [13-17].

Epidemiological studies show that cancer incidence is inversely correlated with the consumption of diets rich in fruits and vegetables [18]. Natural compounds present in the diet, such as resveratrol and curcumin have been shown to be protective against cancer, contributing to decrease cancer risk and progression rate through their effects on signalling pathways related to proliferation and apoptosis [18, 19]. Studies in cell lines and animal models have shown that flavonoids inhibit cell proliferation and induce apoptosis in many types of cancer cells through different signalling pathways, which corroborate the suggestion that dietary choices may limit cancer progression [20-22]. Quercetin (Q) and luteolin (L) (Figure 1) are two flavonoids found in fruits, vegetables

and aromatic plants with high antioxidant activity [23] to which anticancer properties in CRC are attributed [24-27]. In addition, a recent study showed that quercetin reduces the formation of aberrant crypt foci in a rat colon cancer induction model, suggesting the importance of this compound also in the prevention of colon cancer by decreasing cancer initiating events [28]. Although structurally related, the absence of the hydroxyl group at position 3 of L renders it more lipophilic than Q which may confer better access to intracellular targets. In agreement with this, we have previously shown that L is a more potent intracellular antioxidant than Q, and that this was related with its higher lipophilicity [23]. Ursolic acid (UA; Figure 1), a natural pentacyclic triperpenoid carboxylic acid, present ubiquitously in plant foods and also a major constituent in some medicinal plants possesses a wide range of biological activities, such as hepatoprotective and anti-inflammatory properties combined with low toxicity [29-31]. However, contrarily to Q and L, UA is not an antioxidant at relevant cellular levels [32]. Antitumor properties have also been attributed to UA and in colon cancer cells UA has been shown to induce apoptosis and inhibit proliferation [33-35]. Although potential effects on proliferation have been described for these compounds their effects on MAPK/ERK and PI3K pathways have not been established.

In spite of the general benefit of plant rich diets, variation in cancer incidence among individuals with similar dietary habits suggests interactions of food constituents with genetic factors [2, 18]. In the present study we report on the effects of Q, L and UA on two human derived cell lines which harbor different oncogene activating mutations, representative of a large number of CRC: HCT15 has a KRAS (G13D) mutation [36] whereas CO115 has a BRAF (V599E) mutation [37]. These mutations impact on MAPK/ERK and PI3K pathways. The relevance of effects on both these pathways for successful cancer treatment has recently been emphasized [38]. CRC remains a human

malignant tumor often resistant to available treatment and knowledge of anticancer properties of dietary constituents may guide dietary choices for cancer patients with particular genetic backgrounds and possibly also suggest their use in combination with conventional therapy in order to enhance therapeutic effects [39, 40].

Material and methods

Reagents and antibodies

Quercetin (Q), ursolic acid (UA), wortmannin (W) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin (L) was from Extrasynthese (Genay, France) and PD-98059 (PD) from Calbiochem (San Diego, CA). All other reagents and chemicals used were of analytical grade. Stock solutions of Q, L and UA were made in dimethyl sulfoxide (DMSO) and aliquots kept at -20ºC.

Primary antibodies were purchased from the following sources: Cell Signaling (Danvers, MA, USA) the anti-p44/42 MAPK (ERK1/2) total, anti-phospho-Akt (Ser473), anti-Akt total, anti-phospho-PTEN (Ser380/Thr382/383) and anti-PTEN total; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) the anti-phospho-ERK1/2, Raf-B and K-Ras; and Sigma-Aldrich the anti-β-actin. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

Cell culture

HCT15 and CO115 human colon carcinoma-derived 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 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2ml) and twelve (1ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml. Incubations with different concentrations of test compounds were made in serum free medium for 48h for MTT test, BrdU incorporation and TUNEL assay, and for 24h (Akt, ERK and PTEN) or 6h (BRAF and KRAS) for western blot analysis.

Assessment of cell toxicity/proliferation by MTT reduction test

A MTT reduction assay of the tested compounds was performed in order to select concentrations that were not cytotoxic and significantly inhibited cell proliferation. Cells were treated with test compounds for 46h before the 2h incubation with MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04M in isopropanol was then added to dissolve the formazan crystals. The number of viable cells in each well was estimated by the cell capacity to reduce MTT. The results were expressed as percentage relative to the control (cells without any test compound). MTT reduction at the beginning of incubation (t=0h) was subtracted from all the experimental conditions used above, including the control. Since the effects of the compounds were studied after 48h of incubation and cells grow significantly within this time period, the point at the beginning of the incubation allows to distinguish between cell death and inhibition of proliferation. MTT negative values indicate necrotic cell death due to cytotoxicity. Values between 0% and 100% indicate inhibition of cell proliferation.

Assessment of proliferation by BrdU incorporation

After 45h of treatment with test compounds at the chosen concentrations, cells were incubated with bromodeoxyuridine 10μM (BrdU; Sigma-Aldrich) for another 3h.

Both adherent and non-adherent cells were collected from each sample, fixed with 4% paraformaldehyde for 15min at room temperature and 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 cell number (nuclei staining with Hoechst) from a count higher than 500 cells per slide under a fluorescence microscope. Results are presented as mean ± SEM of at least three independent experiments.

Assessment of apoptosis by TUNEL assay

Cells treated with the tested compounds at chosen concentrations for 48h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for 15min at room temperature and attached into a polylysine treated slide using a Shandon Cytospin. Cells were washed in PBS and 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 fluorescence microscope. Results are presented as mean ± SEM of at least three independent experiments.

Protein extraction and western blotting

After treatment with the chosen concentration of test compounds, 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. Twenty micrograms of total protein from each cell lysate 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 (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. 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, using GraphPad Prism 4.0 software (San Diego, CA, USA), and *P*-values ≤ 0.05 were considered statistically significant.

