Original Articles

Phosphorylation-independent mTORC1 inhibition by the autophagy inducer Rottlerin

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Abstract

We recently found that Rottlerin not only inhibits proliferation but also causes Bcl-2- and Beclin 1-independent autophagic death in apoptosis-resistant breast adenocarcinoma MCF-7 cells. Having excluded a role for canonical ~~signalling~~signaling pathways, the current study was aimed to investigate the contribution of the AMPK/mTOR axis in autophagy induction and to search for the upstream ~~signalling~~signaling molecules potentially targeted by Rottlerin. Using several enzyme inhibitors, Western blotting analysis, mTOR siRNA and pull down assay, we demonstrate that the Rottlerin-triggered autophagy is mediated by inhibition of mTORC1 activity through a novel AMPK and mTORC1 phosphorylation-independent mechanism, likely mediated by the direct interaction between Rottlerin and mTOR.

Abbreviations: AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; p70S6K, phosphoprotein 70 ribosomal protein S6 kinase; 4EBP-1, Eukaryotic translation initiation factor 4E-binding protein 1; Bcl2, B-cell lymphoma/leukemia-2; Becl-1, Beclin-1; PP2A, protein phosphatase 2A; FKBP-12, FK-506 binding protein 12; PI3K, phosphoinositide 3-kinase; RSK, p90 ribosomal S6 kinase; SQSTM 1, sequestosome 1; LC3-II, microtubule-associated protein 1 light chain 3-II; TSC, ~~Tuberous~~tuberous sclerosis complex; CZE, capillary zone electrophoresis; PMA, phorbol 12-myristate 13-acetate

Keywords: Rottlerin; AMPK; mTOR; Raptor; Autophagy

Introduction

Autophagy, a highly regulated and dynamic process of cellular degradation, is constitutively active on a basal level helping to keep up tissue homeostasis, but it can be strongly induced in response to a multitude of physiological and

pathological situations such as amino acids or growth factors deprivation, hypoxia, pathogen infection, exposure to chemotherapeutic agents and others [1]. Although this process is induced as a protective response to stress, the prolonged activation of autophagy can lead to cell death by excessive self-degradation of essential cellular components [2].

We have previously presented evidence that purified, commercial Rottlerin kills apoptosis resistant, caspase 3-deficient MCF-7 cells through a non-apoptotic, autophagic death mechanism that was associated towith autophagosome formation, accumulation of LC3-II and degradation of SQSTM1/p62 [3].

Although many open questions on autophagy regulation still exist [4], three main control pathways have been recognized: the AMPK/mTORC1 cascade [5–7], the PI3K(I)/Akt/mTORC1 pathway and the Bcl-2 /Becl-1 (Atg6) axis [8–10].

In our earlier study, we showed that the autophagic process was Bcl-2/Becl-1-independent because MCF-7 cells express Becl-1 at very low basal levels, its expression was not induced by Rottlerin and neither Bcl-2 silencing nor overexpression affected Rottlerin-induced autophagy [3].

We also excluded a role for both PI3K/Akt and ERK/p90 ribosomal S6 kinase (RSK) regulatory pathways [11–13] in the Rottlerin-induced autophagy, since we previously found no changes (decrease) in Akt (Thr 308) and ERK phosphorylation following Rottlerin treatment of MCF-7 cells [14]. Therefore, the current study was focused on the involvement of AMPK and mTORC1 in the autophagic response of MCF-7 cells to Rottlerin.

AMPK is an energy sensor that promotes energy production and limits energy utilization to ensure cellular survival. In fact, AMPK is activated under conditions that deplete cellular ATP and elevate AMP levels, such as glucose deprivation, hypoxia, and mitochondrial uncouplers [15,16].

Recently, an mTOR-independent pathway for activation of ULK1 has been reported: direct phosphorylation of ULK1 by AMPK in response to nutrient depletion leads to ULK1 activation and autophagy induction [17–20].

Therefore, since the Rottlerin uncoupling effects have been documented in different cancer cells [21–23], we hypothesized that AMPK could be the Rottlerin target responsible for MCF-7 autophagic death. Although no literature data have so far appeared in mature cancer cells, in vascular cells and tissues [24] and in breast cancer stem cells [25], Rottlerin has been demonstrated to activate AMPK.

The current study confirmed the working hypothesis that the Rottlerin-triggered autophagy is mediated by inhibition of mTORC1, but, surprisingly, the evidence excluded any role for AMPK and suggested that Rottlerin directly interacts with mTORC1 and inhibits its activity, through a novel phosphorylation-independent mechanism.

Materials and methods

Materials

Rottlerin with purity higher than 95% was obtained from Calbiochem, San Diego, CA. MEM, FBS, antibiotics, DMSO, Okadaic acid, PMA, Temsirolimus (CCI-779), Triflouromethoxyphenylhydrazone (FCCP) and Compound C were from Sigma Aldrich, St. Louis, MO. Antibodies against total and phospho-AMPK (Thr172), total and phospho-ACC (Ser79), total and phospho-p53 (Ser15), phospho-Raptor (Ser722/Ser792), total and phospho-mTOR (Ser2448), total and phospho-p70S6K (Thr389), total and phospho 4EBP-1 (Thr 37/46), total and phospho-Akt (Ser 473), LC3 I/II and β-actin were obtained from Cell Signaling Technology, Danvers, MA. mTOR and control siRNA were from Santa Cruz Biotechnology, Santa Cruz, CA. M-PER Mammalian Protein Extraction Reagent and Halt™ Protease and Phosphatase inhibitor cocktail were from Pierce, Rockford, IL. Equipment and all reagents for protein assay and Western Blotting analysis were from Invitrogen, Carlsbad, CA. Nitrocellulose, ECL Prime Western Blotting Detection Reagent and Hyperfilm™ ECL™ were from GE Healthcare Life Sciences, Uppsala, Sweden.

Cell cultures and treatment

Human breast adenocarcinoma cell line MCF-7 (purchased by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy) werewas grown in a humidified atmosphere (95% air/5% CO_2) at 37 °C in Minimum Essential Medium (MEM) containing 10% fetal calf serum (FBS), Na-Pyruvate (1 mM), glutammin (2 mM) and antibiotics (100 U/mL ml penicillin, 100 µg/mL ml streptomycin, 250 ng/mL ml amphotericin B).

Rottlerin, dissolved in DMSO at a stock concentration of 20 mM, was stored in a dark-colored bottle at –20 °C. Before Rottlerin treatment, cells were grown to about 80% confluence and then exposed to Rottlerin diluted in MEM containing 2.5% FBS. Cells grown in the same medium containing an equal amount of DMSO without Rottlerin were used as control.

