Cancer Letters ■■ (2016) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing

E. Daveri [a], E. Maellaro [b], G. Valacchi [c], F. Ietta [a], M. Muscettola [d], E. Maioli [a,*]

[a] Department of Life Sciences, University of Siena, via Aldo Moro 7, 53100 Siena, Italy
[b] Department of Molecular and Developmental Medicine, University of Siena, Via Aldo Moro 7, 53100 Siena, Italy
[c] Department of Biology and Evolution, University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
[d] Department of Medicine, Surgery and Neuroscience, University of Siena, Strada delle Scotte 4, 53100 Siena, Italy

ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form 16 June 2016
Accepted 18 June 2016

Keywords:
Rottlerin
AMPK
mTOR
4EBP-1
Autophagy
Protein synthesis

ABSTRACT

Earlier studies demonstrated that Rottlerin exerts a time- and dose-dependent antiproliferative effect on SK-Mel-28 melanoma cells during 24 h of treatment, but cytotoxicity due to cell death began only after a 48 h exposure. In the current study, in order to identify the type of cell death in this cell line, which is notoriously refractory to most anticancer therapies, and to clarify the underlying mechanisms of this delayed outcome, we searched for apoptotic, necrotic/necroptotic and autophagic traits in Rottlerin-exposed cells. Although SK-Mel-28 cells are both apoptosis and autophagy competent, Western blotting analysis, caspase activity assay, nuclear imaging and the effects of autophagy, apoptosis and necroptosis inhibitors, indicated that Rottlerin cytotoxicity was due to none of the aforementioned death mechanisms. Nevertheless, in growth arrested cells, the death did occur after a prolonged treatment and most likely ensued from the observed blockage of protein synthesis that reached levels expected to be incompatible with cell survival. From a mechanistic point of view, we ascribed this effect to the documented inhibition of mTORC1 activity; mTORC1 inhibition on the one hand led to a not deadly, rather protective autophagic response but, on the other hand caused a near complete arrest of protein synthesis. Interestingly, no cytotoxicity was found towards normal skin fibroblasts, which only resulted mildly growth arrested by the drug.

Introduction

We recently presented evidence that the natural polyphenol Rottlerin is a potent autophagy inducer. In apoptosis-resistant MCF-7 breast cancer cells, the treatment, at cytotoxic doses, led to autophagic cell death [1]. Autophagy induction was AMPK- and Beclin-1- independent and mediated by direct inhibition of mTORC1 [2], a central controller of several essential cellular functions, the best understood of these being the inhibition of autophagy and the positive regulation of protein synthesis [3].

Autophagy, an important cellular homeostatic process necessary to maintain cell survival under various stresses and changes in intracellular and environmental conditions, functions as a scavenger for misfolded proteins and damaged organelles and/or as a defense against microbes and parasites. Defective autophagy, indeed, is associated with various human pathologies, such as cardiomyopathy, neurodegeneration, autoimmune diseases, infections and cancer [4]. In human cancer, autophagy has become a potential target for pharmacological interventions because it can be also a mechanism of non-apoptotic cell death (type II programmed cell death); excessive autophagy in fact may destroy essential intracellular molecules and structures to a level incompatible with cell life.

It is generally believed that cancers with constitutively activated autophagy, such as SK-Mel-28 melanoma cells used in the current study [5], are profoundly dependent on this process for tumorigenic growth and survival; therefore they should be treated with autophagy-inhibiting drugs, as in the case of pancreatic cancers treated with the autophagy blocker chloroquine (CQ) [6]. Pancreatic cancer, however, also responds to an autophagy inducer, such as gemcitabine, indicating that when autophagy is pharmacologically stimulated beyond a tolerable threshold, it could contribute to cancer cell death even in cells showing elevated basal levels of autophagy [7]. At any rate, because of the 'yin–yang' role of autophagy in survival *versus* cell death, autophagy inducers (Rottlerin included) should be used with caution in anticancer therapies.

Abbreviations: RPMI, Roswell Park Memorial Institute (RPMI); Becl-1, beclin-1; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; mTOR, mammalian target of rapamycin; raptor, regulatory-associated protein of mTOR; p70S6K, p70 ribosomal protein S6 kinase; p90RSK, p90 ribosomal S6 kinase; LKB1, liver kinase B1; SQSTM 1, sequestosome 1; LC3-II, microtubule-associated protein 1 light chain 3-II; PARP, poly(ADP-ribose) polymerase; Z-VAD- FMK, benzyloxycarbonyl-ValAla-Asp (OMe) fluoromethylketone; CQ, chloroquine; 3-MA, 3-methyladenine; Nec-1, necrostatin-1; TCA, trichloroacetic acid; SRB, sulforhodamine; PMSF, phenylmethylsulfonyl fluoride; LDH, lactate dehydrogenase.

* Corresponding author. Tel.: +39 0577 234227; fax: +39 0577 234219.
E-mail address: emanuela.maioli@unisi.it (E. Maioli).

http://dx.doi.org/10.1016/j.canlet.2016.06.018
0304-3835/

However, the recent literature has revealed that Rottlerin is a multitarget drug, able to interfere with both the apoptotic and the autophagic pathways and to exert, as a single agent or as part of combined therapies, a successful anticancer action *in vitro* in a variety of tumor cells [8].

In our earlier study, we demonstrated that Rottlerin exhibited a marked antiproliferative effect on SK-Mel-28 cells during 24 h of treatment, but cytotoxicity, culminating in cell death, occurred only after a 48 h exposure [9]. In order to identify the type of cell death and to clarify the underlying mechanisms of this delayed outcome, we searched in the current study for apoptotic, necrotic/necroptotic and autophagic traits in Rottlerin-exposed cells. A comparative cytotoxicity analysis was also performed in human skin fibroblasts, confirming the already described selectivity of Rottlerin towards cancer cells [10,11].

Materials and methods

Materials

MG132 and Rottlerin were obtained from Calbiochem, San Diego, CA. DMEM, RPMI-1640, FBS, antibiotics, DMSO, Necrostatin, Z-VAD-FMK, chloroquine (CQ), Puromycin, 3-methyladenine (3-MA), LDH assay kit and Ponceau S were from Sigma Aldrich, St. Louis, MO. Antibodies against total and phospho-AMPK (Thr172), total and phospho-ACC (Ser79), total and phospho-Raptor (Ser722/Ser792), total mTOR, total and phospho- p70S6K (Thr389), total 4EBP-1, caspase 9, caspase 8, caspase 3, PARP, Becl-1, Bcl-2, PKCδ, Livin, cyclin D1 and β-actin were obtained from Cell Signaling Technology, Danvers, MA. Antibody against SQSTM1/p62was from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against Puromycin was from Merck Millipore, Darmstadt, Germany. 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.