Results

Effects of Q, L and UA on cell proliferation

In order to choose doses that do not cause significant toxicity (necrosis) and have antiproliferative effects on the two human CRC derived cell lines, HCT15 and CO115, a MTT assay was performed with different concentrations of the tested compounds. The results showed that necrotic cell death (negative values in Figure 2) occurred in HCT15 cells with L at 20µM and in CO115 with UA at 15µM. All the tested compounds inhibited cell proliferation in a concentration-dependent manner in both cell lines as shown by MTT assay (Figure 2). Both reference inhibitors, wortmannin (W) and PD-98059 (PD), at the higher tested concentration did not induce cell toxicity. Based on MTT results, three concentrations of Q, L and UA (2 in case of CO115 cells) that inhibited cell proliferation without significant toxic effects, were selected and used in the following BrdU and TUNEL assays.

For the BrdU incorporation assay, cells were treated with compounds for 48h. As shown in Figure 3, a significant inhibition of proliferation indicated by lower levels of BrdU incorporation was observed for Q, L and UA in both cell lines, in a dose dependent manner. In HCT15 cells, the levels of BrdU incorporation decreased from 22.0% in the control to 5.2%, 3.4% and 9.8% in cells treated with Q 20µM, L 15µM and UA 4µM, respectively (Figure 3a). In CO115 cells, the percentage of cell proliferation significantly decreased from 19.1% in the control to 3.8%, 11.1% and

10.2% in cells treated with Q 15µM, L 12µM and UA 10µM, respectively (Figure 3b). The structurally-related flavonoids, Q and L, showed different responses in the two cell lines: L was a stronger proliferation inhibitor in HCT15 than in CO115, whereas Q was more effective in CO115 than in HCT15. The reference inhibitors, W, a PI3K inhibitor, and PD, a phospho-ERK inhibitor, significantly inhibited cell proliferation in HCT15 and CO115 cells, respectively (Figure 3).

Effects of Q, L and UA on apoptosis

The ability of the test compounds to induce apoptosis was addressed by the TUNEL assay. As shown in Figure 4, all compounds significantly induced apoptosis in both HCT15 and CO115 cells. Apoptotic cells in HCT15 increased from 0.3% in the control to 4.4%, 3.9% and 6.6% in cells treated with the higher concentrations of Q, L and UA, respectively (Figure 4a). In CO115 cells, apoptotic cells increased from 1.9% in the control to 36.2%, 15.1% and 12.4% in cells treated with the higher concentrations of Q, L and UA, respectively (Figure 4b). Between the two flavonoids, Q seems to be a more potent inducer of apoptosis in both cell lines compared to L. In addition, UA showed to be the most effective in HCT15, where it induced 20 times more apoptosis at 4µM when compared with control.

Effects of Q, L and UA on ERK phosphorylation

Activation of MAPK/ERK pathway is representative of a large number of CRC cases and the phosphorylation of ERK is an indicator of this activation. We observed high levels of phospho-ERK in both cell lines (Figure 5). Incubations with L (15µM) and Q (20µM) significantly decreased phospho-ERK protein level in HCT15 cells (Figure 5a), but not in CO115 cells, while UA did not have any effect on either of the

cell lines (Figure 5a,b). A significant reduction of phospho-ERK by PD, a reference inhibitor of MAPK/ERK pathway, was observed in both cell lines. Interestingly, L was a stronger inhibitor of ERK in HCT15 cells than the reference inhibitor PD and than the structure-related compound Q.

Effects of Q, L and UA on Akt phosphorylation

Because MAPK/ERK and PI3K/Akt pathways are both activated by RAS, we also checked if the PI3K/Akt pathway was affected by the test compounds, measuring phospho-Akt and phospho-PTEN expression levels. In HCT15 cells, there were no detectable amounts of phospho-Akt, in incubations with and without serum (Figure 6a). High expression levels of phospho-PTEN were detected in HCT15 cells but were not altered by the test compounds (data not shown). In CO115 cells, phospho-Akt expression was observed and significantly decreased by Q (15µM), L (12µM) and UA (10µM), as shown in Figure 6b. Wortmannin, a reference PI3K inhibitor, also significantly decreased Akt phosphorylation, contrarily to PD that did not alter phospho-Akt levels in CO115 cells. Phospho-PTEN and total PTEN expression were not observed in CO115 cells, in medium with and without serum, in contrast with what was observed with HCT15 cells (Figure 6c). The lack of phospho-Akt in HCT15 and PTEN signal in CO115 cells was reproducible and checked in the presence of a positive reactive sample (Figure 6a,c). These observations seem, therefore, also not to be the result of protein degradation during protein extraction or sample preparation.

Effects of Q, L and UA on KRAS and BRAF expression

To check if the effects of the tested compounds reflect direct effects on KRAS or BRAF, expression of these proteins was also monitored by western blot. As shown in

figure 7, Q and L remarkably decreased the expression of KRAS but not BRAF in both cell lines. UA significantly changed the expression of KRAS only in HCT15 cells, but not as efficiently as the flavonoids. No significant changes were induced by UA in BRAF expression.

Discussion

The effects of quercetin (Q), luteolin (L) and ursolic acid (UA), natural compounds common in diets rich in fruits and vegetables, were studied in two different human colon carcinoma-derived cell lines representative of common CRC cases. We observed that the three tested compounds, at concentrations that did not induce significant cell toxicity (necrosis), inhibited proliferation and induced apoptosis in both cell lines in a concentration-dependent manner. The purpose of the present study was to identify in HCT15 and CO115 cells molecular targets for Q, L and UA related with their antiproliferative and proapoptotic effects. HCT15 and CO115 have activating mutations of KRAS and BRAF, respectively.