In a subset of experiments MCF-7 werewas depleted of PKCδ by prolonged exposure (24 h) to PMA, a PKC activator, before Rottlerin treatment [26]. In another subset of experiments MCF-7 werewas pre-treated for 1 h with 20 µM Compound C (an inhibitor of AMPK) or 0.1 µM Okadaic Acid (a selective inhibitor of PP2A).

Untreated, serum deprived cells were cultured in MEM diluted with PBS (1:2), without Na-Pyruvate, to check the influence of starvation on AMPK activity.

The cytotoxicity of the rapamycin analogue, CCI 779, was evaluated using the SulphorodamineSulforodamine B (SRB) colorimetric assay, as previously described [3].

To check the role of mitochondrial uncoupling on autophagy induction, cells were treated with the chemical uncoupler FCCP.

Quantitative determination of ATP and AMP by capillary zone electrophoresis (CZE)

CZE was done as previously described [23]. The electropherograms were read and ~~analysed~~ analyzed at 254 nm wavelength. The nucleotides in samples were identified by comparison of retention time with internal standards run at the same time. Concentrations of compounds were determined by using the corrected peak areas of known compound~~s~~ concentrations in a calibration curve. The method was linear in the range 2 mM–0.1 mM.

Protein extracts

After treatments, cells were washed with PBS and then lysed with 0.2 ml of M-PER Mammalian Protein Extraction Reagent containing a cocktail of protease and phosphatase inhibitors. After shaking for 10 min, the lysates were collected and transferred to 1.5-ml centrifuge tubes. Cell debris was pelleted by centrifugation at 14,000 × *g* for 15 min. The supernatants were transferred to new tubes, and the protein concentration was measured using Bio-Rad protein assay reagent.

Western blotting analysis

Equal amounts of protein samples were resolved on 6%, 12% or 4–20%, SDS-~~-~~polyacrylamide gels. Proteins were electro-transferred onto nitrocellulose membranes that were blocked in 5% non-fat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature. Then, the blots were probed with appropriate primary polyclonal antibody overnight at 4 °C. After washing, horseradish peroxidase-conjugated IgG was added for 1.5 h at room temperature. The blots were then developed by the ECL Detection Reagents and then exposed on photographic film, according to the manufacturer's instructions. The densitometry of the bands ~~were~~was performed using Image-J software.

mTOR small interfering RNA

MCF-7 cells were grown in six-well plates and transfected with siRNA (30 nM final concentration) for 24 h with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Briefly, siRNA was incubated in Opti-MEM (Invitrogen) for 5 min at room temperature. In another tube, 6 µl of Lipofectamin 2000 was diluted in 200 µl Opti-MEM and incubated for 5 min. The siRNA/Opti-MEM mix was incubated for 30 min before addition to the cells. The medium was removed after 24 hours of transfection and the cells were incubated in serum-free medium for 24 h. The cells were then collected for further experiments and cell lysates were prepared for Western blot analysis. In all siRNA mediated knockdown experiments, cells transfected with non-specific (scrambled) siRNA were used as controls.

Preparation of Rottlerin–-cyanogen bromide (CNBr)-activated Sepharose 4B complex

The procedure was performed following previous reported protocols [27]. The CNBr-activated Sepharose 4B beads (0.3 g) were swelled in 1 mM HCl for 30 min and washed in coupling buffer [0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl]. Rottlerin (1–2 mg), dissolved in 500 µl of coupling buffer was added to CNBr-Sepharose 4B beads and rotated end-over-end overnight at 4 °C. The beads were subsequently transferred to 0.1 M Tris–HCl buffer (pH 8.0) and again rotated end-over-end overnight at 4 °C. Finally, the Rottlerin-conjugated CNBr-activated Sepharose 4B was washed with three cycles of alternating low pH [0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl] and high pH [0.1 M Tris–HCl (pH 8.0) containing 0.5 M NaCl] buffers.

Ex vivo pull-down assay

For the ex vivo pull-down assay, a total of 500 µg of MCF-7 protein extract was incubated with 100 µl (50% slurry) of Rottlerin-~~-~~CNBr-conjugated Sepharose 4B (or CNBr-conjugated Sepharose 4B alone as a control) beads in reaction buffer [50 mM Tris-~~-~~HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 µg/ml bovine serum albumin, 0.02 mM PMSF, 1-~~×~~× protease inhibitor cocktail]. After incubation, with gentle rocking for 3 h at room temperature (or overnight at 4 °C), the beads were washed three times with a buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF. The proteins bound to the beads were eluted with SDS loading buffer and analyzed by Western blotting with specific antibodies.

Statistical analyses

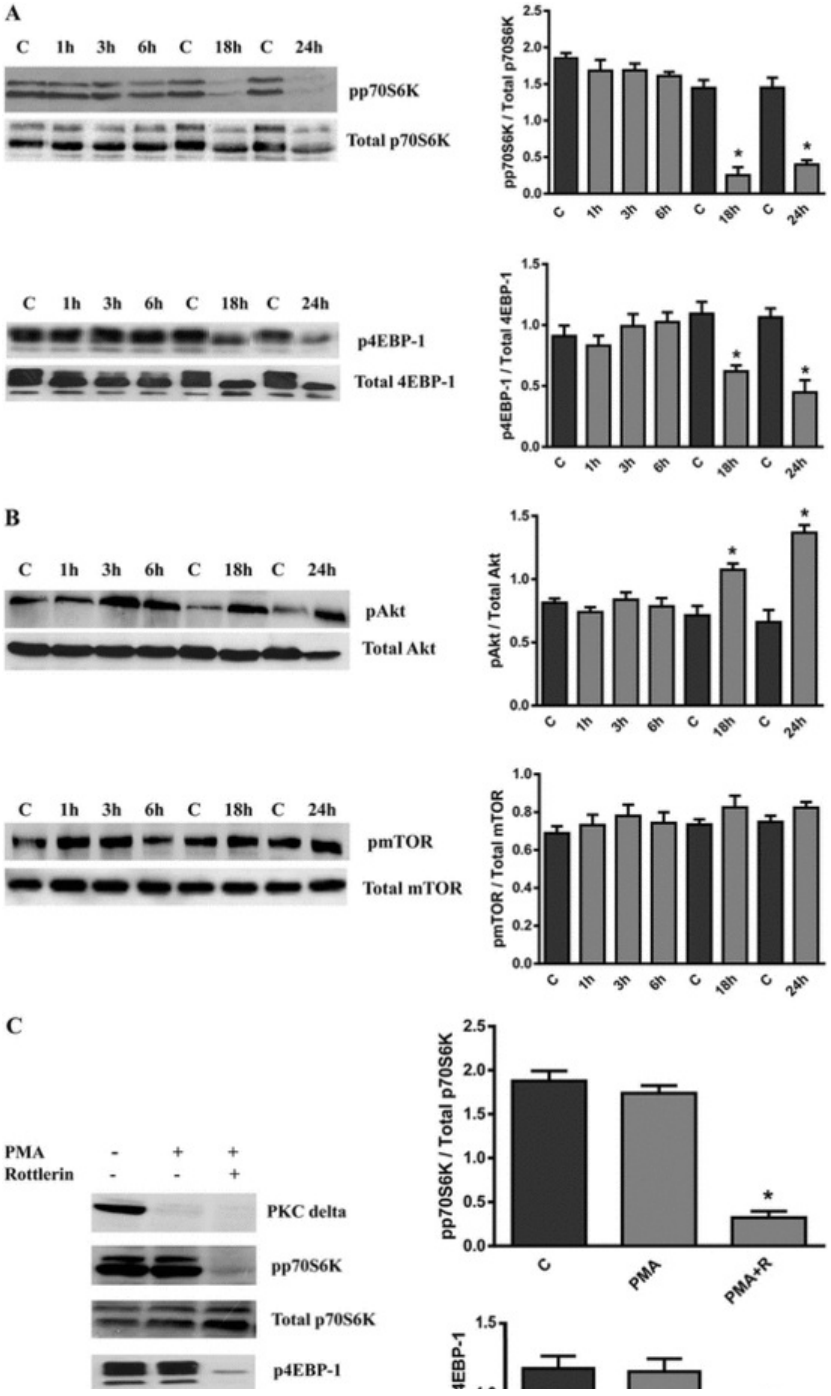
Values are expressed as the mean ± SD. Student's t test was used to determine statistical significance with a threshold of p values less than 0.05.