Cells and culture conditions

Sk-Mel-28 human melanoma cells (from ATCC) were grown and maintained in 25 cm^2 tissue culture flasks in a humidified atmosphere (95% air/5% CO_2) at 37 °C in RPMI-1640 medium, containing 10% FBS, glutamine (2 mM), and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B). Stock solutions of 20 mM Rottlerin, dissolved in DMSO (20 µM final concentration) was stored in a dark colored bottle at –20 °C. After reaching subconfluence, cells were treated with Rottlerin or vehicle (DMSO) in complete medium containing 2.5% FBS for the indicated periods. Human adult dermal fibroblasts were established from healthy volunteers, after written informed consent. The primary cultures of human skin fibroblasts were initiated from a 3 mm skin punch biopsy. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (as reported for SK-Mel-28 cells) and used between passage 3 and 7. Fibroblasts, grown to near confluence, were treated with Rottlerin or vehicle (DMSO) in complete medium containing 2.5% FBS for the indicated periods. In a subset of experiments, cells were pretreated 1 h with the following substances: 2 and 20 µM Z-VAD-FMK (pan-caspase inhibitor), 25 and 50 µM CQ, 5 mM 3-MA (autophagy inhibitors), 10 and 100 µM necrostatin-1 (necroptosis inhibitor). Then, SK-Mel-28 cells were treated with 20 µM Rottlerin for 24–48 h. In a set of experiments, Puromycin was added to the cultured cells (1 µg/ml) just 10 min before cell lysis.

Cell growth and cytotoxicity

Cell growth and cytotoxicity were evaluated by the SRB colorimetric assay, as previously described [9]. The SRB dye, in moderated acid conditions, binds stoichiometrically to basic protein amino acids; the measured optical density correlates well to cell number and is a good indicator of cell growth and/or drug cytotoxicity [12]. Cells were seeded in triplicate on 96-well plates, incubated 4–6 h at 37 °C to allow adherence, and treated with 20 µM Rottlerin for 24–48 h. Following treatment, the medium was removed and the cells were washed twice with PBS and fixed with 100 µL of cold 10% TCA. The plates were incubated at 4 °C for 30 min before being gently washed four times with tap water to remove TCA and dead cells. Then the plates were air-dried and 100 µL of SRB (0.4% w/v SRB dissolved in 1% acetic acid) was added. After 30 min of staining, unbound SRB was removed by four washings with 1% acetic acid. The plates were air-dried again, and 200 µL of 10 mM aqueous Tris base (pH 10.5) was added to solubilize the cell-bound dye. The plate was mixed for 30 min by frequently pipetting up and down to dissolve the dye completely. The optical density (OD) was recorded in a microplate spectrophotometer at 550 nm.

Western blotting analysis

Cell extracts, each containing 30–40 µg of total protein, were resolved on 8% or 12% SDS polyacrylamide gel. Proteins were electrotransferred onto nitrocellulose membranes which were blocked by 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature. Then the blots were probed with primary antibodies overnight at 4 °C. After washing, horseradish peroxidase-conjugated IgG was added for 1.5 h at room temperature. β-actin was used as a loading control. The blots were developed by the ECL reagent and exposed on photographic film.

Measurement of the caspase 3/7 activity

Caspase 3/7 enzymatic activity was measured as previously described [13]. Briefly, the cells were harvested, washed with PBS, suspended (106 cells/100 µL) in ice-cold lysis buffer (20 mM Hepes-NaOH, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 0.2% NP-40, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich)), and sonicated for 10 s (Vibracell Sonicator; amplitude 60, 25 W). The lysates were centrifuged at 14,000 × g for 15 min and the supernatants were used for determination of protein concentration through the Bradford method. 100 µg of cell lysate protein were incubated at 25 °C with 100 µM of the substrate ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-AMC, Alexis) in the assay buffer (lysis buffer, where Hepes and NP-40 are 100 mM and 0.1%, respectively). Cleavage of the fluorogenic substrate was monitored by AMC release in a fluorescence spectrophotometer (excitation/emission wavelengths: 380/460 nm) and caspase activity was expressed as arbitrary units of fluorescence (AUFs)/min/mg protein.

Morphology study in fluorescent microscopy

For analysis of apoptotic cell death, Hoechst 33342 staining was used. The dye freely crosses the plasma membrane, binds specifically to the A-T base region of DNA, and emits fluorescence. SK.Mel-28 cells, cultured on 24-well plates for 24 h and 48 h in RPMI –1640, 2.5% FBS with or without Rottlerin (20 µM), were stained with Hoechst 33342 (10 µg/mL in PBS) in the dark at room temperature for 30 min. After incubation, cells were examined at 355 nm excitation and 460 nm emission by inverted fluorescence microscopy (Nikon Eclipse TE 300, Germany). Cells were also observed under phase contrast microscope and photographed using CCD camera attached to the microscope.

Measurement of proteosynthesis

The incorporation of Puromycin, which becomes covalently linked to the C- terminus of the nascent polypeptides, was used to label newly synthesized proteins, in order to monitor protein synthesis [14]. In SK-Mel-28 cells treated for 24–48 h with 20 µM Rottlerin, Puromycin was added to the medium (1 µg/ml) just 10 min before the end of the experiment. After two washes with PBS, cells were lysed and the labeled polypeptides were visualized by Western blotting analysis using an anti-Puromycin polyclonal antibody.

LDH release assay

To detect the leakage of LDH into cell culture medium, we used a two-steps method. In the first step, LDH reduces NAD to NADH when it catalyzes the oxidation of lactate to pyruvate. In the second step, the newly synthesized NADH converts tetrazolium salt to a colored formazan product that can be specifically detected by colorimetric assay (450 nm).

Statistical analysis

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 selectively acts towards SK-Mel-28 melanoma cells

As shown in Fig. 1A, Rottlerin cytotoxicity (100 × (control – experimental) / control) towards melanoma cells, evaluated by the SRB assay, reached almost 60% after 48 h of exposure, in agreement with previous data [9]. Similar results were obtained by the Trypan Blue exclusion test (not shown). At the same time, a comparative analysis between melanoma cells and skin fibroblasts revealed a modest toxicity (6.7%) against normal cells, for the same Rottlerin dose and exposure time. The observed cytotoxicity is mainly ascribable to the Rottlerin cytostatic effect, because, as shown in Fig. 1B, fibroblast growth (% of control at time 0) decreased from 136% in untreated to 111% in treated cells after 48 h of culture. The observed growth

