

Research Article

Antiproliferative Effect of Rottlerin on Sk-Mel-28 Melanoma Cells

Elena Daveri,¹ Giuseppe Valacchi,² Roberta Romagnoli,¹ Emilia Maellaro,³ and Emanuela Maioli¹

¹Department of Life Sciences, University of Siena, Via Aldo Moro 7, 53100 Siena, Italy

²Department of Biology and Evolution, University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy

³Department of Molecular and Developmental Medicine, University of Siena, Via Aldo Moro 7, 53100 Siena, Italy

Correspondence should be addressed to Emanuela Maioli; emanuela.maioli@unisi.it

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Melanoma is the most aggressive and chemoresistant form of skin cancer. Mutated, constitutively active B-RAF is believed to play a crucial role, although the selective B-RAF inhibition has shown poor clinical success, since phenomena of resistance usually occur, likely arising from additional genetic aberrations, such as loss of function of p53 and PTEN, overexpression of cyclin D1, hyperactivation of NF- κ B, and downregulation of p21/Cip1. Since all of them are present in the Sk-Mel-28 melanoma cells, this cell line could be an ideal, albeit hard to study, model to develop new therapeutic strategies. In the current study, we tested the cytostatic action of Rottlerin on Sk-Mel-28 melanoma cells, on the basis of the known Rottlerin effects on the main proliferative signaling pathways. We presented evidence that the drug inhibits cell growth by an Akt- and p21/Cip1-independent mechanism, involving the dual inhibition of ERK and NF- κ B and downregulation of cyclin D1. In addition, we found that Rottlerin increases ERK phosphorylation, but, surprisingly, this resulted in decreased ERK activity. Pull-down experiments, using Rottlerin-CNBr-conjugated Sepharose beads, revealed that Rottlerin binds to ERK, independently from its phosphorylation status. This direct interaction could in part explain the paradoxical blockage of ERK downstream signaling and growth arrest.

We would like to dedicate this paper to the memory of our friend and colleague, prematurely deceased, Claudia Torricelli, who actively contributed to this project

1. Introduction

Activating mutations of the B-RAF oncogene are present in approximately 5–10% of all human malignancies and cause aberrant cell proliferation. The most frequent mutation of B-RAF is the valine-to-glutamic acid substitution at codon 600 (V600E), which has been observed in several cancers [1]. In particular, about 50% of cutaneous melanomas harbour B-RAF V600E mutations [2] that are responsible for the increased phosphorylation of extracellular activated protein kinases (ERKs) and increased expression of cyclin D1, which is associated with progression through G1/S phase of the cell cycle, as a partner of cyclin-dependent kinases (cdks) [3].

However, B-RAF mutations alone are not sufficient to explain melanomagenesis since also benign nevi frequently

have B-RAF mutations [4, 5]. This observation implies that mutant B-RAF must combine with additional genetic aberrations and/or alterations of other signaling pathways to generate and sustain melanoma [1, 6]. In fact, despite the discovery of selective B-RAF inhibitors, their efficacy in melanoma, as single targeted therapy, was lower than hoped for because of the activation of alternative mitogen activated protein kinases (MAPK) pathways and consequent development of tumour resistance [7].

The challenge is therefore to devise strategies that could prevent therapy escape after treatment with B-RAF inhibitors. To this end, combined treatment with B-RAF and mitogen activated protein kinase kinase (MEK) inhibitors has been proposed, although resistance still develops in most patients, by not yet fully elucidated mechanisms, after

an average of 9.4 months [8]. As the possible mechanisms of resistance to double B-RAF and MEK inhibition is concerned, it must be kept in mind that the activated Ras/Raf/MEK/ERK cascade can lead to opposite proliferative responses. In fact, a moderate ERK activity stimulates cell-cycle progression through cyclin D1 induction, while hyperactivated ERK can cause inhibition of growth by induction of the cdk inhibitor, p21/Cip1 [9–11]. Therefore, the possibility that the simultaneous inhibition of B-RAF and ERK rather suppresses an antiproliferative stimulus must be considered. At the same time, it is also conceivable that the constitutive activity of B-RAF V600E and the concurrent downregulation of p21/Cip1 could cooperate to confer a proliferative advantage, as in the case of Sk-Mel-28 melanoma cells that we used in the current study. This cell line indeed, in addition to the presence of B-RAF V600E variant, mutant p53, and PTEN [12], is characterized by the underexpression of the p21/Cip1 gene [13], likely consequent to the loss of function of p53, along with high expression of miR-106b which directly targets p21/Cip1 [14]. PTEN is a lipid phosphatase that negatively regulates the phosphoinositide kinase-3 (PI3K)/AKT axis, a pathway that plays important roles in melanoma, including resistance to MAPK inhibitors [15]. Moreover, a synergistic effect of the B-RAF mutation with the overexpression of cyclin D1 resulting from genomic amplification [16] contributes to explaining the high growth rate of this cell line and its intrinsic refractoriness to cell-cycle blocking drugs. Sk-Mel-28 cells are therefore a concentrate of genetic alterations that notoriously confer them a tremendous resistance to the most targeted anticancer therapies. It follows that a higher efficacy could be achieved from treatment with multitarget compounds able to affect different processes simultaneously.