Results

Rottlerin inhibits mTORC1 but not mTORC2 activity

We checked the mTORC1/2 activity by monitoring the phosphorylation status of their effectors, by Western blotting analysis. As shown in Fig. 1A, 20 µM Rottlerin treatment effectively suppressed basal levels of phosphorylated p70S6K (Thr 389) and phosphorylated 4EBP-1 (Thr 37/46), targets of mTORC1 at 18 h and 24 h time points. Conversely, the phosphorylation of Akt in Ser 473 (target of mTORC2) increased upon treatment, indicating that the drug inhibits mTORC1 but not mTORC2, which, conversely, appears to be activated (Akt phosphorylation). Several studies reported that inhibition of mTORC1 by rapalogs increases mTORC2 activity through a poorly defined mechanism involving AKT activation, possibly via upregulation of receptor tyrosine kinases such

as insulin-like growth factor-1 receptor (IGF-R1). Active Akt, in turn, phosphorylates and activates mTORC2 (Ser 2448), thereby closing the loop ([28] and references therein).



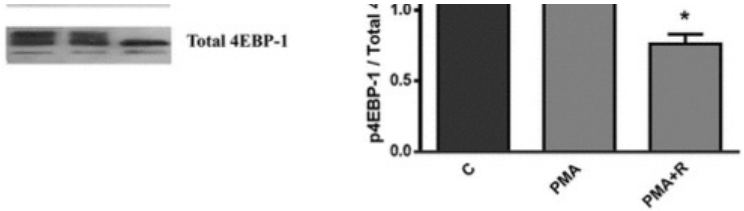


Fig. 1 Effect of Rottlerin on mTORC1 and mTORC2 activity. (A) Western blotting of total and phosphorylated p70S6K (Thr 389), total and phosphorylated 4EBP-1 (Thr 37/46); (B) total and phosphorylated Akt (Ser 473), total and phosphorylated mTOR (Ser 2448) after 20 μM Rottlerin treatment for 1–24 h; (C) Western blotting of total and phosphorylated p70S6K (Thr 389) and total and phosphorylated 4EBP-1 in PKCδ-depleted cells. The blots are representative of multiple independent experiments. Densitometry results are expressed as phospho-protein/total protein ratio. *$p < 0.05$.

In addition, consistent with Akt activation, likely mediated by the described mTORC1 feedback loop on the mTORC2/Akt axis, mTOR phosphorylation in Ser 2448, the site targeted by Akt, was slightly increased. This result, which apparently conflicts with the drop in the phosphorylated p70S6K and 4EBP-1 levels, strongly indicates that Rottlerin inhibition of mTORC1 occurs independently from Akt and phosphorylation events.

Rottlerin inhibits mTORC1 activity in a PKCδ-independent manner

Rottlerin is marketed as a selective inhibitor of PKCδ, although this designation has long been debated [29]. To clarify whether the Rottlerin effect on phosphorylated p70S6K and 4EBP-1 levels was PKCδ-dependent, cells were transiently depleted of PKCδby prolonged PMA treatment [26]. As shown in Fig. 1B, in PKCδ-depleted cells, Rottlerin retained its ability to decrease p70S6K and 4EBP-1 phosphorylation after 18 h treatment. This result excluded any PKCδ involvement in the Rottlerin outcome.

Rottlerin inhibits mTORC1 activity independently from the AMPK signaling pathway

To understand the mechanism through which Rottlerin inhibits mTORC1, the role of AMPK in this process was examined.

We first evaluated whether and, if so, how much mitochondrial uncoupling by Rottlerin increases AMP levels in non-starved MCF-7 cells. As illustrated in Table 1, in agreement with other studies [21], a drop in ATP levels was observed after 20 min, followed thereafter by a prompt, albeit slow, recovery, due both to compensatory metabolism and to the activity of adenylate kinase, which catalyzes the reaction 2 ADP → ATP + AMP. Indeed, the AMP levels and the AMP/ATP ratio increased early and roughly doubled within 40 min after Rottlerin treatment. This prompted us to analyze the levels of phospho-AMPK and its downstream substrate phospho-ACC. The Western blotting results showed that the phosphorylation status of AMPK and ACC in treated cells did not differ significantly from controls at any time (Fig. 2).

Table 1 Effect of Rottlerin on ATP and AMP levels. MCF-7 cells were treated with 20 μM Rottlerin and adenylates were determined by CZE. ATP and AMP concentrations were expressed as pmol/10^6 cells and the AMP/ATP ratio was calculated at the indicated time points. Values are the average of three independent experiments and are expressed as mean ± SD.

	Control	20 min	30 min	40 min
AMP	264.29 ± 26.81	353.70 ± 38.23*	467.77 ± 51.12*	475.12 ± 39.56*
ATP	2877.78 ± 264.61	830.32 ± 67.03*	1472 ± 185.23*	2308 ± 201.36*
AMP/ATP	0.092 ± 0.002	0.426 ± 0.04*	0.318 ± 0.03*	0.206 ± 0.04*

* $p < 0.05$.