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

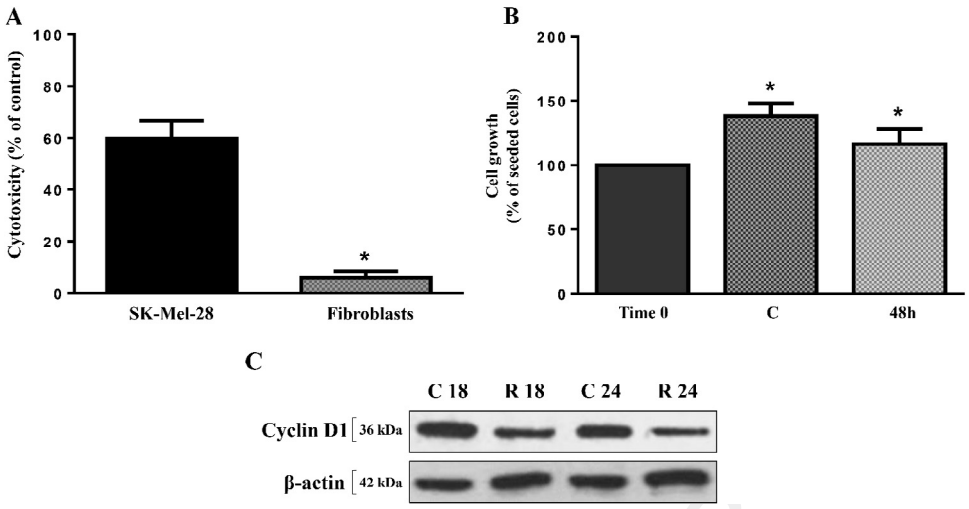

Fig. 1. Rottlerin is cytotoxic for melanoma cells but not for skin fibroblasts. (a) Rottlerin cytotoxicity towards SK-Mel-28 cells and fibroblasts after 48 h exposure, evaluated by the SRB assay. % cytotoxicity: 100 × (cell control – experimental) / cell control. *$p < 0.05$ with respect to SK-Mel-28. (b) Fibroblast survival after 48 h exposure, expressed as % of seeded cells (control). *$p < 0.05$ with respect to control. Controls were exposed to DMSO alone. Results are the means ± SD of at least three independent experiments in quadruplicate. (c) Western blot analysis of cyclin D1 after 24–48 h of 20 μM Rottlerin treatment; DMSO treated cells were used as control; β-actin was used as loading control. Representative of three independent experiments.

inhibition (19% with respect to untreated cells) can be ascribed to the observed downregulation of Cyclin D1 at 18 h and 24 h (Fig. 1C).

SK-Mel-28 melanoma cells do not die by apoptosis

Nuclear morphology examination indicated that Rottlerin neither induced chromatin condensation, excluding an apoptotic mode of cell death, nor caused other visible alterations, such as enlarged, irregular and multilobed nuclei or multiple micronuclei that are typical signs of mitotic catastrophe (Fig. 2A). Consistent with non-apoptotic cell death, no activation of caspases 3, 8 and 9 was observed, because, despite the loss of pro-caspase proteins, cleaved fragments were not found (Fig. 2B). Similarly to caspases, the PARP cleavage fragments were also absent, in spite of the loss of full-length PARP protein. Consistently, the caspase 3/7 activity showed no increase after 48 h of treatment (Fig. 3A). A further evidence of non-apoptotic death comes from the use of a pan-caspase inhibitor (Z-VAD-FMK), which failed to prevent Rottlerin-induced cytotoxicity (Fig. 3B) and loss of pro-caspase 3 protein and PARP (Fig. 3C). Interestingly, as noted in Fig. 2B, the protein levels of pro-caspases, PARP and even β-actin dramatically decreased after 48 h.

SK-Mel-28 melanoma cells do not die by autophagy

As shown in Fig. 4A, Rottlerin increased autophagic markers after 24 h and 48 h of treatment. In fact, Western blotting analysis showed that the lipidated LC3 II form increased and the p62 protein levels decreased.

In order to identify the Rottlerin mechanism of action, the major autophagic factors AMPK/mTORC1 and Beclin-1 were investigated. As shown in Fig. 4B, Rottlerin did not stimulate autophagy by Beclin-1 upregulation; rather Beclin-1 levels decreased at 24, 30 and 48 h of treatment. Conversely, Rottlerin caused AMPK activation (phosphorylation) and consequently phosphorylation of its substrates ACC (Fig. 4B) and raptor (Fig. 4C); the phosphorylation of both substrates peaking at 24 h. AMPK activation led to mTORC1 inhibition, as documented by the concomitant decrease of the phosphorylated β and γ isoforms of the mTORC1 target 4EBP-1 (Fig. 4C).

Substrates for mTORC1 also include p70S6K, which, once activated by phosphorylation, regulates protein translation and elongation. However, we did not find any phospho-p70S6K band in SK-Mel-28 cells lysate (not shown) and even the total p70S6K levels were nearly undetectable by Western blotting analysis (Fig. 4C).

Notably, a marked decrease in protein levels of AMPK, ACC and Raptor occurred at 48 h; for ACC and Raptor, such a decrease was also evident at 30 h. This protein loss makes difficult and potentially misleading any data normalization based on β-actin or phospho-protein/total protein ratio.

Although the autophagic AMPK/mTORC1 pathway was clearly activated after 24 h, autophagy is not the cause of Rottlerin toxicity because the autophagy inhibitor CQ, which blocks the late acidification steps of the process, failed to prevent Rottlerin-induced cytotoxicity (Fig. 5A). Moreover, in agreement with other studies on cancer cells [6], our results demonstrated that SK-Mel-28 cells are sensitive to autophagy blocker; CQ alone at both 25 μM and 50 μM doses exhibited around 90% cytotoxicity after 48 h. It is also evident that Rottlerin, as an autophagy inducer, counteracted the CQ action by lowering its cytotoxicity from 90% to 65% after 48 h. These results are also consistent with the absence of any effect following treatment with 5 mM 3-MA, which inhibits the class III PI3K (Vps34)/Beclin-1 complex and thus autophagosoma formation [15], in the Rottlerin cytotoxicity (Fig. 5B). Western blotting of p62 confirmed the inhibitory effects of both CQ and 3-MA, alone and in combination with Rottlerin, on the autophagic flux, although the effect of CQ was more evident than that of 3-MA (Fig. 5C).

On the whole, these results indicated that, although Rottlerin is able to further stimulate the basal autophagy of SK-Mel-28 cells, this effect is not deadly but presumably protective against toxic insults.