Such a compound might be Rottlerin, a natural polyphenol with a broad range of activities [17]. Importantly, despite its pleiotropic effects *in vitro*, Rottlerin is nontoxic *in vivo* [18, 19]. Among the molecules potentially inhibited by Rottlerin, relevant to this study on Sk-Mel-28 cells, are Akt [20], ERK [21, 22], and the nuclear factor κ B (NF- κ B), which, among other transcription factors, regulates the expression of proproliferative molecules, such as cyclin D1 [23–25]. Interestingly, NF- κ B is constitutively active in melanoma cells harbouring mutant p53, including Sk-Mel-28 cells [26].

Against this background, the challenge was to use Rottlerin as a cytostatic against Sk-Mel-28 melanoma cells, focusing the study on changes in key molecules potentially involved in cell-cycle arrest, such as cyclin D1 and p21/Cip1. The major upstream signaling pathways, such as ERK, Akt, and NF- κ B cascades, were also investigated. We provide evidence that Rottlerin blocks Sk-Mel-28 cell proliferation through an Akt- and p21/Cip1-independent mechanism, involving the dual inhibition of NF- κ B nuclear migration and ERK activity, which converges on cyclin D1 downregulation and cell-cycle arrest.

2. Materials and Methods

2.1. Materials. Rottlerin with purity higher than 95% was obtained from Calbiochem, San Diego, CA. RPMI-1640, FBS, antibiotics, DMSO, paraformaldehyde, Triton X-100,

DABCO, Ponceau S, Trypan blue, and FITC were from Sigma Aldrich, St. Louis, MO. Antibodies against total and phospho-Akt (Thr 308), total and phospho-ERK, total and phospho-p90RSK (Thr573), p21/Cip1, cyclin D1, PARP, and β -actin were obtained from Cell Signaling Technology, Danvers, MA. Antibody against SQSTM1/p62 was from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against p65 NF- κ B was from Millipore, Temecula, 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.

2.2. Cells and Culture Conditions. Sk-Mel-28 human melanoma cells (from ATCC) were grown and maintained in 25 cm² tissue culture flasks in a humidified atmosphere (95% air/5% CO₂) at 37°C in RPMI-1640 medium, containing 10% FBS, glutamine (2 mM), and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B). Rottlerin, dissolved in DMSO at a stock concentration of 20 mM, was stored in a dark coloured bottle at –20°C. After reaching subconfluence, cells were treated with the indicated concentrations of Rottlerin or vehicle (DMSO) in complete medium containing 2.5% FBS for the indicated periods.

2.3. Cell Proliferation and Viability. Cell proliferation was evaluated by the Sulforhodamine (SRB) colorimetric assay as previously described [27]. This assay measures cellular protein content to determine cell density. Cells were seeded in triplicate on 96-well plates, incubated 4–6 h at 37°C to allow adherence, and treated with Rottlerin: 20 μ M for 24–72 h or increasing concentration (0.1–100 μ M) for 24 h. Following treatment, the medium was removed, and the cells were washed twice with PBS and fixed with 100 μ L of cold 10% trichloroacetic acid (TCA). The plate was incubated at 4°C for 30 min before being gently washed four times with tap water to remove TCA and dead cells. It was allowed to dry in air; then, 100 μ L 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 plate was air dried again, and 200 μ L of 10 mM aqueous Tris base (pH 10.5) was added to solubilise 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.

Cell viability was assessed by Trypan blue staining. Sk-Mel-28 cells were seeded in triplicate on 6-well plates and incubated for 4–6 h at 37°C to allow adherence. After incubation with 20 μ M of Rottlerin for 24–72 h, cells were trypsinized, resuspended in phosphate buffer saline (PBS), and stained with 0.4% Trypan blue dye solution (v/v in PBS). The total cell number and the number of cells which excluded Trypan blue (considered viable) were counted in a Burkler chamber within 5 min after staining.

