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ORIGINAL ARTICLE

Resveratrol induces SIRT1- and energy–stress-independent inhibition of tumor cell regrowth after low-dose platinum treatment

My Björklund · Jeanette Roos · Vladimir Gogvadze · Maria Shoshan

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Abstract
Purpose To investigate resveratrol (RSV) as a calorie restriction (CR) mimetic potentiator of platinum-based cancer drugs.
Methods In ovarian carcinoma cell lines, the potentiating effects of RSV were assessed in sulforhodamine B-based growth assays and clonogenic assays. Flow cytometry was used to detect cell cycle effects, siRNA transfections for determining the involvement of SIRT1, and Western blotting for the assessment of altered protein expression and of autophagy. Intracellular ATP levels were detected with a commercial kit.
Results Single-dose RSV co-treatment with cisplatin or carboplatin at inefficiently low doses had the clinically interesting effect of preventing regrowth of cancer cells after drug withdrawal. Of three cell lines tested, metastatic cells with low bioenergetic cellular index (i.e., more glycolytic) were particularly sensitive to combination treatment leading to PUMA induction, acute apoptosis, and autophagy. However, inhibition of regrowth and complete loss of clonogenicity was seen also without these events, in other cells. The underlying mechanism(s) was independent of effects reported to underlie the CR-mimetic cancer-preventive potential of RSV. Thus, SIRT1, estrogen receptors, AMPK activation or upregulation of mitobiogenesis, β-F_1-ATPase or PTEN were not involved, and ATP levels did not decrease.
Conclusions RSV is an excellent candidate for potentiation of platinum treatment, rather than a cancer therapeutic drug in its own right. While SIRT1-dependent and lifespan-promoting effects of RSV are well-documented and may dominate in normal cells, the observed potentiation of platinum drugs does not require these mechanisms. We suggest that the responses of cancer cells to RSV differ greatly from those of normal cells.

Keywords Chemotherapy · Platinum · Resveratrol · SIRT1 · Ovarian carcinoma

Introduction

Tumor progression is now known to correlate with extensive alterations in energy metabolism, notably downregulation of mitochondrial production of ATP, i.e., oxidative phosphorylation (OxPhos) and the mitochondrial β-F_0F_1-ATPase, and increased aerobic glycolysis. Accordingly, mitochondrial downregulation selects for an aggressive and resistant phenotype [28]. Moreover, low OxPhos activity and low mitochondrial density contribute to chemoresistance by failing to provide reactive oxygen species in response to cancer drugs including cisplatin [18, 27, 30]. A bioenergetic protein signature (BEC index), involving expression of the β-ATPase F_1 subunit correlates with prognosis in several carcinomas [9, 13]. Increased glycolysis helps meet tumor cell demands not only for ATP but also reducing equivalents, fatty acid, purine, and amino acid

M. Björklund and J. Roos equally contributed to this work.

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M. Björklund · J. Roos · M. Shoshan (✉)
Department of Oncology-Pathology,
Cancer Center Karolinska, Karolinska Institutet,
171 76 Stockholm, Sweden
e-mail: mimmi.shoshan@ki.se

V. Gogvadze
Institute of Environmental Medicine (IMM),
Karolinska Institutet, 171 77 Stockholm, Sweden

syntheses to support increased macromolecule synthesis and proliferation [29, 31]. This scenario of altered energy metabolism has prompted strategies for restoration of mitochondrial function and/or inhibition of glycolysis to improve cancer treatment.

Resveratrol (RSV) is a polyphenol with phytoestrogen features that attracts considerable interest as a chemopreventive, longevity-promoting and now also as an anticancer drug [5]. Its chemopreventive properties are ascribed to effects similar to those of calorie restriction (CR). RSV is frequently used as an activator of the protein deacetylase SIRT1, a NAD^+-dependent metabolic sensor which activates PGC-1α, a major upregulator of mitochondrial biogenesis [33], suggesting that this mechanism at least partially explains the pro-mitochondrial and pro-longevity effects of RSV [10, 16]. Pro-longevity in normal cells is moreover supported by SIRT1-mediated autophagy, as shown with RSV and other drugs [5, 23, 24, 26]. It is unclear, however, whether SIRT1 has different roles in normal and cancer cells, and whether it has oncogenic or tumor-suppressive functions [7]. Similarly, the role of autophagy is complex and can serve both a tumor-suppressive and a pro-survival function in tumor cells under metabolic stress [35]. Suppression rather than stimulation of autophagy could thus be a cancer therapeutic strategy [35]. Other effects of RSV with relevance for anticancer treatment have been reported, e.g., the upregulation of PTEN and inhibition of AKT [1, 4, 34] increased p53 activity [1], but paradoxically also inhibition of the β-ATPase [11]. Altogether, it remains unclear which effects are important for the cancer therapeutic effects of RSV and which for its cancer-preventive effects.