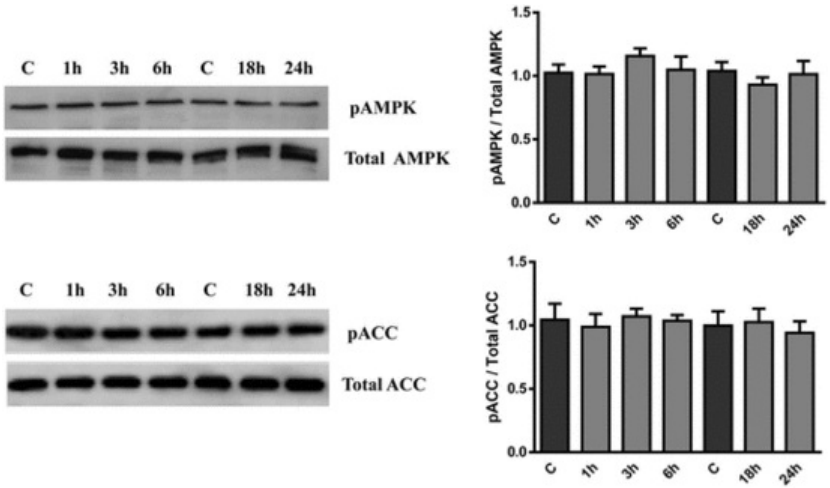


Fig. 2 AMPK-independent inhibition of mTORC1 activity by Rottlerin. Western blotting of total and phosphorylated AMPK (Thr172) and total and phosphorylated ACC (Ser79) after 20 μM Rottlerin treatment for 1–24 h. Representative of multiple independent experiments. *$p < 0.05$.

In addition, we analyzed the phosphorylation level of Raptor (Ser 722/792) and p53 (Ser 15), two sites targeted by active AMPK. The Western blotting results failed to show any phospho-Raptor and phospho-p53 bands in both control and treated cells (data not shown).

As a control, we checked the phosphorylation levels of AMPK in starved cells and found that, although the activation of phospho-AMPK and phospho-ACC occurred after 6–18 h starvation, no decrease in p70S6K and no autophagy induction (Western blotting of LC3 I/II) were observed (Fig. 3). We also measured SQSTM1/p62 levels after 18 and 24 h starvation and no decrease/degradation was found (not shown).

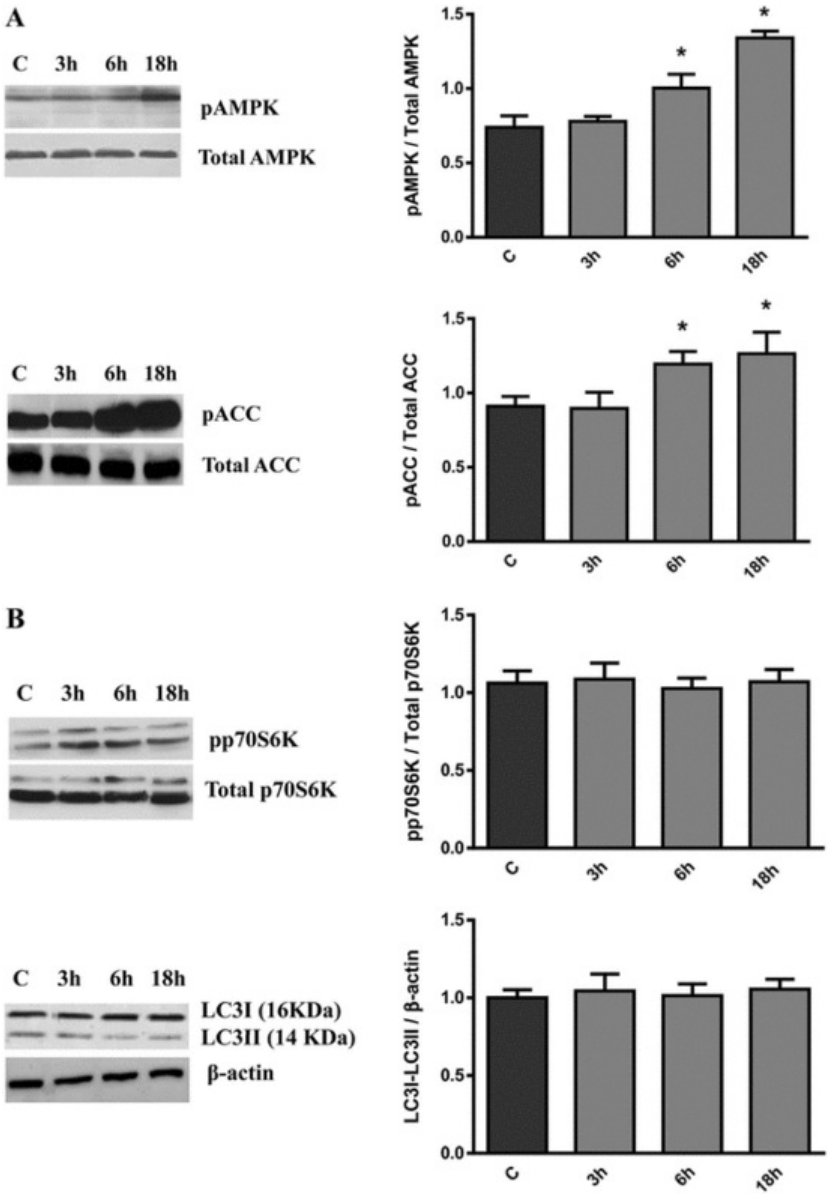


Fig. 3 Starvation-activated AMPK is not a sufficient stimulus to inhibit mTORC1 and to trigger autophagy. Western blotting of total and phosphorylated AMPK, total and phosphorylated ACC, total and phosphorylated p70S6K and LC3 I/II after 1–18 h starvation. β-actin was used as loading control. Representative of two independent experiments. *$p < 0.05$.

These results indicate that starvation-activated AMPK alone is not enough to inhibit mTORC1 and to trigger autophagy in MCF-7 cells.

To further exclude the involvement of AMPK in the mTORC1 inhibition by Rottlerin, we checked the phosphorylation level of p70S6K in the presence of an AMPK inhibitor (compound C). As expected, compound C decreased AMPK and ACC phosphorylation levels (Fig. 4A) but did not revert the inhibitory effect of 20 μM Rottlerin treatment for 18 h on p70S6K phosphorylation and, in addition, the same inhibitory effect was obtained even with 5 μM Rottlerin. (Fig. 4B).