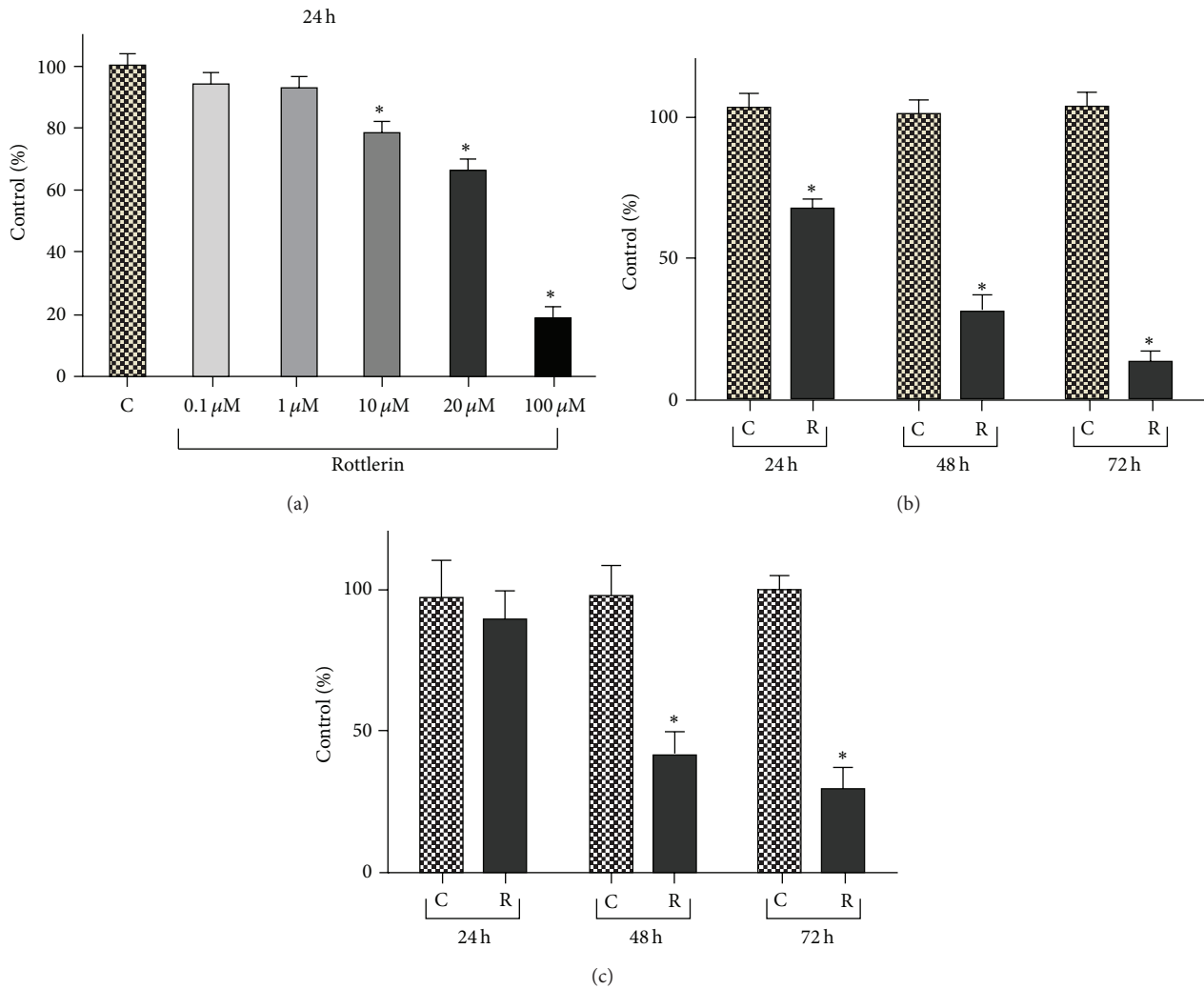


FIGURE 1: Rottlerin inhibits proliferation of Sk-Mel-28 melanoma cells. (a) Rottlerin treatment for 24 h reduced cell density, evaluated by the SRB assay, in a dose-dependent manner. (b) Time-dependent decrease of cell density after 24–72 h exposure to 20 μM Rottlerin. Results are the means ± SD of at least three independent experiments in triplicate and expressed as % of control. (c) Cell viability, evaluated by Trypan blue staining, after 24–72 h exposure to 20 μM Rottlerin. The percentage viability (live cell count/total cell count) was calculated and expressed as % of control. Results are the means ± SD of three independent experiments. Controls were exposed to DMSO alone; * $P < 0.05$.

2.4. Western Blotting Analysis. Cell extracts, each containing 30–40 μg of total protein, were resolved on 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 polyclonal 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 and exposed on photographic film. Immunoreactive bands were quantified by Image J analysis software.