We showed earlier that 2-deoxyglucose, an inhibitor of glycolysis, potentiates platinum treatment in ovarian cancer cell lines and in ascitic tumor cells, and that potentiation correlated with a glycolytic phenotype and low levels of β-F_1-ATPase [12]. The present work was prompted by RSV-mediated inhibition of glycolysis [15] and upregulation of mitochondrial function [16], both suggesting that RSV should potentiate platinum drugs.

Epithelial ovarian carcinoma (EOC) is the fifth leading cause of cancer death in women After debulking surgery, platinum drugs (cisplatin or carboplatin) combined with a taxane are administered. However, relapse to disseminated and platinum-resistant disease is common, and the 5-year survival for EOC is only 40–45% [17].

We found that RSV co-treatment with low-dose cisplatin or carboplatin has the clinically interesting effect of potently preventing post-treatment regrowth of EOC cells even after drug withdrawal. We then focused on determining whether the observed RSV effects involve its CR-mimetic features. The role of SIRT1 was particularly addressed using siRNA-mediated downregulation.

Materials and methods

Cell culture, drug treatment, and assays

The epithelial ovarian carcinoma cell lines A2780, SKOV-3, and CaOv-4 were cultured in RPMI1640 medium (11 mM glucose) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin, and 2 mM L-glutamine (all from Nordic Biolabs AB, Täby, Sweden) and were kept at +37°C in 5% CO_2. For experiments, cells were seeded in microplates, and after overnight incubation, they were treated with freshly diluted cisplatin or carboplatin (Platinol®, Bristol-Myers-Squibb, and Karolinska Hospital Pharmacy, respectively), or resveratrol (Sigma–Aldrich Sweden AB, Stockholm, Sweden) or combinations thereof. The half-maximal inhibitory concentrations (IC_{50}) for each drug and post-treatment regrowth were based on quantification of total cellular protein in controls and treated samples using the sulforhodamine B (SRB)-based TOX6 kit (Sigma–Aldrich Sweden AB). To determine post-treatment regrowth, cells were treated for 48 h, whereafter they were allowed to recuperate in drug-free medium for 72 h. Results at 48 h and 48 + 72 h were calculated as fold increase in SRB absorbance from $t = 0$ h. For clonogenic assays after 48 h treatment, cells were seeded in 9-cm dishes (600 per dish) and cultured in drug-free medium for 2–3 weeks, with media changes every 3rd day. Colonies were stained with Coomassie Blue. Other drugs used were LY294002, 2-deoxyglucose, and metformin (all from Sigma–Aldrich Sweden AB). ATP levels were detected using ATP Colorimetric Assay Kit from BioVision (Labinova AB, Upplands Väsby, Sweden), according to manufacturer′s instructions.

Western blotting

Whole-cell lysates were made in lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris HCl). Protease and phosphatase inhibitor cocktails (P2714 and P5726; Sigma–Aldrich) were added freshly. For LC3 analyses, cells were treated with pepstatin A and protease inhibitor cocktail containing E64a in order to prevent degradation of LC3-II [22]. Samples were loaded for gel electrophoresis at 20–30 μg/sample, and after electrophoresis, they were blotted onto PVDF membranes. Transfer was confirmed using Ponceau S staining. Membranes were blocked for 1 h in 5% milk/PBS-Tween or 3% BSA/TBS-Tween. All antibodies were diluted in blocking agent. Membranes were incubated overnight at +4°C with rabbit primary antibodies to: ATP5B (Atlas Antibodies AB, Stockholm, Sweden, 1:1,000), Hsp60, GAPDH (both from Abcam, Cambridge, UK, 1:4,000), SIRT1 (Sigma-Aldrich, 1:400), phospho-AMPK (1:2,000), AMPK (1:1,000), PTEN (1:500), LC3B (1:1,000), PUMA