SK-Mel-28 melanoma cells do not die by primary necrosis/necroptosis

Primary necrosis is referred to as accidental cell death, a form of non-regulated, non-specific and uncontrolled cell death that occurs quickly as a consequence of extreme physicochemical damage. Secondary necrosis can occur as a consequence of other forms of cell death, which culminate in plasma membrane rupture. In order to evaluate necrosis, LDH activity assay has been performed on media

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

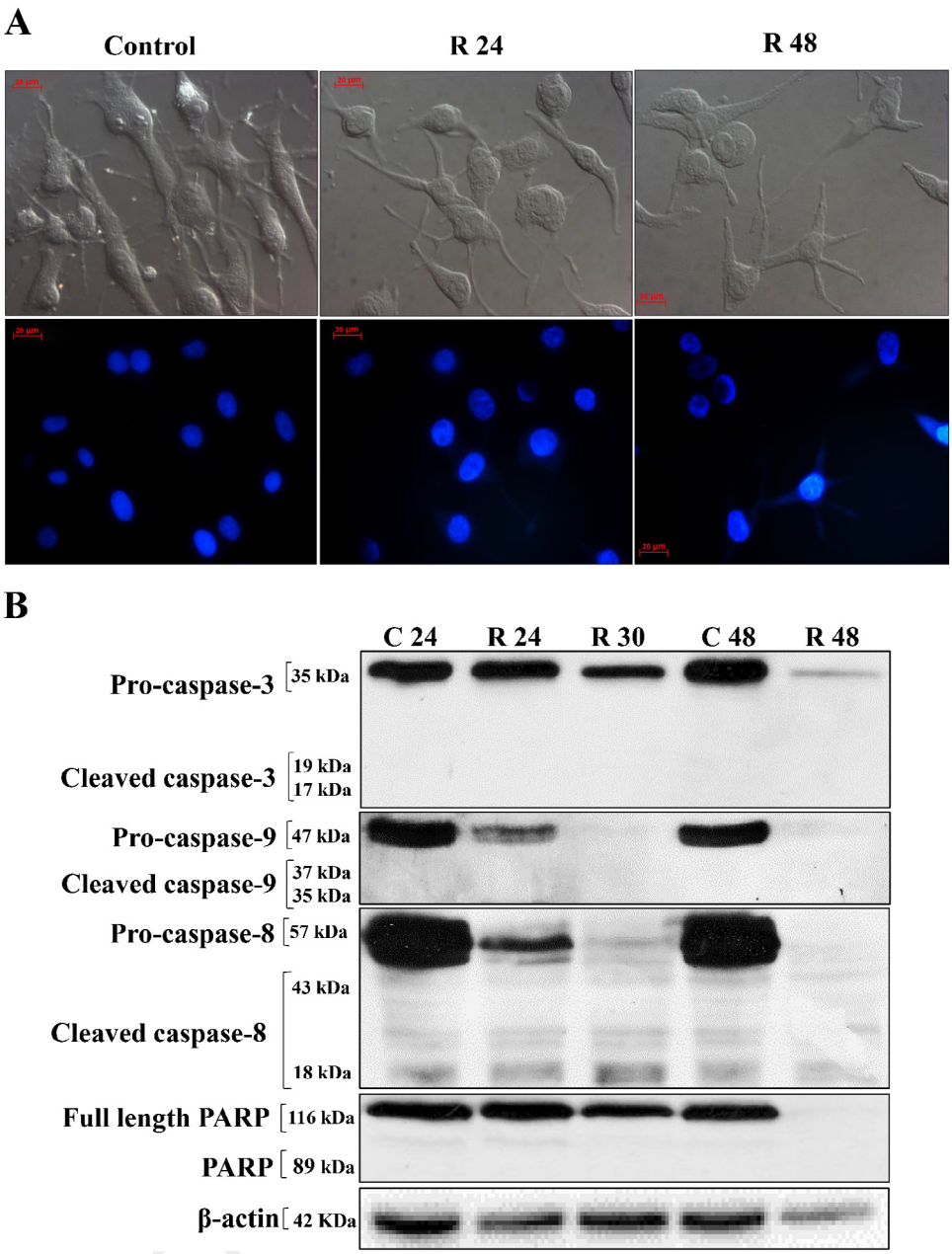

Fig. 2. Rottlerin did not induce apoptosis in SK-Mel-28 cells. (a) Nuclear staining with Hoechst 33342 after 24 h and 48 h of treatment. Controls were exposed to DMSO alone. (b) Western blot analysis of caspase 3, caspase 9, caspase 8 and PARP after 24–48 h of 20 μM Rottlerin treatment. DMSO treated cells were used as control; β-actin was used as loading control. Representative of four independent experiments.

from treated and untreated cells. Although the LDH assay does not distinguish between primary and secondary necrosis, the results of the current study, indicating plasma membrane rupture after 48 h of treatment (Fig. 5D), suggest that LDH release is the result of a programmed interplay of signaling pathways that damages the cell beyond any possible repair.

In addition to apoptosis and autophagy, necroptosis is an emerging form of programmed cell death induced by stimulation of death receptors [16]. Although necroptosis and necrosis share a few features, necroptosis exhibits a complex signaling pathway that is dependent on the key upstream kinase, the receptor interaction protein kinase 1 and 3 (RIP1 and RIP3) and can be specifically inhibited by necrostatins [17].

To answer the question of whether necroptosis plays a role in Rottlerin-induced cell death, we used the RIP1 kinase inhibitor Nec-1. As shown in Fig. 5E, pre-treatment with Nec-1 failed to rescue Rottlerin-induced cell death, indicating that the drug did not engage necroptotic pathways.

Rottlerin inhibits eIF4F complex-mediated mRNA translation

In addition to the proteins reported above, the results shown in Fig. 6A indicated that other proteins, such as mTOR, PKCδ, Bcl-2 and Livin are downregulated after 48 h of treatment. The puromycin-based assay revealed that a general inhibition of protein synthesis was already evident after 24 h of treatment and persisted at 48 h (Fig. 6 B). The global blockage of the translational machinery most probably occurred at the initiation step. Initiation indeed is the rate limiting step of mRNA translation and is commenced by the binding of the eukaryotic translation initiation factor 4F (eIF4E) complex to