2.5. Nuclear p65 NF-κB Immunostaining. Through the canonical NF-κB signaling pathway, IKK phosphorylates IκB proteins, thus allowing the release of free p50/p65 NF-κB dimers that translocate to the nucleus. For

immunocytochemical experiments, cells were cultured and treated on sterile glass coverslips placed in a 24-well microplate. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 40 min at room temperature (RT). After washes with PBS, 0.5% Triton X-100 (v/v in PBS) was added for 5 min at 4°C and then washed with PBS. Cells were incubated with blocking buffer (1% BSA in PBS) for 45 min prior to incubation for 1 h at RT with primary antibody anti-p65, diluted 1:100 in PBS with 0.5% BSA. Cells were washed and incubated for 45 min with FITC-conjugated secondary antibody diluted in PBS containing 0.5% BSA. Cells were washed and incubated for 10 min in DAPI diluted 1:10,000 in PBS. Finally, the coverslips were mounted on glass slides using DABCO. Images were acquired on a Carl Zeiss Axioplan 2 imaging microscope using an AxioCam HR CCD camera and AxioVision 3.1 software (Carl Zeiss, Göttingen, Germany).

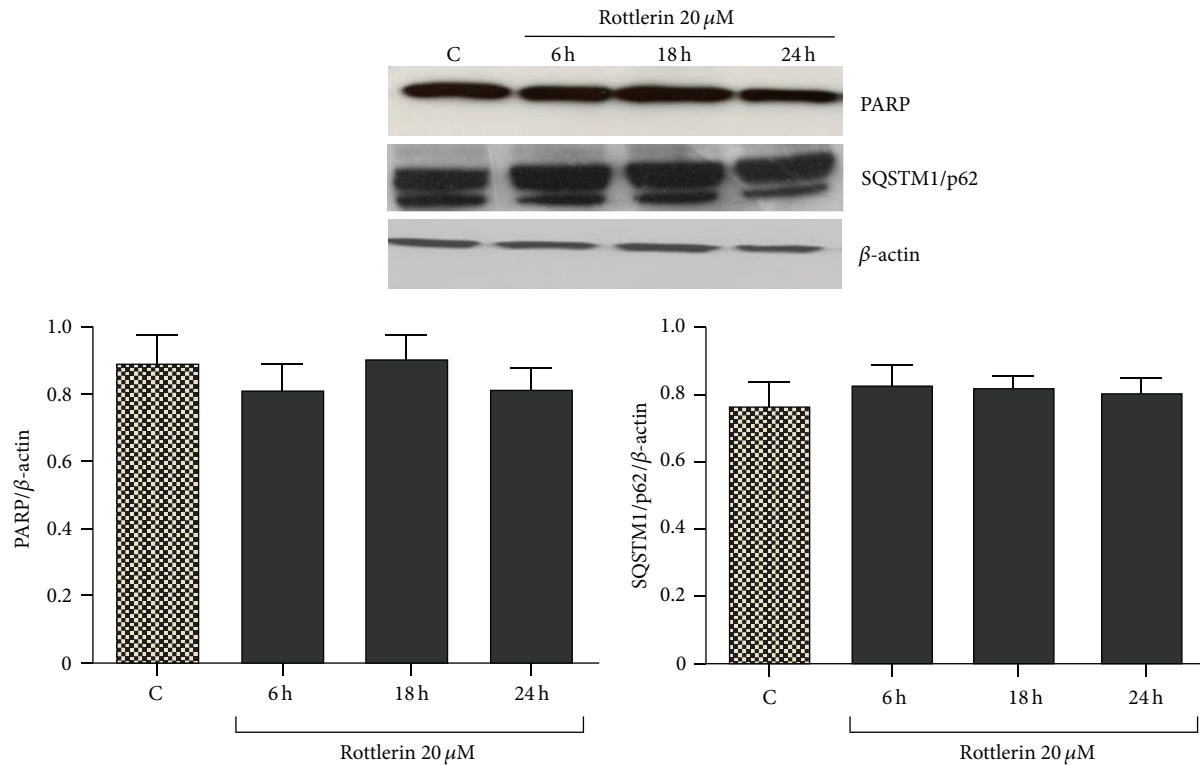


FIGURE 2: Effect of Rottlerin on apoptosis and autophagy. Western blotting of PARP and SQSTM1/p62 in Sk-Mel-28 cell lysate after 6–24 h treatment with 20 μ M Rottlerin. β -actin was used as loading control. A representative western blot of three independent experiments is shown. Densitometry results are expressed as protein/ β -actin ratio.

2.6. Preparation of Rottlerin-Cyanogen Bromide- (CNBr-) Activated Sepharose 4B Complex. The procedure was performed following the previously reported protocol [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 NaHCO₃ (pH 8.3) and 0.5 M NaCl). Rottlerin (2 mg), dissolved in 500 μ L 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.