Q and L decreased the expression of phospho-ERK in the KRAS mutated HCT15 cell line but not in the BRAF mutated CO115 cell line. These results suggest that the BRAF mutation in CO115 cells overrides any inhibitory effect of Q and L on phospho-ERK, indicating that these flavonoids act on KRAS upstream of BRAF (Figure 8). This was further confirmed by a decrease in the expression of KRAS but not BRAF induced by both flavonoids. Our findings corroborate recent reports where quercetin treatment resulted in a reduction of Ras protein levels in colon cell lines expressing oncogenic Ras [41, 42]. A recent study in skin epidermal cell line, showed a different effect of quercetin, which in these cells inhibited both Raf and MEK activity [43]. In addition, we observed in HCT15 cells that Q and L decreased phospho-ERK levels as

efficiently as PD-98059 (PD), a specific inhibitor of MEK downstream of RAF [44]. Inhibition of proliferation and induction of apoptosis by Q and L in HCT15 cells does however not seem to be due to phospho-ERK inhibition alone since PD inhibited phospho-ERK but was without effect on cell proliferation and induction of apoptosis. This finding is in agreement with other reports [10, 45], which showed that tumor cells carrying KRAS mutation do not rely only on MAPK/ERK pathway to proliferate. Since HCT15 cell proliferation was inhibited by wortmannin (W), a PI3K inhibitor, it seems that inhibition of proliferation by Q and L treatment could be through inhibition of PI3K dependent pathways. Contrarily to a previous report [46], we did not detect phospho-Akt in HCT15 cells, which could be explained by the high levels of phospho-PTEN observed (Figure 8). It is known that other downstream targets of PI3K besides Akt also contribute to cell proliferation and apoptosis, such as PKC, which is known to be inhibited by Q and L [47-51]. PKC isozymes have been shown to be commonly deregulated in colon cancer and other natural compounds, such as curcumin, have also shown to inhibit PKC in CRC cells [17]. Thus, inhibition of proliferation in HCT15 cells by Q and L seems to be due to effects on KRAS, affecting not only the MAPK/ERK pathway but also other alternative pathways, such as PI3K/PKC pathway. However, apoptosis induced by Q and L in HCT15 cells does not seem to be due to inhibition of PI3K, since W did not induce apoptosis in this cell line. Other apoptotic targets of these compounds should be considered.

Studies have shown that BRAF mutated cell lines rely more on MAPK/ERK pathway for proliferation than KRAS mutated cell lines [10, 45]. However, our results show that the dependence of CO115 cells on MAPK/ERK pathway for proliferation is not exclusive, since all tested compounds inhibit proliferation without affecting phospho-ERK levels. CO115 cells, besides harbouring a BRAF mutation, also present

291 high PI3K activity [52]. In agreement with this, a high expression of phospho-Akt was
292 observed while PTEN was not detectable. Our results show a significant decrease in
293 phospho-Akt expression by Q, L and UA. These suggest an inhibition of PI3K activity,
294 in addition to an inhibition of KRAS (Figure 8). Several studies have shown that
295 quercetin and analogs are potent inhibitors of PI3K activity [53, 54]. However, W
296 although inhibiting phospho-Akt, did not inhibit proliferation or induce apoptosis. The
297 effect of the natural compounds on PI3K may, therefore, only partially explain their
298 antiproliferative and proapoptotic activities in CO115 cells. The inhibition of phospho-
299 Akt and phospho-ERK by Q and L has been reported in human hepatoma cell line
300 (HepG2) and brain tumors [55-57].

301 Regarding UA, our results show that this compound does not affect phospho-
302 ERK expression, being only effective in inhibiting phospho-Akt in CO115 cells. It
303 seems that UA does not affect significantly KRAS (although it decreased expression
304 levels in HCT15 cells) and has PI3K as one of its molecular targets. Interestingly, it was
305 the most efficient proliferation inhibitor and inducer of apoptosis in HCT15 cells, which
306 do not express phospho-Akt. Contrarily to the effects of the antioxidants Q and L, the
307 antitumor properties of UA through redox-sensitive pathways are most likely not the
308 result of its reactive oxygen species scavenging ability, since it was previously shown
309 that UA is inactive as free radical scavenger [32]. Interestingly, all tested compounds
310 showed to be more efficient than the reference compounds, PD and W, in inhibiting cell
311 proliferation and inducing apoptosis. Their wider range of molecular targets is therefore
312 advantageous in the control of tumor progression [26] and their importance of
313 modulating several signal transduction pathways associated with carcinogenesis is once
314 again reinforced.

In summary, the antiproliferative and proapoptotic effects of Q and L seem to be, at least in part, due to effects on KRAS through regulation of both MAPK/ERK and PI3K pathways. The BRAF mutation overrides the compounds` inhibition of KRAS on the MAPK/ERK pathway but not on the PI3K pathway. UA seems to act on PI3K where Q and L may also act, independently of KRAS mutation. The results of this study suggest, therefore, the applicability of these phytochemicals in dietary strategies and as possible adjuvants in CRC therapy both in KRAS and BRAF gene mutation profiles.

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Figure legends

Fig. 1. Chemical structures of quercetin (Q), luteolin (L) and ursolic acid (UA).

Fig. 2. Effect of different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA), for 48h on MTT reduction in HCT15 (a) and CO115 (b) cells. Wortmannin (W) and PD-98059 (PD) were used as reference inhibitors of PI3K/Akt and MAPK/ERK pathways, respectively. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

Fig. 3. Effect on cell proliferation of different concentrations of quercetin (Q), luteolin (L), ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48h in HCT15 (a) and CO115 (b) cells, using the BrdU incorporation assay. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

Fig. 4. Effect on apoptosis of different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48h in HCT15 (a) and CO115 (b) cells, using the TUNEL assay. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

Fig. 5. Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 24h on phospho-ERK expression in HCT15 and CO115 cells, using western blot. β-Actin was used as loading control. (a) HCT15 cells were treated with L 15μM (L15), Q 20μM (Q20) and

UA 4 μM (UA4) in serum free medium. (b) CO115 cells were treated with 12μM L (L12), 15μM Q (Q15) and 10μM UA (UA10) in serum free medium. Wortmannin 1μM (W1) and PD-98059 50μM (PD50) were used as reference inhibitors of PI3K and MEK, respectively, in both cell lines. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and **P≤ 0.01 when compared to control.