Table 1 Cell line features and IC_{50}:s

	A2780			SKOV-3	CaOv-4
	Wt-p53. Untreated primary tumor			p53-deficient. From ascites	Mutant p53* Fallopian tube metastasis
		Mock transfected	SIRT1 siRNA		
Cisplatin (μM)	4	3	3	4	9
Carboplatin (μM)	52	45	45	50	170
RSV (μM)	38	33	28	54	37
RSV (μM) in the absence of glucose	36	ND	ND	55	34

The IC_{50} values are based on SRB assays after 72 h incubation. Each value is the average of IC_{50}:s from at least two separate experiments. *ND* no data

Mock- and siRNA-transfected A2780 cells were seeded at 36 h post-transfection. Knockdown efficiencies are shown in Fig. 4a

* Mutation V147D [36]; this is not a hot spot mutation

(1:1,000) (all from Cell Signaling Technology, InVitro Sweden AB, Stockholm, Sweden), BAX (Becton–Dickinson AB, Stockholm, Sweden 1:1,000), or mouse primary antibody to p53 (1:1,000; Santa Cruz Biotechnology Inc., Heidelberg, Germany), and then for 1 h at room temperature with HRP-conjugated anti-rabbit (1:2,500) or anti-mouse IgG (1:2,000)(both from Abcam). For detection of phospho-AMPK, a positive control from Cell Signaling Technology was used. Probes were developed with Western Lightning Plus-ECL (PerkinElmer, Upplands Väsby, Sweden). Images were captured using FUJI LAS-1000 or Kodak M35 X-omat processor.

Flow cytometry

For cell cycle analyses, cells were treated for 48 h, harvested and fixed in 70% ethanol for 24 h (−20°C), resuspended in PBS containing sodium citrate (1 mg/ml), NP-40 (0.3%), propidium iodide (20 μg/ml; all from Sigma–Aldrich), and RNase A (100 μg/ml, Fermentas AB, Sweden), and incubated for 1 h at room temperature. For analysis of cell death, harvested cells were incubated for 10 min with annexin-V FLUOS (Roche Molecular Biochemicals) and propidium iodide (Sigma–Aldrich) in incubation buffer (10 mM HEPES/NaOH pH7.4, 140 mM NaCl, 5 mM $CaCl_2$). For both types of analysis, 10,000 events/samples were analyzed using a FACS-Calibur flow cytometer and CellQuest software (BD Biosciences).

siRNA transfection

A2780 cells at 60% confluence were transfected with siRNA specific for SIRT1 (Invitrogen Ltd, Paisley, U.K.), or with manufacturer′s negative control (scrambled siRNA), or mock transfected, for 5 h using Lipofectamine-2000 in OptiMEM Reduced Serum Medium (Invitrogen AB) as recommended by the manufacturer. After incubation in antibiotic-free medium for 48 h, cells were reseeded for SRB proliferation assays. SIRT1 protein was evaluated by Western blotting.

Results

Differential cell cycle and cell death responses to RSV and cisplatin in EOC cell lines

The cell lines used here represent different stages of EOC and p53 backgrounds (Table 1). For each one, the growth-inhibitory concentrations (IC_{50}) over 72 h of RSV, cisplatin, and carboplatin were determined. RSV IC_{50}′s were found not to differ greatly (Table 1). Within 48 h, 40 μM RSV had induced robust S-phase accumulation in both A2780 and CaOv-4 cells, although CaOv-4 cells lacked the G1 peak seen in A2780 (Fig. 1a). In CaOv-4, RSV led to 46% cell death (6.5-fold background), while A2780 showed no or little cell death. p53-null SKOV-3 cells showed an intermediate response (Fig. 1b). At 24 h, 20 μM RSV had induced 2-fold cell death only in CaOv-4 cells (not shown).

CaOv-4 cells were the most platinum-resistant (Table 1) and showed more G2/M arrest in response to 2 μM cisplatin (Fig. 1a). With combined RSV + cisplatin, S-phase arrest dominated in both A2780 and CaOv-4 (Fig. 1a).