2.7. Ex Vivo Pull-Down Assay. For the ex vivo pull-down assay, a total of 500 μ g of Sk-Mel-28 protein extract was incubated with 100 μ L (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 μ g/mL bovine serum albumin, 0.02 mM PMSF, 1x protease inhibitor cocktail). After incubation, with gentle rocking for 3 h at room temperature, 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 of interest, bound to the beads,

were eluted with SDS loading buffer and analysed by western blotting with specific antibodies.

2.8. Statistical Analyses. Values are expressed as the mean \pm SD. Student's *t*-test was used to determine statistical significance with a threshold of *P* values less than 0.05.

3. Results

3.1. Effect of Rottlerin on Sk-Mel-28 Cells Proliferation. As shown in Figure 1(a), the cell density, evaluated by SRB assay after 24 h of treatment, decreased progressively with increasing Rottlerin doses and became statistically significant starting from 10 μ M Rottlerin (78%). A cell number reduction to 66% was observed at the dose of 20 μ M and an almost total cell loss was obtained with 100 μ M Rottlerin (18%). Therefore, the dose of 20 μ M was used for the time course evaluation.

As shown in Figure 1(b), a time-dependent decrease in cellularity was observed, with a modest cell recovery of 26% at 48 h and a very low recovery (15%) at 72 h. Therefore, the dose of 20 μ M and the time exposure of 24 h, which yielded a mean of 68% cell recovery, were chosen for the subsequent molecular analyses on cell extracts.

No evident cell detachment was observed after 24 h but, as shown in Figure 1(c), circa 13, 60 and 70% cell death were revealed by the Trypan blue exclusion test, performed on both adherent and floating cells after 24, 48, and 72 h, respectively.

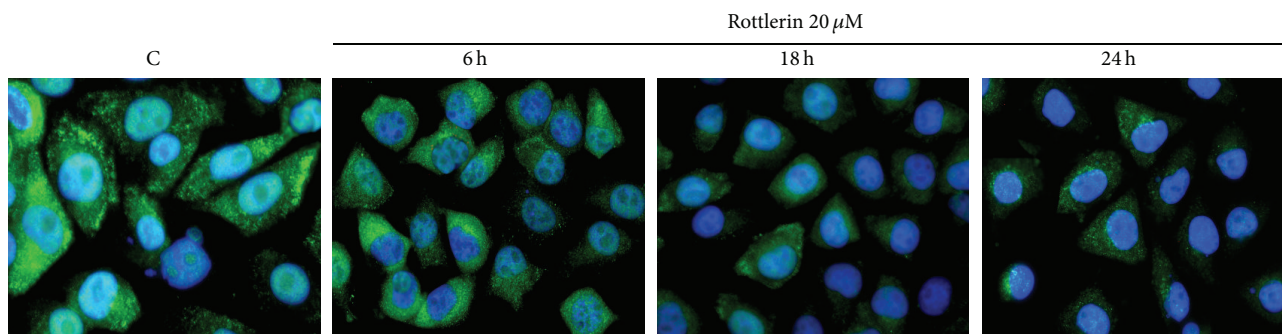


FIGURE 3: Effect of Rottlerin on NF- κ B nuclear translocation. Cells were treated with 20 μ M Rottlerin for 6–24 h, stained with anti-p65 NF- κ B subunit antibody, and revealed with FITC-conjugated secondary antibody. The merge images of anti-p65 labeling (green) and nuclear staining with DAPI (blue) show that both stainings overlap in the control and after 6 h of Rottlerin treatment, while the p65 expression is restricted in the cytosol after 18–24 h (original magnification 50x). Representative images of two independent experiments.

3.2. Rottlerin Cytotoxicity on Sk-Mel-28 Cells. Sk-Mel-28 cells, exposed to 20 μ M Rottlerin for 24 h, did not display signs of apoptosis and autophagy, as deduced by the lack of PARP cleavage (a marker of apoptosis) and SQSTM1/p62 degradation (a hallmark of autophagic flux) (Figure 2). The observed decrease in cell number is therefore mainly ascribable to inhibited proliferation.

3.3. Effect of Rottlerin on NF- κ B Nuclear Translocation. In Figure 3, it is possible to appreciate that in untreated cells and in cells exposed to 20 μ M Rottlerin for 6 h, the fluorescence is distributed between the cytoplasm and the nucleus, whereas after 18–24 h of Rottlerin treatment, the fluorescence is confined to the cytoplasm. This result, in agreement with previous findings [24, 25], indicates that Rottlerin interferes with the basal NF- κ B activation process also in Sk-Mel-28 cells.