Fig. 6. Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 24h on phospho-Akt expression and phospho-PTEN levels in HCT15 and CO115 cells, using western blot. β-Actin was used as loading control. (a) HCT15 cells were cultured in medium without and containing 2% or 10% serum (FBS) and CO115 cells were cultured in serum free medium. (b) CO115 cells were treated with UA 10μM (UA10), Q 15μM (Q15) and L 12μM (L12) in serum free medium. Wortmannin 1μM (W1) and PD-98059 50μM (PD50) were used as reference inhibitors of PI3K and MEK, respectively. (c) CO115 cells were cultured in medium without and containing 2% or 10% FBS and HCT15 cells were cultured in serum free medium. Values are mean ± SEM of at least 3 independent experiments. ***P≤ 0.001 when compared to control.

Fig. 7. Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 6h on KRAS and BRAF expression in HCT15 (a) and CO115 (b) cells, using western blot. β-Actin was used as loading control. Images and values are representative of three independent experiments.

Fig. 8. Schematic representation of possible targets of quercetin (Q), luteolin (L) and ursolic acid (UA) on MAPK/ERK and PI3K/Akt pathways in HCT15 and CO115 cell lines, which may lead to inhibition of cell proliferation and induction of apoptosis.

Figure 1

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Figure 2

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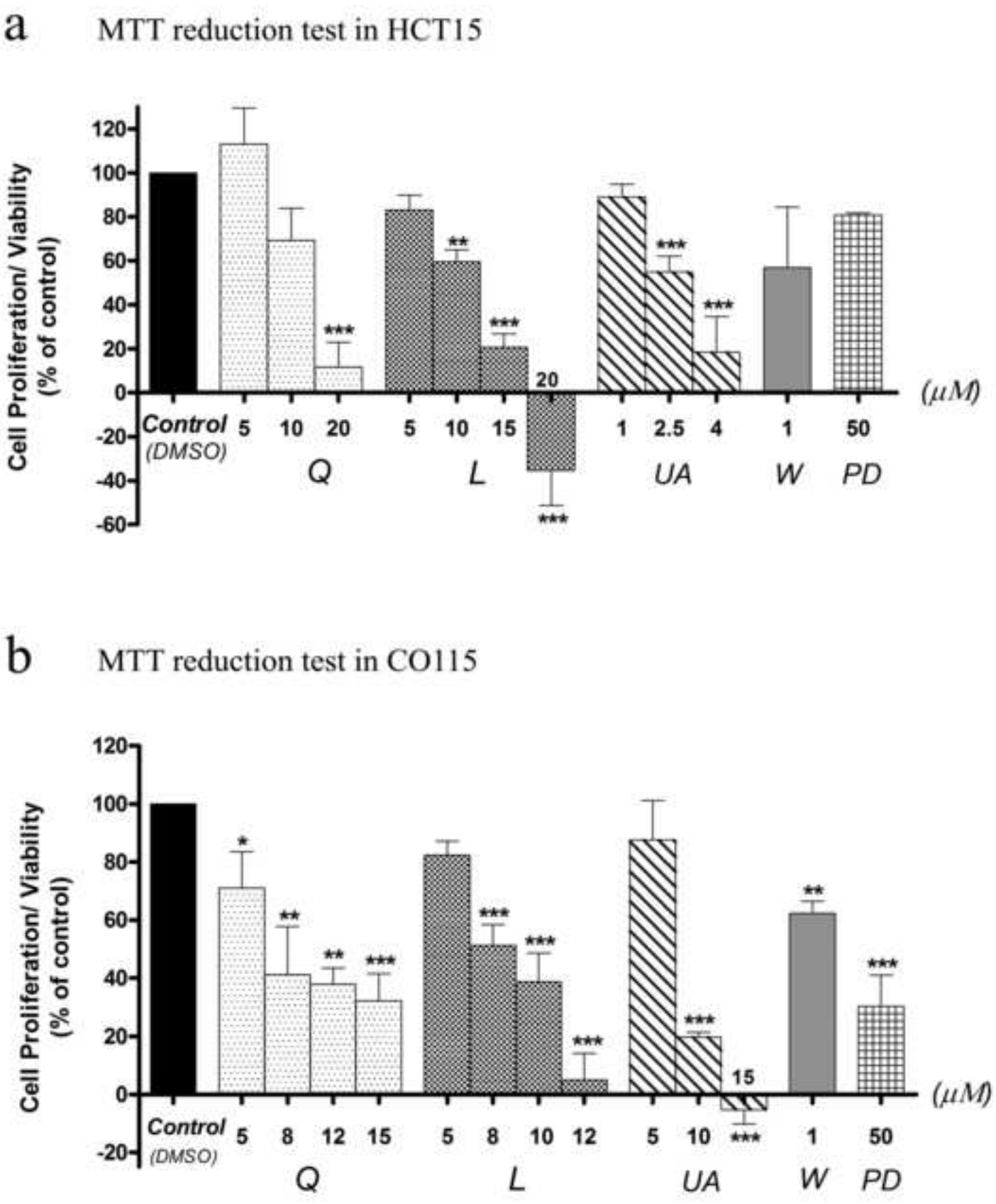