RSV co-treatment prevents post-treatment regrowth

Apoptosis (cell death) was investigated as induced by 2 μM cisplatin (0.5 × IC_{50} in A2780 and SKOV-3, and 0.2 × IC_{50} in CaOv-4), 40 μM RSV, and combinations thereof. In all cell lines, combination treatment did not increase apoptosis compared with either cisplatin or RSV alone (Fig. 1b). However, while 48 h is sufficient for acute apoptosis to develop, it is not always enough to allow development of

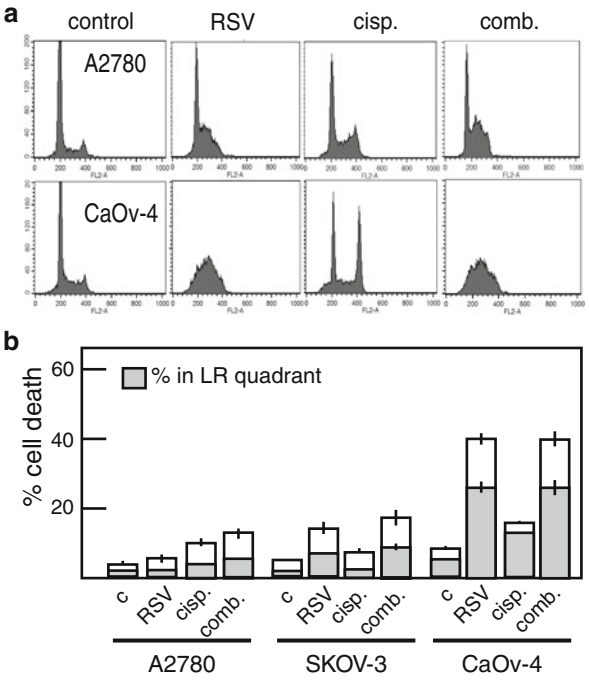
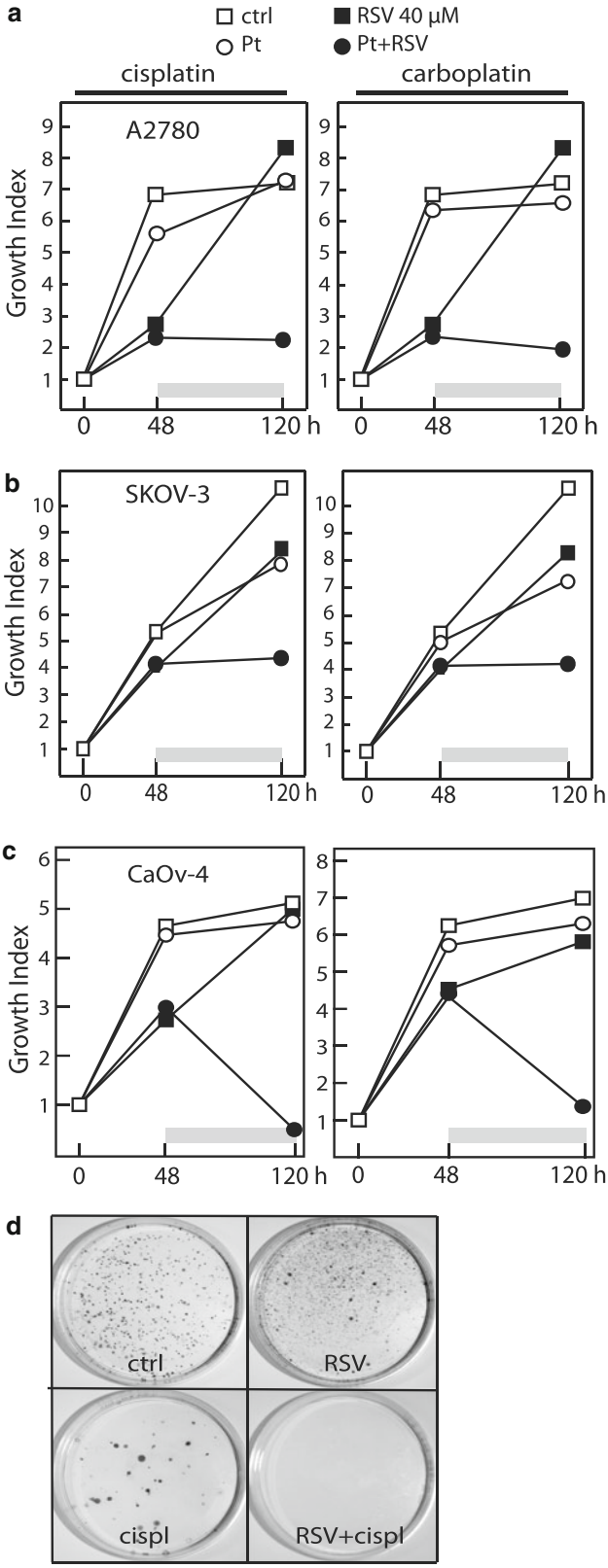