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

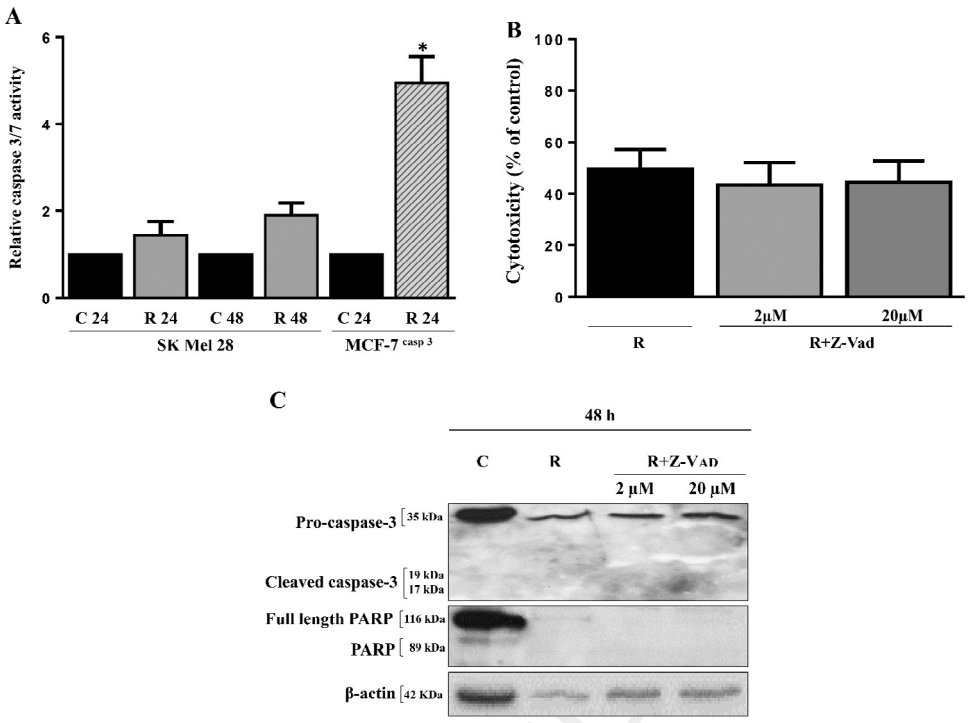

Fig. 3. Rottlerin did not activate caspase 3/7 in SK-Mel-28 cells. (a) caspase 3/7 activity after 24–48 h of 20 μM Rottlerin treatment. Caspase 3-tranfected MCF-7 cells were used as positive control. Controls were exposed to DMSO alone. (b) Rottlerin cytotoxicity after 48 h in the presence of a pan-caspase inhibitor. Results are the means ± SD of at least three independent experiments in quadruplicate and are expressed as a percentage of the control (DMSO). (c) Western blot analysis of caspase 3 and PARP after 48 h of 20 μM Rottlerin treatment in the presence of a pan-caspase inhibitor. Controls were exposed to DMSO alone. β-actin was used as loading control. Representative of three independent experiments.

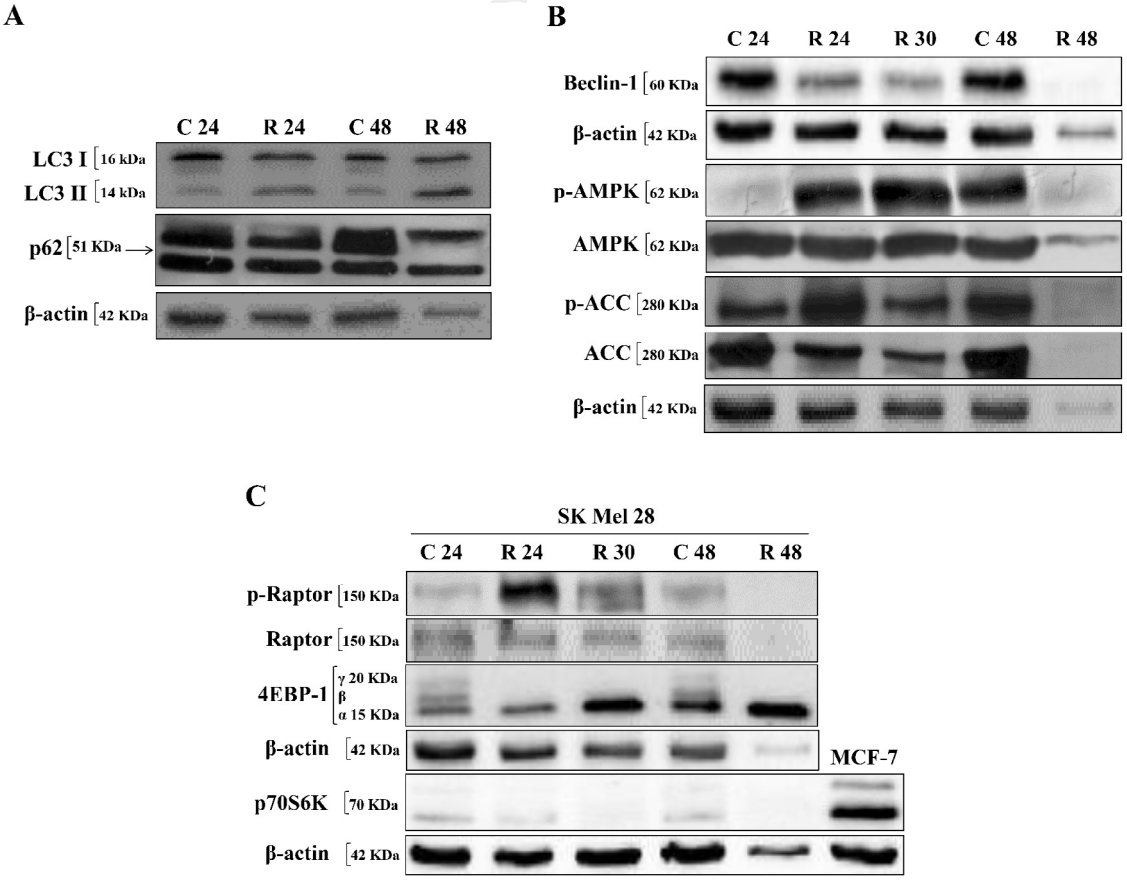

Fig. 4. Rottlerin induced autophagy. (a) Western blotting analysis of the autophagy markers LC3I-II and SQSTM1/p62, (b) Becl-1, total and phosphorylated AMPK, total and phosphorylated ACC, (c) total and phosphorylated raptor, total 4EBP-1 and total and phosphorylated p70S6K after 20 μM Rottlerin treatment for 24–48 h. MCF-7 cells lysate was used as positive control. Controls were exposed to DMSO alone. β-actin was used as loading control. Representative of three independent experiments.