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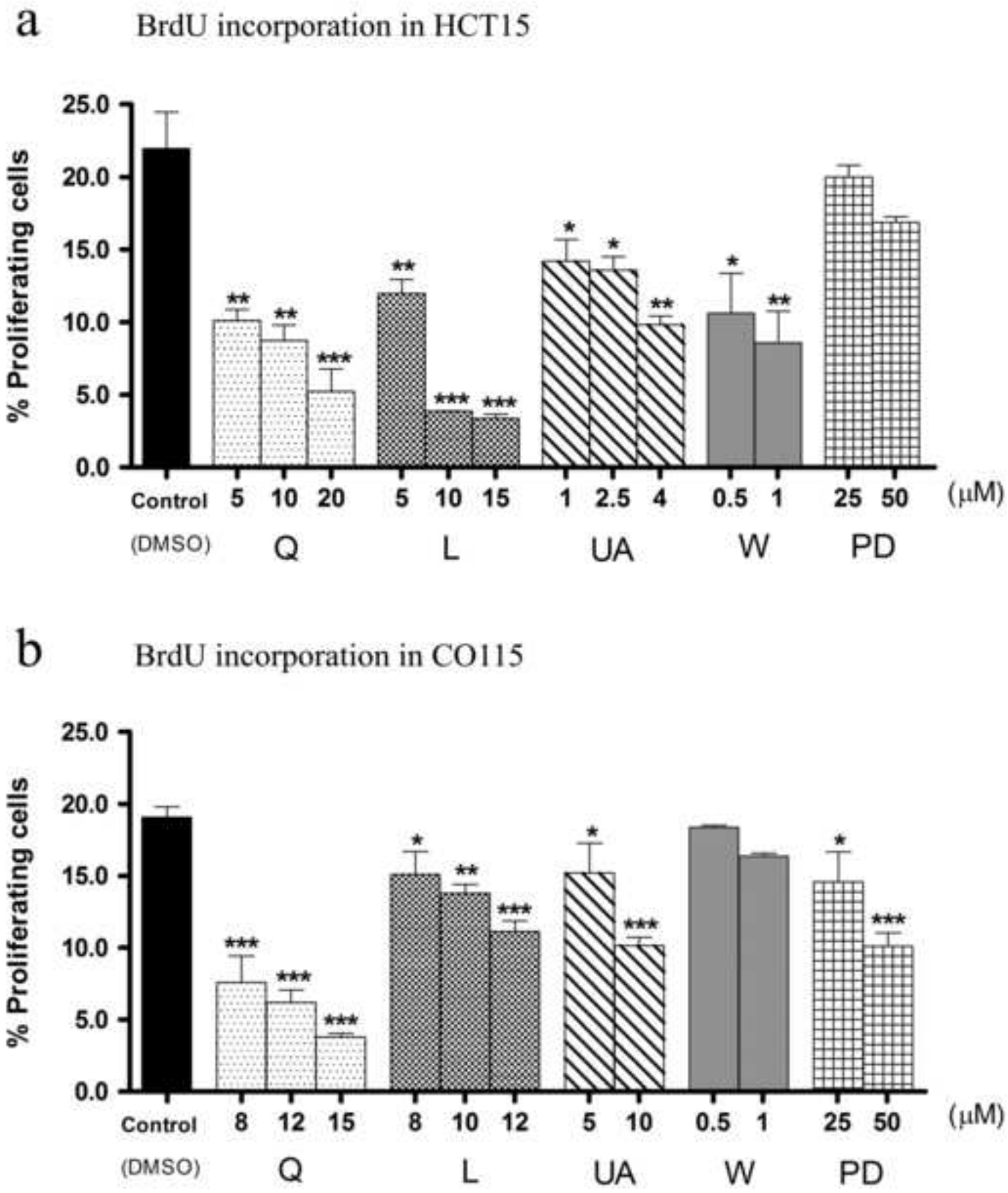

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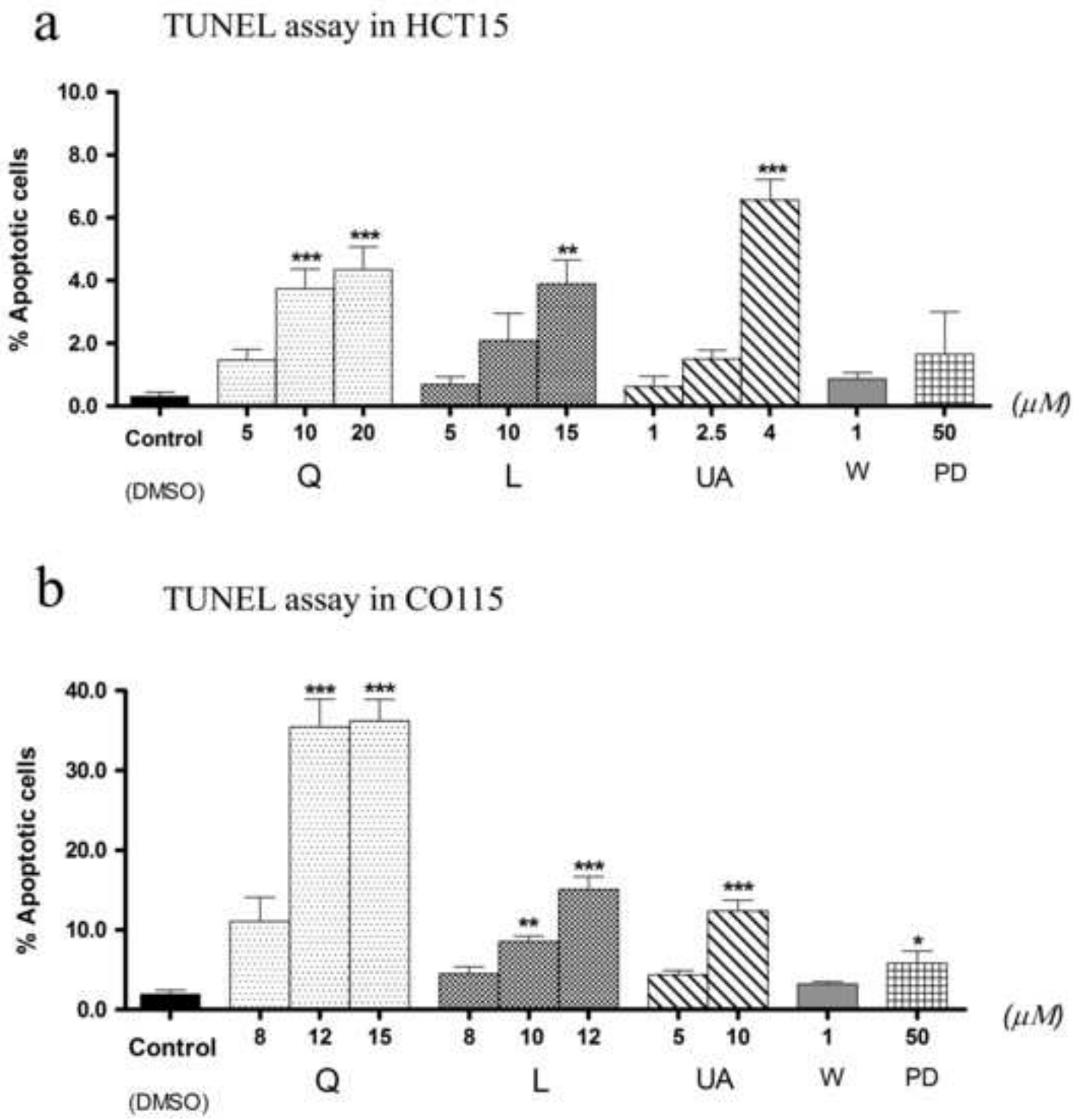