Fig. 1 Cell cycle and cell death responses to RSV. **a** Cell cycle analyses of A2780 and CaOv-4 cells at 48 h after addition of cisplatin (cisp., 2 μM), RSV (40 μM), or the combination (comb.) thereof. **b** AnnexinV-PI analysis of cell death in three EOC cell lines treated as indicated for 48 h. For each treatment, the entire *bar* represents the total percentage of dying cells, i.e., cells in all quadrants of the flow cytometry dot plot except the *lower left*. In each *bar*, the *gray* section indicates percent primary apoptosis, i.e., %cells in the *lower right* (LR) quadrant. The SEM of each value is indicated

effects involving DNA damage, metabolic stress, and autophagy. Therefore, and because post-treatment regrowth of surviving cells is a major clinical problem, we investigated the effects of RSV on post-platinum regrowth capacity. After 48 h with platinum, RSV, or combinations thereof, cells were allowed to recuperate in drug-free medium over 3 days. This protocol confirmed that low-dose platinum (2 μM cisplatin; 16 μM carboplatin corresponding to 0.3 × IC_{50} in A2780 and SKOV-3, and 0.1 × IC_{50} in CaOv-4) was ineffective and allowed rapid regrowth of surviving cells (Fig. 2a–c). Secondly, it demonstrated the reversibility of RSV action, and, thirdly, that combination-treated cells could not resume growth (Fig. 2a–c).

Clonogenic assays showed that 48 h combination treatment completely prevented regrowth also in A2780 cells (Fig. 2d, and Supplementary material). Note the selection for large and fast-growing colonies in the cisplatin-treated sample and the lack of survivors in the combination treatment (Fig. 2d). Regrowth was similarly inhibited in CaOv-4 cells treated with cisplatin or carboplatin in combination with RSV even at 20 μM (Supplementary material).



The data demonstrate that a single co-treatment with RSV potentiates the long-term effects of cisplatin and carboplatin at inefficiently low doses. Moreover, although

◀ **Fig. 2** Prevention of post-platinum regrowth by RSV. Cells were incubated with drugs for 48 h, and post-incubated in drug-free medium for another 72 h, represented by the *gray bar* in each *graph*. Growth index is based on the SRB assay. *Pt* platinum, cisplatin, or carboplatin as indicated. SEM's of quadruplicate samples were smaller than the symbols used. The experiments were repeated at least twice with similar results. **a** A2780; **b** SKOV-3; **c** CaOv-4. **d** Clonogenic regrowth in A2780 cells. After 48 h treatments, cells were reseeded at 600 cells/9-cm dish and incubated in drug-free medium until visible colonies had formed, or approximately 2 weeks, and were stained with Coomassie Blue

acute apoptosis may be involved, it is not required for the prevention of regrowth, as exemplified by A2780 cells.

RSV treatment does not affect SIRT1, β-F_1-ATPase, Hsp60, or PTEN expression or respiration

RSV is often used as a SIRT1 activator, and SIRT1 promotes mitochondrial biogenesis. Baseline and RSV-induced expression of SIRT1 and mitochondrial proteins β-F_1-ATPase and Hsp60 were therefore investigated. Baseline SIRT1 was high in A2780 and low in CaOv-4 (Fig. 3a), i.e., low in the cells with the highest cell death response to RSV. At 24 h, 20–40 μM RSV had not altered expression of any of the three proteins (Fig. 3a, b). Furthermore, although both SIRT1 and β-F_1-ATPase were highest in A2780, there was no correlation in the other two cell lines (Fig. 3b). Altogether, this shows that SIRT1 levels do not correlate with RSV sensitivity and that RSV does not increase mitobiogenesis.