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

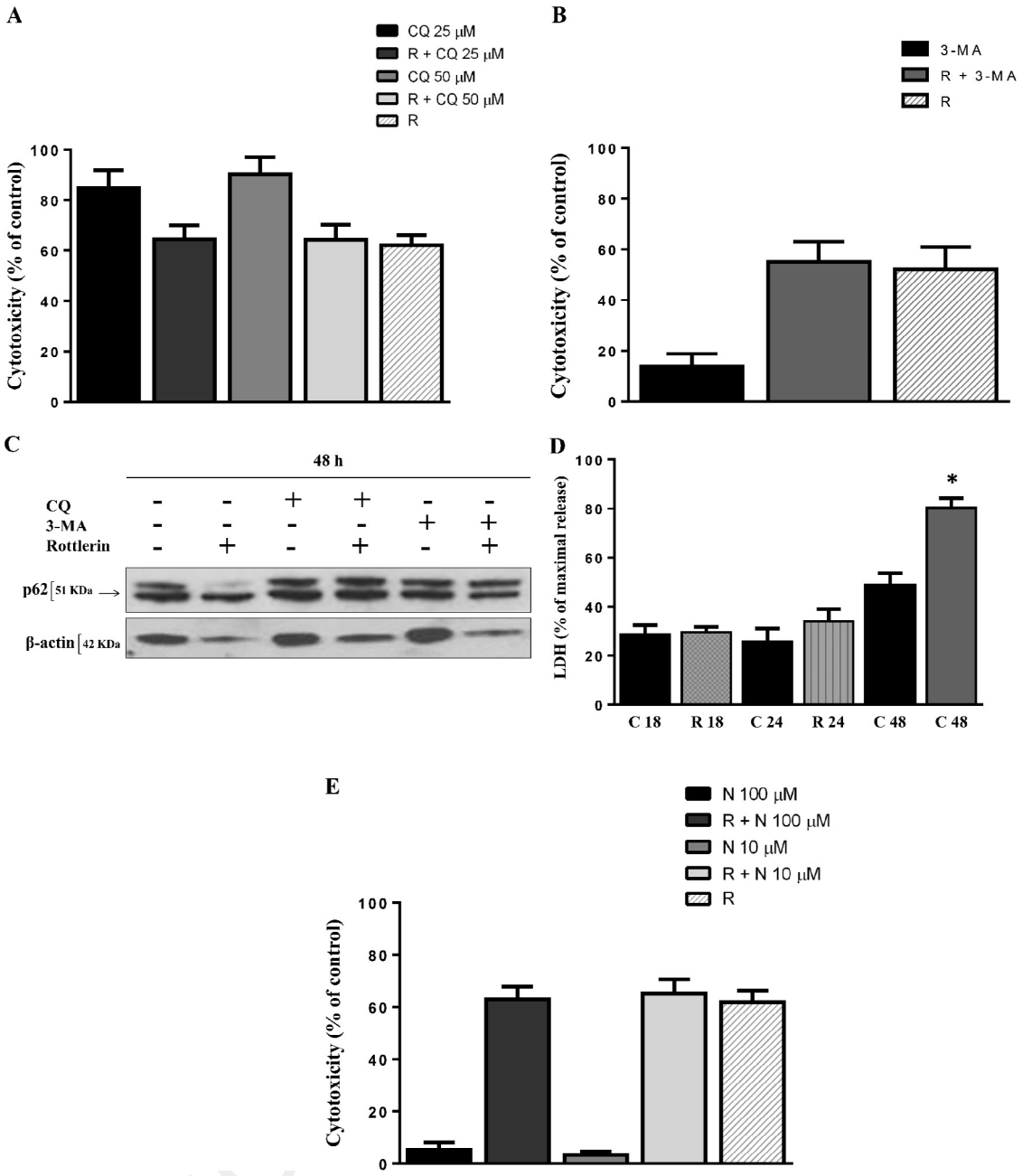

Fig. 5. Rottlerin induced non-autophagic and non-necroptotic SK-Mel-28 cell death. Rottlerin cytotoxicity after 48 h treatment in the presence of the autophagy inhibitor CQ (a) and the autophagy inhibitor 3-MA (b). Western blotting of p62 after 48 h Rottlerin treatment in the presence of CQ and 3-MA (c). Representative of two independent experiments. LDH release after 24–48 h treatment. The percentage of LDH release was calculated by comparing it with the maximal release of the positive control, which was treated with Triton X-100 (d). Rottlerin cytotoxicity after 48 h treatment in the presence of the necroptosis inhibitor Nec-1. *$p < 0.05$ with respect to control (e). Results are the means ± SD of at least three independent experiments in quadruplicate and are expressed as a percentage of the control (DMSO). Equal amounts of DMSO were added in CQ- and 3-MA-treated cells.

the cap-structure of mRNA. eIF4E is controlled by 4E-BP. In fact, 4EBP-1 phosphorylation by mTORC1 leads to dissociation of the 4EBP-1/eIF4E complex, and free eIF4E will then initiate protein synthesis. As shown in Fig. 4C, Rottlerin decreased the expression of phosphorylated 4E-BP1 β and γ and increased the levels of the fastest-migrating unphosphorylated 4E-BP1 α isoform.

Treatment with the proteasome inhibitor MG132 failed to reverse β-actin and Becl-1 downregulation, indicating that Rottlerin mainly acts at the level of protein synthesis (Fig. 6 C). Thus, these findings highlight mTORC1 inhibition and subsequent protein synthesis arrest as key events orchestrated by Rottlerin in SK-Mel-28 cell killing.

Discussion

In this study, we reported that Rottlerin exerts cytotoxicity towards SK-Mel-28 melanoma cells after 48 h of treatment, although cell growth was arrested much earlier by the drug [9].

Because the efficacy of anticancer agents is often limited by their negative side effects on normal cells, the toxicity of Rottlerin in non-transformed cells was addressed first. The results were encouraging in that the compound, at the same dose and exposure time, did not show significant toxicity against human skin fibroblasts, thus confirming the already-described selectivity of Rottlerin towards cancer cells [10,11].

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

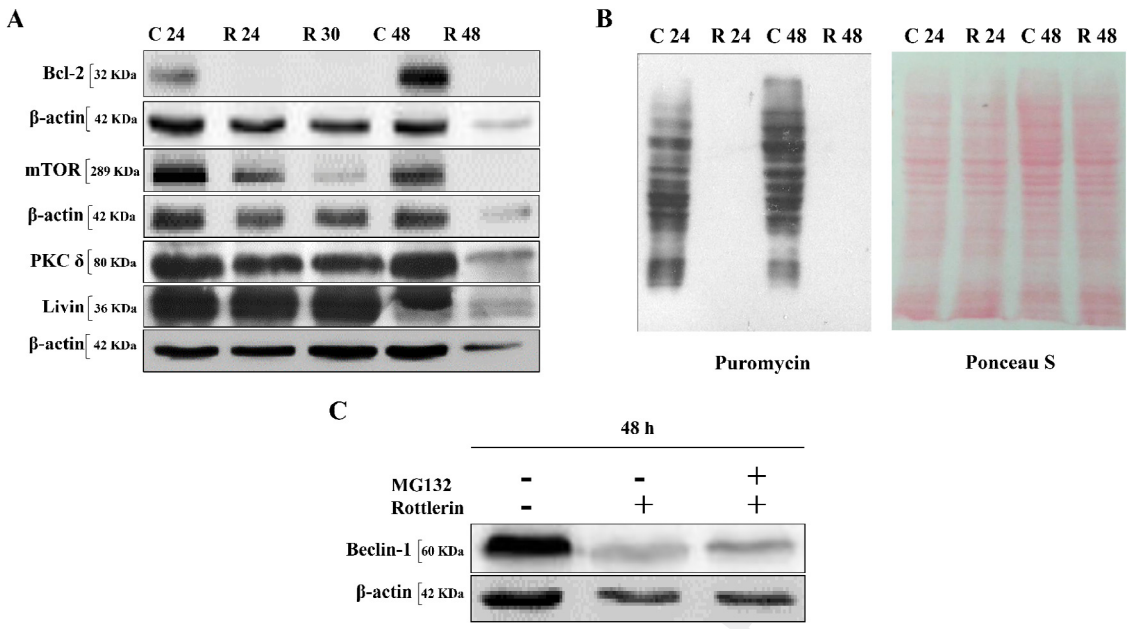

Fig. 6. Rottlerin downregulated a panel of unrelated proteins. (a) Western blotting analysis of Bcl-2, mTOR, PKCδ and Livin, after 24–48 h Rottlerin treatment. Controls were exposed to DMSO alone. β-actin was used as loading control. Representative of three independent experiments (b) Western blotting analysis of Puromycin-labeled proteins (left panel). Ponceau S staining (right panel) was used as loading control. (c) Western blotting analysis of Becl-1 and actin after 48 h Rottlerin treatment in the presence of the proteasome inhibitor MG132. Controls were exposed to DMSO alone. Representative of three independent experiments.