3.4. Effect of Rottlerin on Cyclin D1 and p21/Cip1 Levels. Following Rottlerin exposure, the levels of cyclin D1 dropped to undetectable levels after only 6 h of treatment (Figure 4), an effect already observed in other cell types [24, 28, 29].

In agreement with other studies on Sk-Mel-28 cells [13, 14], the basal levels of the cell-cycle inhibitor p21/Cip1 were undetectable by western blotting. The protein expression did not change during the 6–24 h of Rottlerin treatment (Figure 4).

3.5. Effect of Rottlerin on ERKs and Akt Activation. As shown in Figure 5(a), phospho-ERKs levels were markedly increased after 6–24 h of 20 μ M Rottlerin treatment. Increased ERK phosphorylation was not accompanied by enhanced downstream signaling, as evidenced by the decrease of phospho-p90RSK1, the cytoplasmic target of ERK (Figure 5(b)). Conversely, Rottlerin did not significantly modify the phospho-Akt levels (Figure 5(c)).

3.6. Rottlerin Binds to ERKs. Incubation of the Sk-Mel-28 cell lysate with 2 mg Rottlerin-coupled Sepharose 4B led to the formation of a Rottlerin/ERKs complex. As shown in

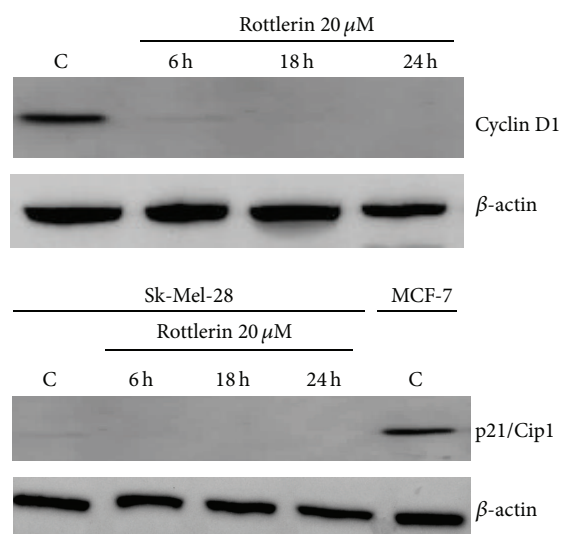


FIGURE 4: Effect of Rottlerin on cyclin D1 and p21/Cip1 levels. Western blotting of cyclin D1 and p21/Cip1 in Sk-Mel-28 cell lysate after a 6–24 h treatment with 20 μ M Rottlerin. Western blotting of p21/Cip1 in MCF-7 cells was used as positive control. β -actin was used as loading control. Representative western blots of three independent experiments are shown.

Figure 6(a), ERKs were pulled down by Rottlerin-Sepharose beads, but not by unconjugated Sepharose beads used as negative control. Of note, Rottlerin was able to equally bind unphosphorylated and phosphorylated proteins.

The membrane stained with Ponceau S (Figure 6(b)) indicates that while no (visible) proteins are bound to Sepharose beads, Rottlerin can bind several proteins, thus exhibiting a not fully selective behaviour, in agreement with the notion that this compound is a multitarget drug.

4. Discussion

Recently, Rottlerin has been revealed to be a promising antitumor compound for its *in vitro* and *in vivo* activity against a wide array of cancers [30, 31].