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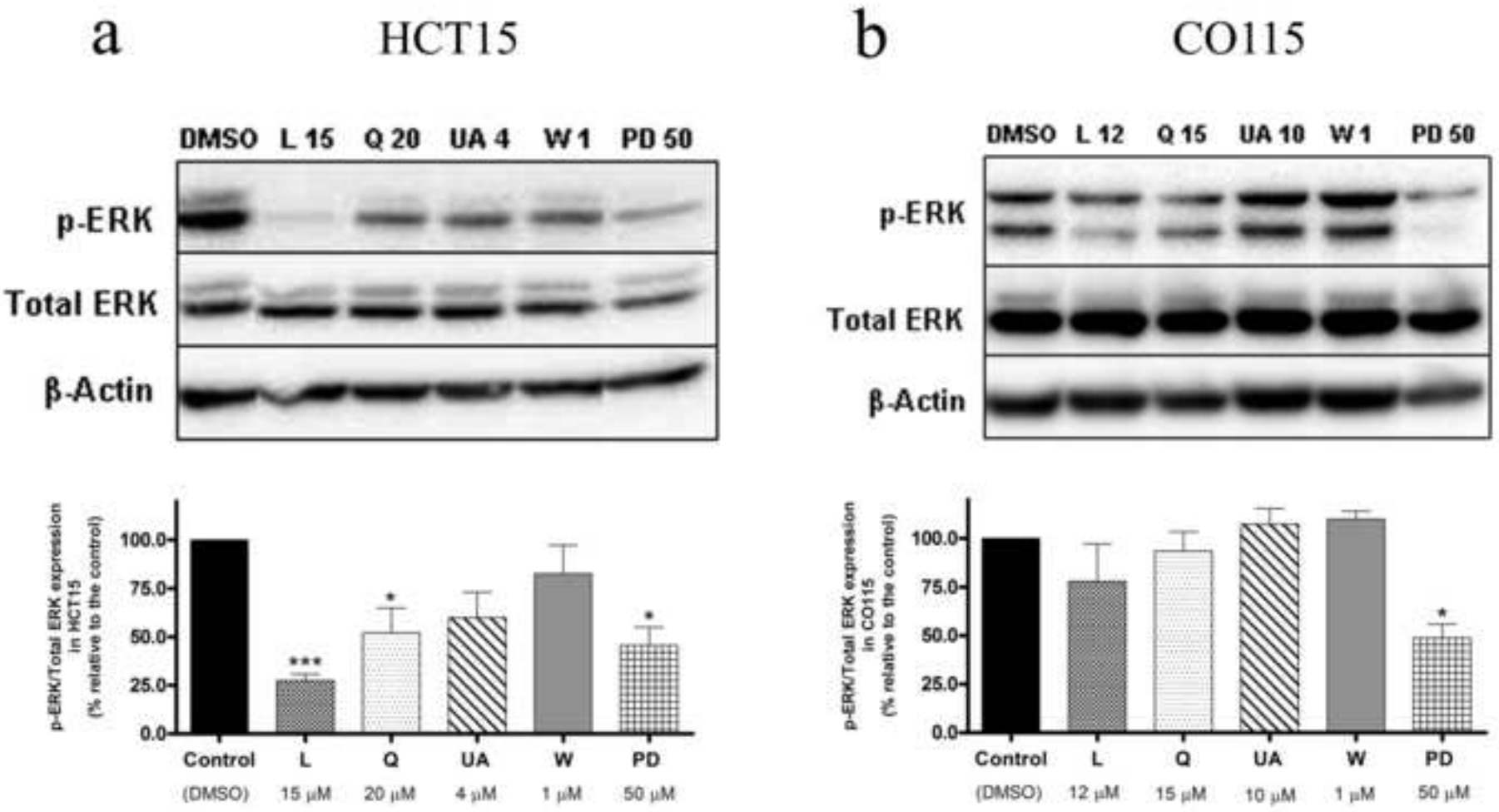