Although the mechanisms underlying the different sensitivity remain to be determined, it has been suggested that mitochondrial depolarization by Rottlerin, leading to ATP depletion, prevents energy-consuming programmed cell deaths in non-transformed cells, where mitochondria are the major source of ATP production, while in (pancreatic) cancer cells, ATP production is mostly dependent on glycolysis and thus mitochondria uncoupling has a minor effect on the energy balance [11].

Moreover, the modest cytostatic effect on skin fibroblasts, likely dependent on cyclin D1 downregulation, could even be seen as another Rottlerin protective mechanism against melanoma progression. In fact, because skin melanoma usually develops at the junction between epidermis and dermis, malignant cells come in direct contact with dermal, cancer-associated fibroblasts, which are known to play important roles in melanoma development and spreading [18].

Although Rottlerin may exert its anticancer effect by inducing apoptosis in apoptosis-competent cells [1], it did not induce morphological (condensed chromatin) and biochemical (PARP and caspases cleavage, increased caspase 3/7 activity) traits typical of an apoptotic form of cell death in SK-Mel-28 cells. Consistently, a pan-caspase inhibitor did not rescue cells from death. The cell resistance to apoptotic stimuli is probably due to the particular genetic profile of this cell line, which is characterized by the presence of B-RAF V600E variant, mutant p53 and PTEN, downregulation of p21/Cip1, overexpression of Cyclin D1 and hyperactivation of NFκB [19,20]. In addition, the lack of active p53 along with p21/Cip1 downregulation are also expected to compromise other tumor-suppression mechanisms, such as chemotherapy-induced premature senescence [21].

Necroptosis, an alternative form of programmed cell death, was also excluded, on the basis of the finding that the necroptosis inhibitor, necrostatin, failed to prevent Rottlerin-induced cell death. The late increase of LDH release, observed after 48 h of treatment, is ascribable to the occurrence of secondary necrosis, as the result of Rottlerin-induced irreparable cell damage. In addition, the nuclear morphology of Rottlerin-treated SK-Mel-28 cells did not show any peculiar sign of mitotic catastrophe, an alternative mode of cell death that occurs during or after a faulty mitosis.

Conversely, consistent with the autophagy-promoting property of Rottlerin, we found that SK-Mel-28 basal autophagy was strengthened by the drug. Mechanistically, Rottlerin induced autophagy through AMPK-mediated mTORC1 inhibition. In fact, AMPK has a triple inhibitory effect on mTORC1: AMPK phosphorylates TSC2 at Thr-1227 and Ser-1345 and increases the inhibitory activity of the TSC1/2 complex towards mTORC1 [22]; AMPK directly phosphorylates mTOR at Thr-2446, thereby inhibiting its activity [23]; and AMPK phosphorylates the mTOR binding partner Raptor at Ser722 and Ser792, followed by 14-3-3 binding to Raptor and inhibition of mTORC1 [24]. AMPK activation is based on its phosphorylation at Thr172 by LKB1 [25]. Interestingly, LKB1 can be phosphorylated by ERK and p 90RSK in malignant melanoma cells that express the B-RAF V600E mutation (like SK-Mel-28 cells) and such phosphorylation results in LKB1 inactivation [26]. Therefore, the earlier finding that Rottlerin inhibits ERK activity and p90RSK phosphorylation in SK-Mel-28 cells [9], thus relieving the cell of a crucial AMPK inhibitory pathway, likely contributed to AMPK activation, in addition to the well-recognized Rottlerin mitochondrial uncoupling effect [27].

Nevertheless, autophagy induction by Rottlerin was not lethal for SK-Mel-28 melanoma cells, because cytotoxicity was not modified by the autophagy blocker CQ. Rather, CQ exhibited by itself a marked cytotoxicity, in agreement with other studies performed on cancer cells with high basal levels of autophagy [6]. The use of a less toxic autophagy inhibitor (3-MA) confirmed that autophagy was not the mechanism of Rottlerin-induced cell death.

On the whole, these results indicate that Rottlerin kills SK-Mel-28 cells through non-canonical routes. The only dramatic alteration that was found after 48 h and that could lead to cell demise is the drop in the levels of several proteins (most of them involved in malignancy-related signaling pathways), which was not caused by increased proteasomal degradation but rather mirrored the general inhibition of protein synthesis that was revealed by the Puromycin-based assay.

Please cite this article in press as: E. Daveri, et al., Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.018

Mechanistically, the protein synthesis arrest could be explained by the observed inhibition of the mTORC1/4EBP-1 cascade and consequent blockage of the initiation step of mRNA translation.

Data from the literature further support this view, because Rottlerin has also been reported to induce phosphorylation of the eukaryotic translation initiation factor-2α (eIF2α) (leading to inhibition of its translational activity) in HT29 human colon carcinoma cells, through endoplasmic reticulum stress and consequent induction of eIF2α kinases [28].

Furthermore, it is worth recalling that, in addition to or alternative to AMPK activation, Rottlerin can inhibit mTORC1 by direct binding, independent from upstream kinases, as demonstrated in a previous study performed on MCF-7 breast cancer cells [2].

In summary, this study discloses a novel mechanism of Rottlerin-induced cancer cells death, i.e. inhibition of cap-dependent protein translation via mTORC1/4EBP-1 inhibition. Recently, eIF4F inactivation is emerging as a promising approach for anti-cancer intervention [29–31], which could be particularly important in those cancer cells where the canonical modes of cell death are expected to be defective. The current results also emphasized the versatility of Rottlerin as anticancer drug in a variety of cellular models, which greatly differ from each other in terms of genetic/biochemical profile and chemosensitivity. This is especially relevant in melanoma where heterogeneity of cellular populations is thought to be responsible for drug refractoriness, even with targeted therapeutics and selection of resistant phenotypes [32].

Acknowledgments

The authors thank Dr. Roberta Romagnoli, Dr. Barbara Del Bello, Dr. Allavena Giulia and Laura Cresti for their technical support. This work is part of the research project “Rottlerin repositioning: from folk medicine to modern perspectives in cancer therapy,” supported by the Istituto Toscano Tumori (ITT), Regione Toscana (Grant to E. Maioli).

Conflicts of interest

The authors declare no conflicts of interest.

References

[1] C. Torricelli, S. Salvadori, G. Valacchi, K. Souček, E. Slabáková, M. Muscettola, et al., Alternative pathways of cancer cell death by rottlerin: apoptosis versus autophagy, Evid. Based Complement. Alternat. Med. 2012 (2012) 980658, doi:10.1155/2012/980658.