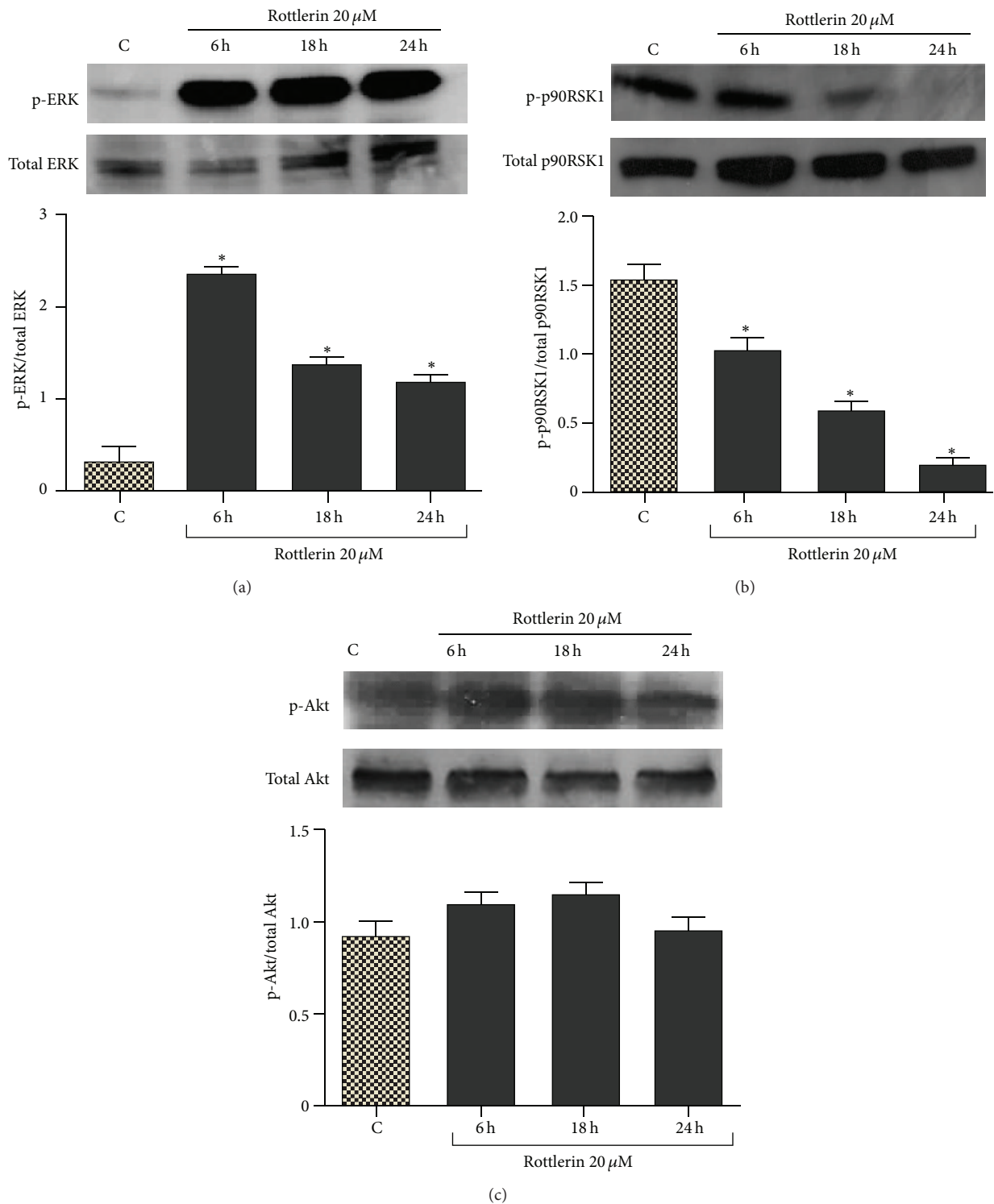


FIGURE 5: Effect of Rottlerin on ERKs and Akt activation. (a) Western blotting of phospho- and total ERKs, (b) phospho- and total p90RSK, and (c) phospho- and total Akt after 6–24 h of 20 μ M Rottlerin treatment. The blots are representative of three independent experiments. Densitometry results are expressed as phosphoprotein/total protein ratio; * $P < 0.05$.