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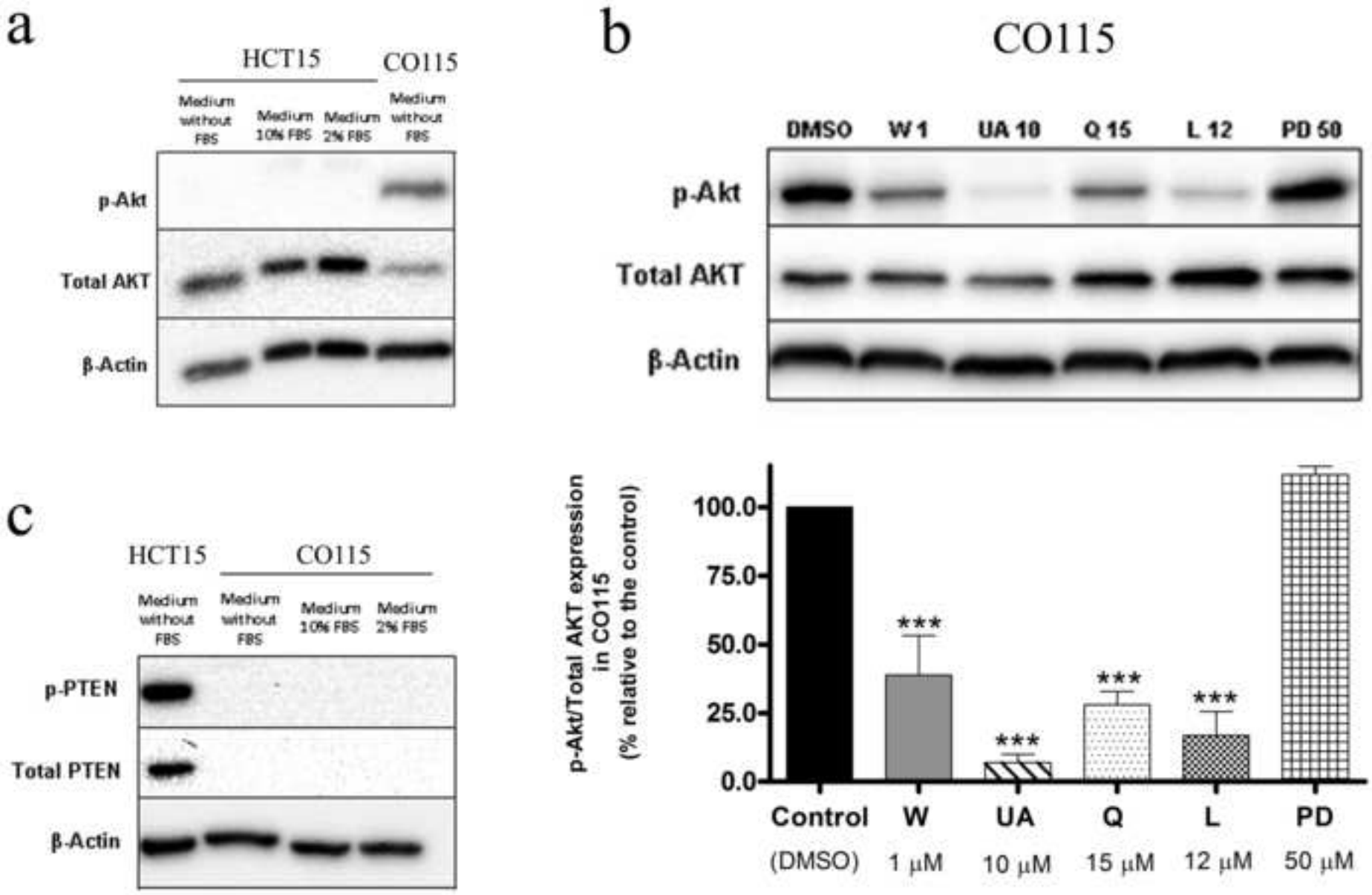

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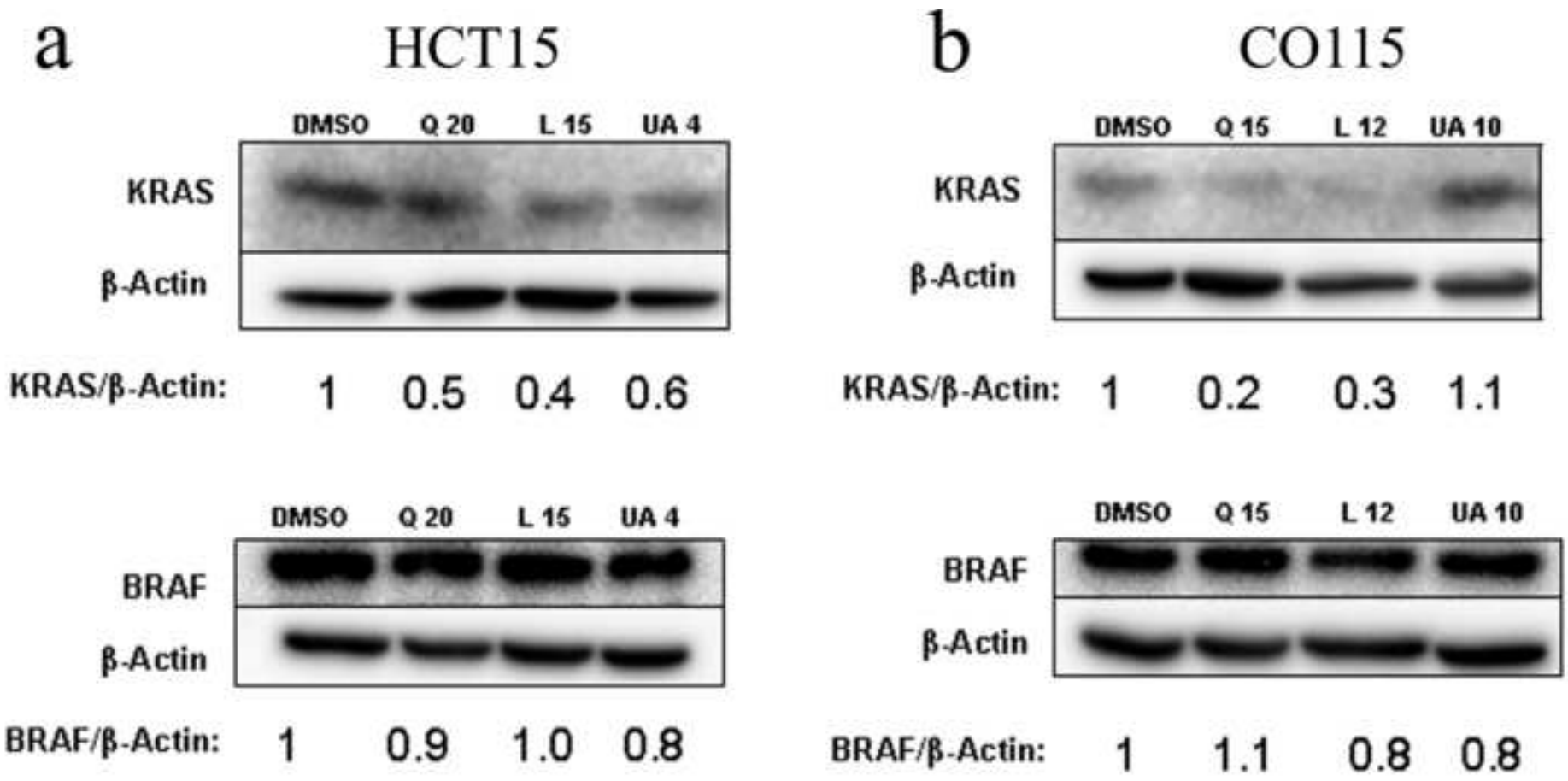