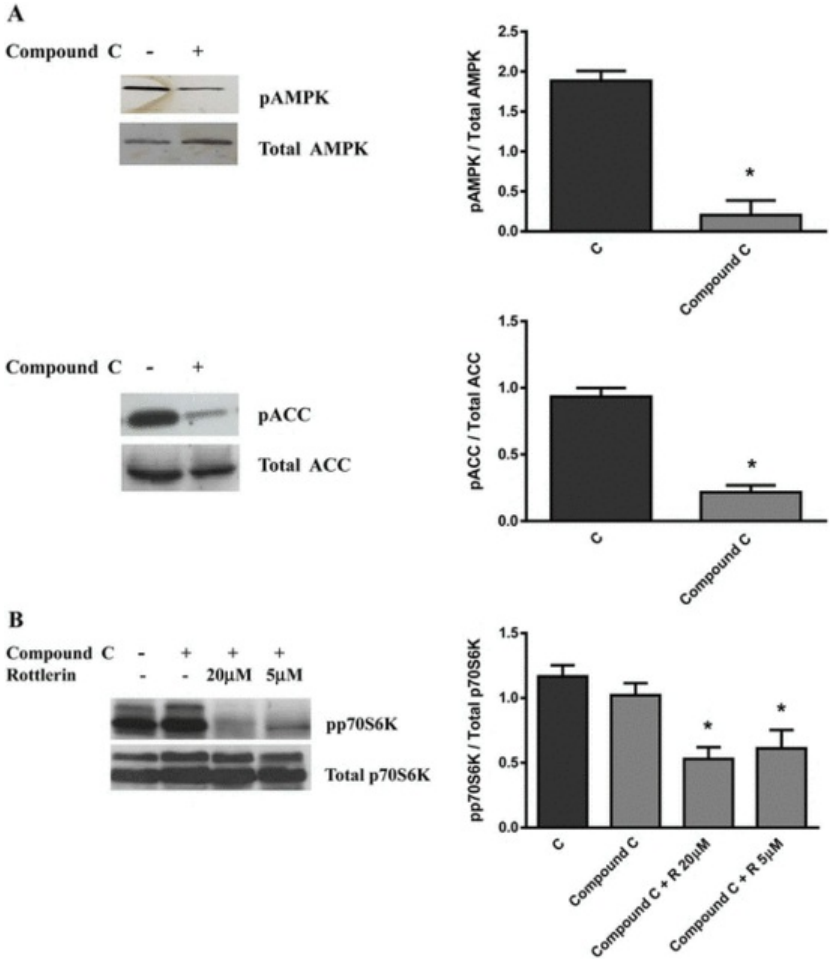


Fig. 4 AMPK-independent inhibition of p70S6K phosphorylation by Rottlerin. (A) Western blotting of total and phosphorylated AMPK (Thr172) and total and phosphorylated ACC (Ser79) in MCF-7 cells pre-treated with 20 μM compound C for 30 min and then cultured for an additional 20 h in the presence of Rottlerin. (B) Western blotting of total and phosphorylated p70S6K (Thr 389) after 5 and 20 μM Rottlerin treatment for 24 h in the presence of the AMPK inhibitor, compound C. Representative of two independent experiments. *$p < 0.05$.

Together, these data unequivocally demonstrated that Rottlerin inhibits mTORC1 signaling independently from the known AMPK signaling pathways.

Rottlerin induces autophagy independently from the mitochondrial uncoupling

Since inhibitors of the mitochondrial electron transport chain are autophagy inducers [30] and Rottlerin has been previously described to induce autophagy via mitochondrial uncoupling [22], we tried to mimic the Rottlerin autophagic effect by the use of the mitochondrial uncoupler FCCP. As shown in Fig. 5A, FCCP failed to increase the lipidated form of LC3, further indicating that autophagy is not triggered by the Rottlerin mitochondria targeting in MCF-7 cells. Conversely, FCCP treatment resulted in nuclear chromatin condensation, typical of apoptosis, evidenced by DNA staining with Hoechst 33342, performed as previously described [3] (Fig. 5B).

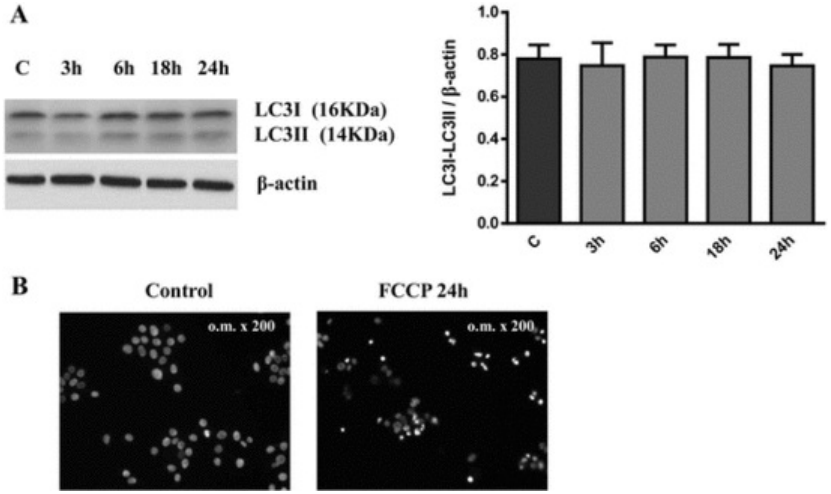


Fig. 5 Mitochondrial uncoupling-independent induction of autophagy. (A) Western blotting analysis of the autophagosomal marker LC3I-II after 20 μM FCCP treatment for 3–24 h. β-actin was used as loading control. Representative of two independent experiments; (B) ~~Nuclear~~nuclear staining with Hoechst 33342 after 24 h of exposure to 20 μM FCCP.

Rottlerin inhibits the mTOR signaling pathway independently from PP2A phosphatase activation

Protein phosphatase 2A (PP2A) is a trimeric complex consisting of a catalytic subunit (C), and the regulatory subunits A and B. PP2A not only regulates mTOR phosphorylation but is also involved in the direct dephosphorylation and inactivation of p70S6K [31]. Therefore, to explore if PP2A plays a role in the Rottlerin-induced p70S6K dephosphorylation, MCF-7 cells were pretreated with ~~the~~ okadaic acid, a potent PP2A inhibitor, for 20 min. Then, the cells were treated with 20 μM Rottlerin for 18 h and the cell lysates analyzed by Western blotting. As expected, inhibition of PP2A by okadaic acid enhanced the mTORC1-induced phosphorylation of p70S6K. Nevertheless, PP2A inhibition did not prevent the inhibitory effect of Rottlerin on p70S6K phosphorylation (Fig. 6). This result indicates that Rottlerin inhibits mTOR signaling independently from PP2A activation.