[2] C. Torricelli, E. Daveri, S. Salvadori, G. Valacchi, F. Ietta, M. Muscettola, et al., Phosphorylation-independent mTORC1 inhibition by the autophagy inducer Rottlerin, Cancer Lett. 360 (2015) 17–27.

[3] X. Wang, C.G. Proud, The mTOR pathway in the control of protein synthesis, Physiology (Bethesda) 21 (2006) 362–369.

[4] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (2008) 27–42.

[5] X.H. Ma, S. Piao, D. Wang, Q.W. McAfee, K.L. Nathanson, J.J. Lum, et al., Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma, Clin. Cancer Res. 17 (2011) 3478–3489.

[6] S. Yang, X. Wang, G. Contino, M. Liesa, E. Sahin, H. Ying, et al., Pancreatic cancers require autophagy for tumor growth, Genes Dev. 25 (2011) 717–729.

[7] H. Mukubou, T. Tsujimura, R. Sasaki, Y. Ku, The role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation, Int. J. Oncol. 37 (2010) 821–828.

[8] E. Maioli, C. Torricelli, G. Valacchi, Rottlerin and cancer: novel evidence and mechanisms, ScientificWorldJournal (2012) 350826, doi:10.1100/2012/350826.

[9] E. Daveri, G. Valacchi, R. Romagnoli, E. Maellaro, E. Maioli, Antiproliferative effect of rottlerin on sk-Mel-28 melanoma cells, Evid. Based Complement. Alternat. Med. (2015) 545838, doi:10.1155/2015/545838.

[10] J.H. Lim, J.W. Park, K.S. Choi, Y.B. Park, T.K. Kwon, Rottlerin induces apoptosis via death receptor 5 (DR5) upregulation through CHOP-dependent and PKC delta-independent mechanism in human malignant tumor cells, Carcinogenesis 30 (2009) 729–736.

[11] I. Ohno, G. Eibl, I. Odinokova, M. Edderkaoui, R.D. Damoiseaux, M. Yazbec, et al., Rottlerin stimulates apoptosis in pancreatic cancer cells through interactions with proteins of the Bcl-2 family, Am. J. Physiol. Gastrointest. Liver Physiol. 298 (2010) G63–G73.

[12] W. Voigt, Sulforhodamine B assay and chemosensitivity, Methods Mol. Med. 110 (2005) 39–48.

[13] D. Moretti, B. Del Bello, G. Allavena, A. Corti, C. Signorini, E. Maellaro, Calpain-3 impairs cell proliferation and stimulates oxidative stress-mediated cell death in melanoma cells, PLoS ONE 10 (2) (2015) e0117258.

[14] D.K. Eggers, W.J. Welch, W.J. Hansen, Complexes between nascent polypeptides and their molecular chaperones in the cytosol of mammalian cells, Mol. Biol. Cell 8 (1997) 1559–1573.

[15] A. Petiot, E. Ogier-Denis, E.F.C. Blommaart, A.J. Meijer, P. Codogno, Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells, J. Biol. Chem. 275 (2000) 992–998.

[16] T. Vanden Berghe, N. Vanlangenakker, E. Parthoens, W. Deckers, M. Devos, N. Festjens, et al., Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features, Cell Death Differ. 17 (2010) 922–930.

[17] A. Degterev, J. Hitomi, M. Germscheid, I.L. Ch'en, O. Korkina, X. Teng, et al., Identification of RIP1 kinase as a specific cellular target of necrostatins, Nat. Chem. Biol. 4 (2008) 313–321.

[18] L. Zhou, K. Yang, T. Andl, R.R. Wickett, Y. Zhang, Perspective of targeting cancer-associated fibroblasts in melanoma, J. Cancer 6 (2015) 717–726.

[19] F.G. Haluska, H. Tsao, H. Wu, F.S. Haluska, A. Lazar, V. Goel, Genetic alterations in signaling pathways in melanoma, Clin. Cancer Res. 12 (2006) 2301s–2307s.

[20] Y. Ueda, A. Richmond, NF-κB activation in melanoma, Pigment Cell Res. 19 (2006) 112–124.

[21] G. Saretzki, Cellular senescence in the development and treatment of cancer, Curr. Pharm. Des. 16 (2010) 79–100.

[22] K. Inoki, T. Zhu, K.L. Guan, TSC2 mediates cellular energy response to control cell growth and survival, Cell 115 (2003) 577–590.

[23] S.W. Cheng, L.G. Fryer, D. Carling, P.R. Shepherd, Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status, J. Biol. Chem. 279 (2004) 15719–15722.

[24] D.M. Gwinn, B. David, D.B. Shackelford, D.F. Egan, M.M. Mihaylova, A. Mery, et al., AMPK phosphorylation of raptor mediates a metabolic checkpoint, Mol. Cell 30 (2008) 214–226.

[25] A. Woods, S.R. Johnstone, K. Dickerson, F.C. Leiper, L.G. Fryer, D. Neumann, et al., LKB1 is the upstream kinase in the AMP-activated protein kinase cascade, Curr. Biol. 13 (2003) 2004–2008.

[26] B. Zheng, J.H. Jeong, J.M. Asara, Y.Y. Yuan, S.R. Granter, L. Chin, et al., Oncogenic B-RAF negatively regulates the tumor suppressor LKB1 to promote melanoma cell proliferation, Mol. Cell 33 (2009) 237–247.

[27] S.P. Soltoff, Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta, Trends Pharmacol. Sci. 28 (2007) 453–458.

[28] J.H. Lim, J.W. Park, S.H. Kim, Y.H. Choi, K.S. Choi, T.K. Kwon, Rottlerin induces pro-apoptotic endoplasmic reticulum stress through the protein kinase C-delta-independent pathway in human colon cancer cells, Apoptosis 13 (2008) 1378–1385.

[29] S. Assouline, B. Culjkovic, E. Cocolakis, C. Rousseau, N. Beslu, A. Amri, et al., Molecular targeting of the oncogene eIF4E in acute myeloid leukemia (AML): a proof-of-principle clinical trial with ribavirin, Blood 114 (2009) 257–260.

[30] S.Y. Ko, H. Guo, N. Barengo, H. Naora, Inhibition of ovarian cancer growth by a tumor-targeting peptide that binds eukaryotic translation initiation factor 4E, Clin. Cancer Res. 15 (2009) 4336–4347.

[31] C.J. Lin, R. Sukarieh, J. Pelletier, Silibinin inhibits translation initiation: implications for anticancer therapy, Mol. Cancer Ther. 8 (2009) 1606–1612.

[32] R. Somasundaram, J. Villanueva, M. Herlyn, Intratumoral heterogeneity as a therapy resistance mechanism: role of melanoma subpopulations, Adv. Pharmacol. 65 (2012) 335–359, doi:10.1016/B978-0-12-397927-8.00011-7.