Figure 8

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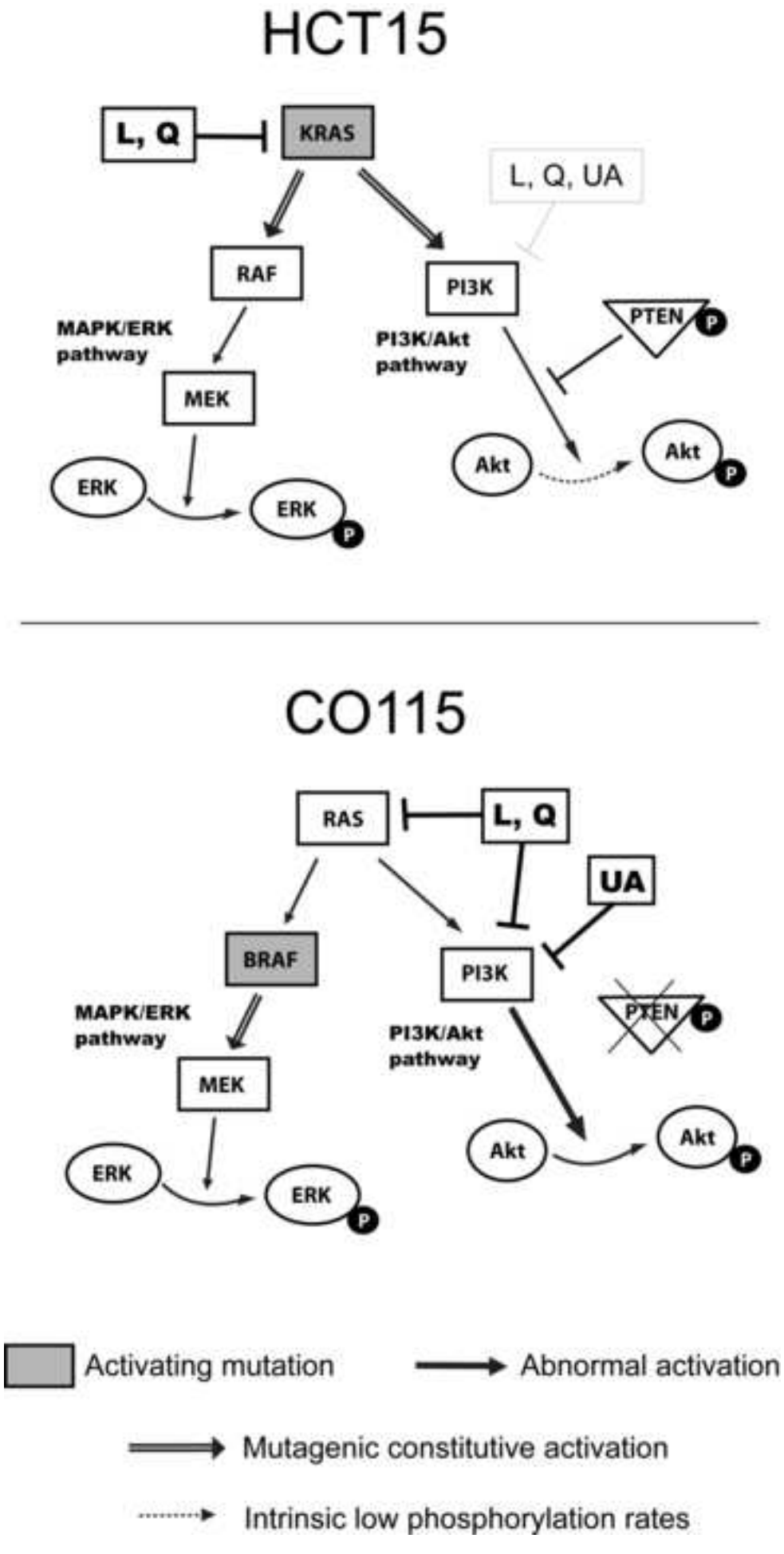

Conflicts of Interest Statement

The authors do not have conflicts of interest.