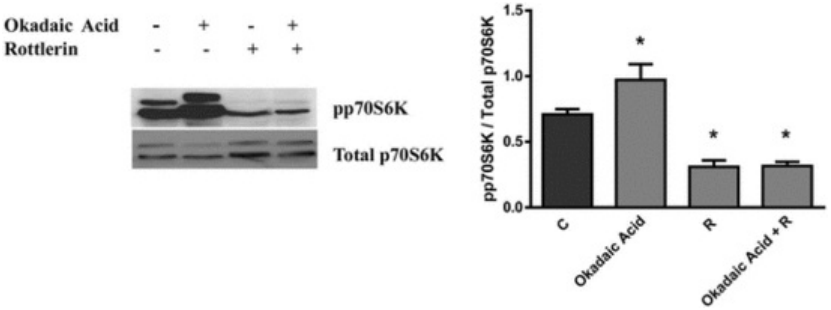


Fig. 6 PP2A-independent inhibition of mTORC1 activity. Western blotting of total and phosphorylated p70S6K (Thr 389) after 20 μM Rottlerin treatment for 18 h in cells pre-treated with the PP2A inhibitor, okadaic acid (0.1 μM). Representative of two independent experiments. *$p < 0.05$.

Cause–effect relationship between mTORC1 inhibition and autophagic cell death

To confirm that the mTORC1 pathway is the main regulator of autophagy in MCF-7 cells and that the blockage of mTORC1 stimulates the autophagic flux, independently from upstream signaling molecules, the rapamycin analogue CCI 779 was used, in parallel to Rottlerin. As shown in Fig 7A, a strong and comparable inhibition of p70S6K and 4eBP-1 phosphorylation was observed in both treatments after 24 h, accompanied by an increase of lipidated LC3II form and cell death (Fig. 7B).

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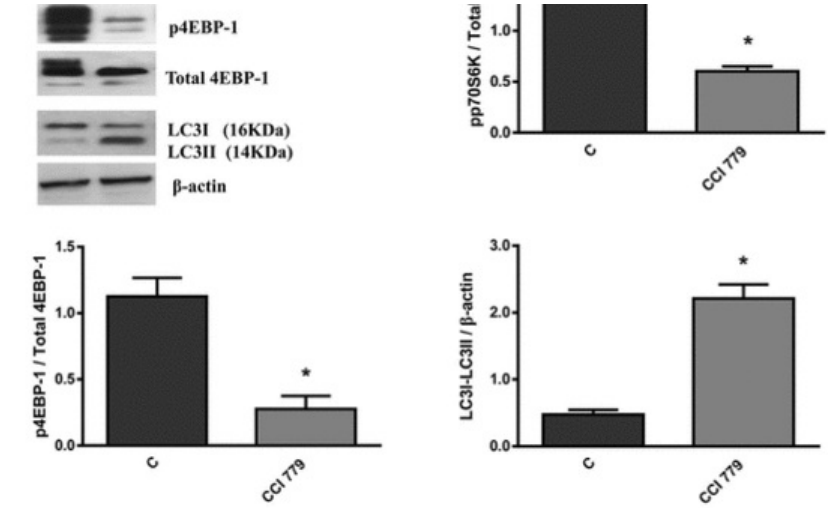

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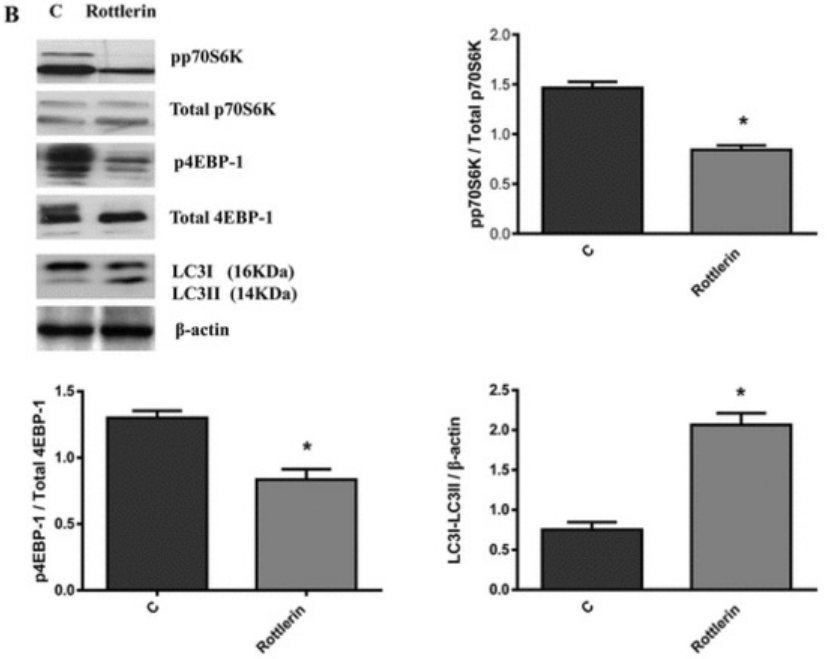