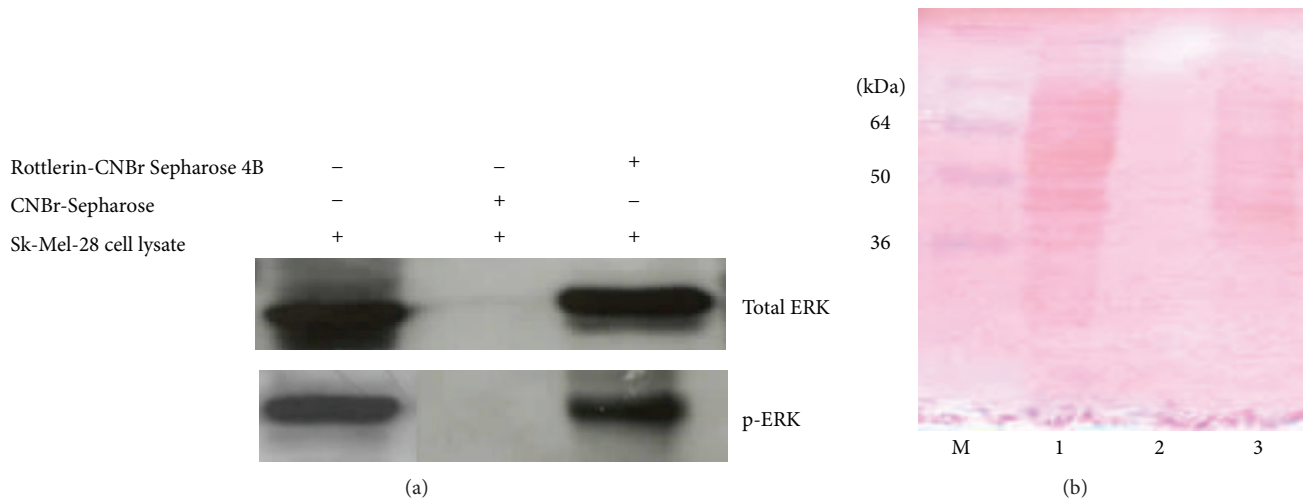


FIGURE 6: Rottlerin binds to ERKs. (a) Western blotting of total and phospho-ERKs eluted from the Rottlerin-Sepharose beads. Unconjugated Sepharose beads were used as negative control. (b) Membrane stained with Ponceau S. Lane M: molecular weight markers; lane 1: cell lysate; lane 2: unconjugated Sepharose beads; lane 3: Rottlerin-conjugated Sepharose beads. Representative of two independent experiments.

The current study shows, for the first time, that Rottlerin has antiproliferative effects in Sk-Mel-28 melanoma cells. A 24 h treatment with 20 μ M Rottlerin caused a reduction in cell density to 68%, a significant decrease, albeit lower than that observed in other cancer cells, such as MCF-7 breast cancer cells [32]. No signs of apoptosis (PARP cleavage) or autophagy (SQSTM1/p62 degradation) were found after 24 h exposure, although a modest cytotoxicity (circa 13%) was revealed by Trypan blue staining. Prolonged treatment, for 48–72 h, resulted in a marked cell loss, most likely due to delayed and marked cytotoxic effects.

In line with our earlier results obtained in breast cancer cells [24], also in melanoma cells, Rottlerin potently downregulated cyclin D1 protein levels and inhibited cell proliferation.

For a mechanistic study, since the cyclin D1 gene can be regulated by different transcription factors, such as AP-1, Sp-1, and NF- κ B [33, 34], we searched for the responsible upstream signaling pathways, focusing on ERK and Akt. It is well known that AP-1 is a major target of the ERK cascade [35] and Sp-1 can be phosphorylated by ERK in two major sites, both critical for Sp-1 transcriptional activity [36]. ERK has also an indirect effect on cyclin D1 expression, based on the ability of its cytoplasmic target, p90RSK1, to activate NF- κ B by phosphorylation of I κ B α on Ser-32 [37]. Also, Akt can activate NF- κ B, through phosphorylation of IKK on Thr-23 [38, 39].

Thus, the Ras/ERK and the PI3K/Akt axes are the major pathways that regulate the cyclin D1 gene expression.

In agreement with previous studies on different cells [24, 28, 29], the observed decrease of cyclin D1 was accompanied (and caused) by the inhibition of NF- κ B nuclear migration.

Regarding the involved upstream pathways, our results do not support a role for Akt in the Rottlerin mediated effect, neither on the NF- κ B activation pathway nor on the

downregulation of cyclin D1, as phospho-Akt levels (Thr 308, target of PI3K) were unaffected by the drug.

Conversely and surprisingly, Rottlerin treatment resulted in a marked and early (6 h) increase in ERK phosphorylation, a finding that was in complete incongruity with the decrease of p90RSK1 phosphorylation, the inhibition of NF- κ B, and the lack of p21/Cip1 induction. As far as p21/Cip1 is concerned, although it is basically downregulated in Sk-Mel-28 cells, the gene is functionally active and can be induced in certain circumstances by p53-independent mechanisms [40–42]. Therefore, the apparent ERK activation resulted, paradoxically, in suppressed downstream signaling.

In the search for a plausible explanation of these results and on the basis of the recent finding that Rottlerin can inhibit protein kinases, such as the mammalian target of rapamycin (mTOR), by a direct binding [27], we applied the previously employed methodology to verify a possible physical interaction between Rottlerin and ERK. We found, by ex vivo pull-down assay, that Rottlerin complexes with and likely inhibits ERK, regardless of its phosphorylation status, thus independently from upstream signaling events, just as in the case of mTOR.

In this context, this preliminary finding might help explain the observed lack of function of phospho-ERK on one hand and expands the list of the Rottlerin-modulated signaling molecules on the other hand.

In conclusion, whatever the inhibitory mechanism, the results of the current study suggest that Rottlerin may offer a promising mono- or combined-therapeutic approach for a subpopulation of melanomas with constitutively active Ras/Raf mutants, cyclin D1 overexpression, and high NF- κ B activity, for which a limited number of effective drugs are currently available. Studies are in progress to verify/confirm whether Rottlerin treatment longer than 24 h is also able to kill Sk-Mel-28 cells and to assess the mode of cell death.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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