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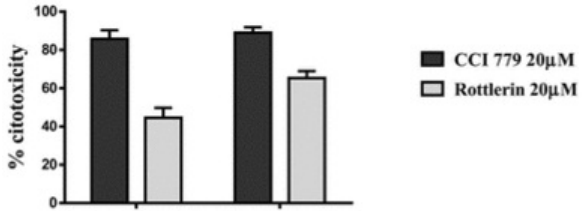
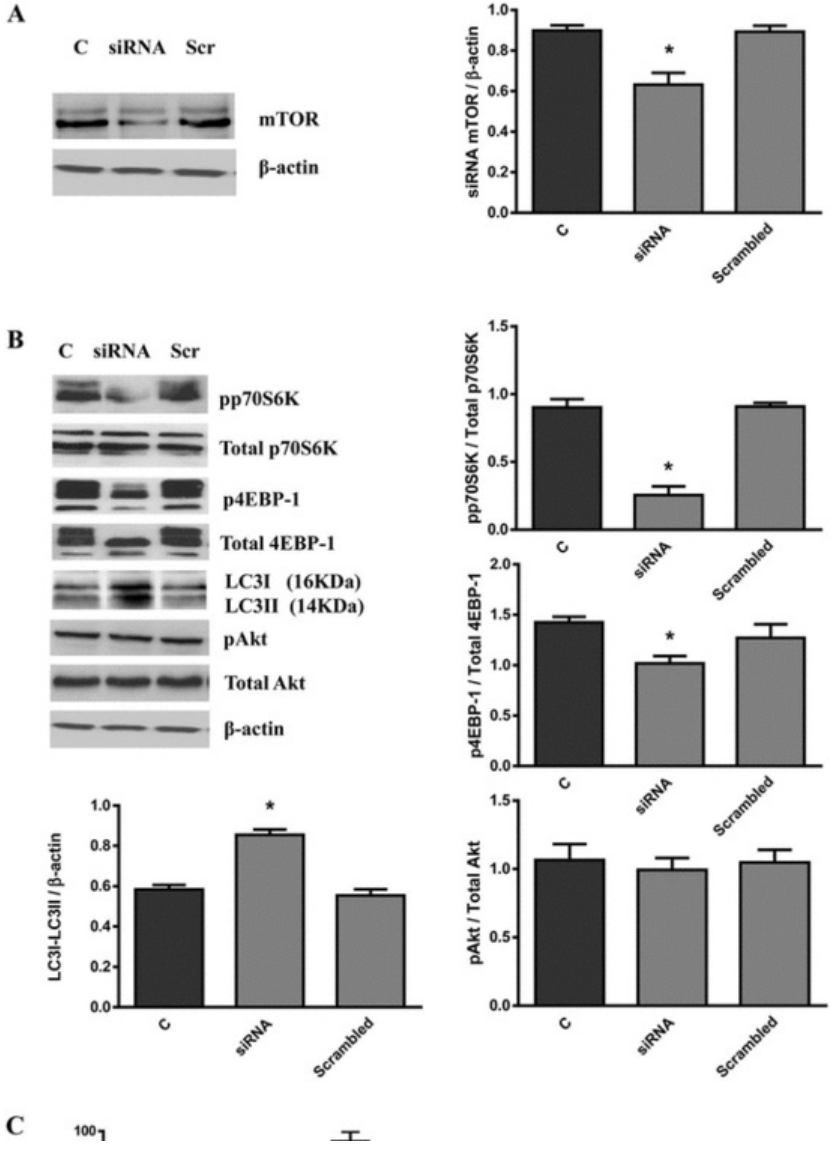


Fig. 7 Cause–effect relationship between mTORC1 inhibition and autophagic cell death. (A) Western blotting of total and phosphorylated p70S6K (Thr 389), total and phosphorylated 4EBP-1 (Thr 37/46) and LC3I/II after 20 μM CCI 779 and 20 μM Rottlerin treatment for 24 h. β-actin was used as loading control; (B) SRB assay after 20 μM CCI 779 and 20 μM Rottlerin treatment for 24 h; % Cytotoxicity: (100 × (Cell control − Experimental)/Cell control. Representative of three independent experiments. *$p < 0.05$.

To examine if mTOR silencing has the same impact of CCI779 on mTOR targets and cytotoxicity, cells were transfected with mTOR siRNA. As shown in Fig. 8A, depletion of mTOR was associated with decreased phosphorylation of the mTOR downstream targets, p70S6K and 4EBP1, increased LC3II levels and cell death. These findings confirm the cause–effect relationship between mTORC1 inhibition/silencing and autophagic death in MCF-7 cells. To determine if the combination of siRNA against mTOR with Rottlerin has a collaborative cytotoxic effect, cells transfected with siRNA against mTOR for 24 h were exposed to 2 and 20 μM Rottlerin for a further 48 h. The results of the SRB assay showed that the combination of mTOR silencing with Rottlerin has an additive effect.



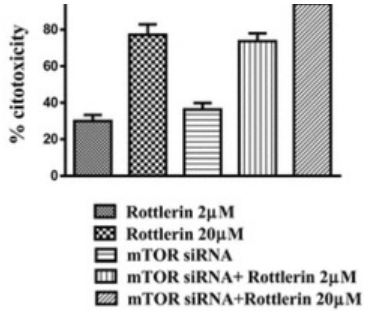


Fig. 8 Effect and efficiency of mTOR siRNA mediated knockdown. (A) Shown is a representative Western Blot with mTOR, p70S6 and phospho-p70S6, 4EBP-1 and phospho-4EBP, LC3 and Akt and phospho-Akt specific antibodies. (B) SRB assay after 2 and 20 μM Rottlerin treatment for 48 h of mTOR silenced MCF-7 cells; % Cytotoxicity: (100 × (Cell control − Experimental)/Cell control. Representative of three independent experiments. *$p < 0.05$.

Indeed, as shown in Fig. 8B, the cytotoxic effect of Rottlerin was enhanced by the combination with mTOR siRNA and this effect was significant compared with Rottlerin alone or mTOR siRNA alone. Of note, the same cytotoxic effect of 20 μM Rottlerin alone can be achieved with 2 μM Rottlerin in mTOR depleted cells.

Moreover, as it was expected, while mTOR inhibition by Rottlerin activated Akt (Fig. 1A), mTOR siRNA did not affect the phosphorylation of this upstream signaling molecule (Fig. 8A).

Rottlerin binds to mTOR

Incubation of the MCF-7 lysate with 2 mg Rottlerin-coupled Sepharose for 3 h at room temperature resulted in a tight Rottlerin/mTOR complex. As shown in Fig. 9, mTOR was pulled down by Rottlerin–Sepharose 4B beads, but not by Sepharose 4B beads used alone as negative control. According to the recommended procedure [32], we also observed that the binding was specific for mTOR, both in the phosphorylated and non-phosphorylated forms, since no binding occurred between Rottlerin and p53 used as negative protein. The same qualitative results were obtained with 1 mg Rottlerin-coupled Sepharose and overnight incubation at 4 °C (not shown).

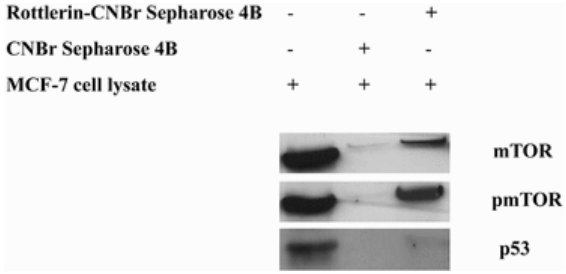


Fig. 9 Rottlerin selectively binds to mTOR. Whole cell lysates were incubated with Rottlerin-conjugated CNBr-activated Sepharose 4B beads. After precipitation and elution in SDS, the levels of bound mTOR, phosphorylated-mTOR and p53 were monitored by Western blot analysis. Representative of three independent experiments.

Discussion

In recent years, many studies have reported the anticancer potential of Rottlerin in different cancer cell models. Rottlerin has been shown to affect multiple signaling pathways and to inhibit cell proliferation [33], invasion [34–36] and angiogenesis [37].

In addition, Rottlerin controls different genes involved in cell death pathways. Although Rottlerin-promoted cell death has been mainly ascribed to apoptosis [38,39], other studies have described autophagy induction in different cancer cells through different mechanisms [22,40,41].

In an earlier study [3], we found that Rottlerin induced a non-canonical Bcl-2/Beclin 1-independent autophagic death in non-starved MCF-7 cells, but the precise mechanism was not fully understood. Since the knowledge of the mode of action of a potential anticancer agent provides helpful information for its future use, we further investigated on the molecular mechanism underlying the observed Rottlerin effect.

Knowing that the Akt–mTOR is the major pathway that negatively regulates the autophagic process, we first determined the activity of mTORC1 and mTORC2 by monitoring the phosphorylation status of their substrates, p70S6K at

Thr389, p4EBP-1 at Thr37/46 and Akt at Ser473, respectively. The decreased phosphorylation of the mTORC1 targets that was observed both in cells expressing functional PKCδ and in cells where PKCδ was transiently downregulated, demonstrated that Rottlerin inhibits mTORC1 in a PKCδ-independent manner. Moreover, Rottlerin, likely through feedback mechanisms triggered by mTORC1 inhibition [28], increased Akt (Ser 473) and mTORC1 (Ser 2448) phosphorylation, which, however, did not result in enhanced mTORC1 kinase activity and autophagy inhibition thereby demonstrating the irrelevance of mTORC1 phosphorylation events in the presence of Rottlerin.

The key role played by mTORC1 inhibition in autophagy induction in MCF-7 cells was demonstrated by the use of CCI 779 and mTOR siRNA, which exhibited roughly the same potency in inhibiting the phosphorylation of mTORC1 substrates, increasing LC3II levels and inducing cytotoxicity.

Moreover, we found that mTOR siRNA and Rottlerin work in synergy, since the Rottlerin-induced cytotoxic effect was enhanced in mTOR silenced cells. This result could also be ascribed to the lack of Akt activation following mTOR siRNA, due to the concomitant inhibition of both mTORC1 and mTORC2.

The next step was to identify the molecule responsible for the inhibition of the mTORC1 signaling and then induction of autophagy. Although AMPK was the most likely candidate, the results revealed that Rottlerin, despite the rise in AMP and AMP/ATP ratio, did not affect AMPK activity, but was able to block p70S6K phosphorylation even in the presence of the AMPK inhibitor compound C.

The finding that starvation-activated AMPK neither decreased p70S6K phosphorylation nor induced LC3 I/II, provided a further evidence that AMPK plays an irrelevant role in regulating mTORC1 activity and autophagy in MCF-7 cells.

We also considered the Ser/Thr protein phosphatase PP2A as a potential target of Rottlerin, because PP2A has been reported as a major negative regulator of p70S6K and a major target of mTORC1. In fact, mTORC1 increases p70S6K (and 4EBP1) phosphorylation both directly and via inhibition of PP2A [42]. The result excluded PP2A as a Rottlerin target since the PP2A inhibitor, okadaic acid, had no effect on the Rottlerin-induced inhibition of p70S6K phosphorylation.

Therefore, in the light of these results, we concluded that Rottlerin likely inhibits mTORC1 by phosphorylation-independent mechanisms.

Relevant precedents already exist for this phenomenon. For example, Rapamycin, which has been used in this study solely to establish a cause-effect relationship between mTORC1 inhibition and autophagy induction, inhibits mTORC1 activity by an allosteric mechanism. In fact, it is well known that Rapamycin, by forming complexes with FKBP-12, causes Raptor dissociation and inhibition of mTORC1 kinase activity. Further examples of allosteric inhibitors of mTORC1 are curcumin [43] and fenretinide [44].

Our ex vivo pull-down assay, demonstrating that Rottlerin binds to mTOR, suggested an allosteric mechanism of action that might be responsible for the observed inhibition of mTORC1 kinase activity. The finding that Rottlerin also binds to phosphorylated mTOR, further supports the conclusion that mTOR inhibition occurs independently from upstream phosphorylation events. In addition, based on the different effect of Rottlerin on mTORC1 and mTORC2 activityactivities, it can be hypothesized that the drug affects the binding between mTOR and its partner FKBP-12. Although further studies are needed to confirm the functional impact of this direct interaction, the finding raises the possibility that Rottlerin, by direct binding, might perturb a variety of signallingsignaling pathways, which could help explain-ing the pleiotropic activities of this compound [29]. Docking studies and pull down assays have shown that other natural polyphenols are able to modulate many target molecules, both protein kinases [32] and non-kinase proteins [45–47] by direct interaction.

Nevertheless, only another natural polyphenol, isoangustone A, isolated from the licorice root, has been demonstrated, by pull down assay, to directly bind mTOR [48].

Interestingly, like Rottlerin, this novel flavonoid exhibits antitumor properties in different cancer cells [49].

In closing, by combining our results and the current knowledge, we hypothesize that Rottlerin induces autophagy in MCF-7 cells by directly inhibiting mTORC1, in a PKCδ-, ERK-, PI3K/AKT-, Bcl2/Beclin- and AMPK-independent fashion. The identification of mTORC1 as a novel target of Rottlerin sheds new light on the labyrinth of the Rottlerin-modulated signaling pathways and implicates that the drug may represent a potential new class of agents for the treatment of cancers where the mTORC1 signaling plays a crucial role.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Highlights

- Rottlerin inhibits proliferation and causes autophagic death in apoptosis-resistant MCF-7 breast cancer cells.
- Autophagy is induced by PKCδ-, PP2A, - ERK-, PI3K/AKT-, Bcl2/Beclin- and AMPK–independent mTORC1 inhibition.
- Rottlerin inhibits mTORC1 but not mTORC2 activity.
- Ex vivo pull-down assay (Rottlerin–conjugated Sepharose 4B beads) demonstrated that Rottlerin directly binds mTOR and likely disturbs its interaction with FKBP-12/raptor.

Queries and Answers

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Query: Highlights should only consist of 125 characters per bullet point, including spaces. The last highlight provided is too long; please edit it to meet the requirement.
Answer: Ex vivo pull-down assay demonstrated that Rottlerin directly binds mTOR.

Query: Please provide the grant number for 'Istituto Toscano Tumori' if any.
Answer: Istituto Toscano Tumori, grant 2010.

Query: Please indicate where the closing parenthesis should be in the equation "(100 × (Cell control – Experimental)/Cell control" in the legend of Fig. 7.
Answer: Please, delete the first parenthesis

Query: Please indicate where the closing parenthesis should be in the equation "(100 × (Cell control – Experimental)/Cell control" in the legend of Fig. 8.
Answer: See Q4

Query: "*$p < 0.05$" has been set as a footnote to Table 1 as per journal style.

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