Assessment of Hsp60 allowed estimation of a relative BEC index or ratio of β-F_1-ATPase/Hsp60. A low ratio reflects a more glycolytic (progressed) phenotype [9, 13]. CaOv-4 cells had the lowest BEC index and A2780 the highest (Fig. 3b), possibly reflecting that CaOv-4 originate from a metastasis while A2780 are from a primary tumor and show a differentiation marker expression profile that is closer to that of the normal ovarian surface epithelium (manuscript in preparation). Glycolysis promotes pro-survival signaling via AKT [20]. RSV is reported to block AKT signaling in breast carcinoma cells by inducing PTEN expression [34]. Here, RSV had no effect on PTEN levels (Fig. 3b). The presence or absence of glucose did not affect RSV IC_{50}'s (Table 1), showing that glycolysis/glucose uptake is not protective. Finally, if RSV potentiates platinum by inhibiting AKT signaling, the PI3K inhibitor LY294002 should have a similar effect as RSV. However, the post-platinum regrowth of CaOv-4 cells was unaffected by 50 μM LY294002 (Fig. 3c). Neither was regrowth affected by LY294002 at 10 μM and present throughout the entire 120 h (not shown).

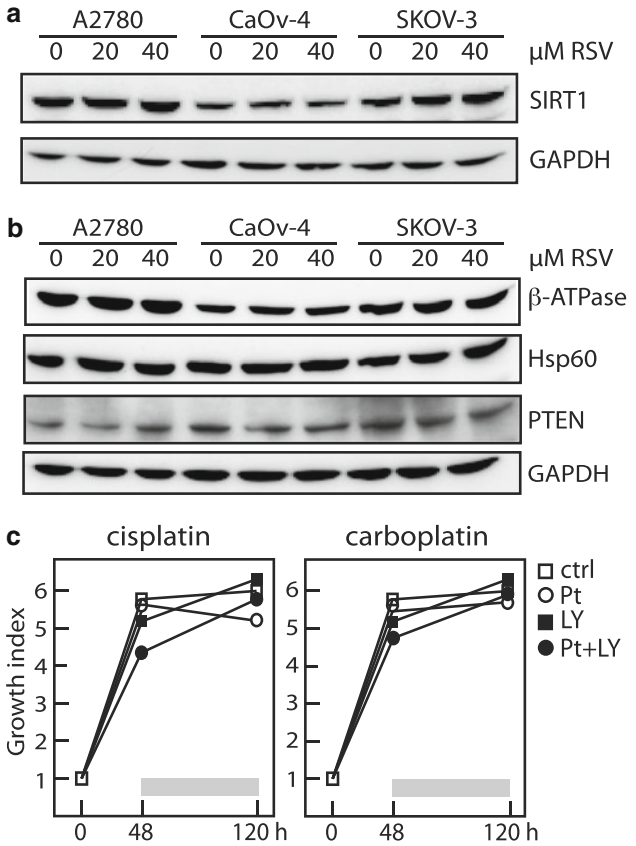


Fig. 3 No effect of RSV on SIRT1, β-F_1-ATPase, Hsp60 or PTEN expression. **a**, **b** Western blots after 24 h incubation with indicated concentrations of RSV. **c** To see if PI3K inhibitor LY294002 (LY; 50 μM) prevents post-platinum outgrowth, CaOv-4 cells were incubated as indicated by symbols for 48 h and were then allowed to recuperate in drug-free medium for another 72 h, represented by the *light gray bar* in each graph. SEM's of the quadruplicates were too small to be shown

If β-F_1-ATPase is inhibited, this should affect respiration. A2780 cells showed similar levels of respiration states in control and combination-treated cells. Samples of combination-treated CaOv-4 cells contained both viable and dying cells but showed identical respiration as in control cells (Supplementary material). We conclude that respiration effects are not involved in the potentiating effect of RSV.

SIRT1 is not required for RSV potentiation of platinum

SIRT1 expression in A2780 cells was knocked down using siRNA transfection. SIRT1 knockdown persisted for up to 6 days. Only successfully transfected cells were used in subsequent experiments (Fig. 4a). Compared with mock transfected, SIRT1-siRNA-transfected cells had a slightly lower IC_{50} for RSV (Table 1). In regrowth assays, knockdown cells were slightly more sensitive than mock transfected to combination

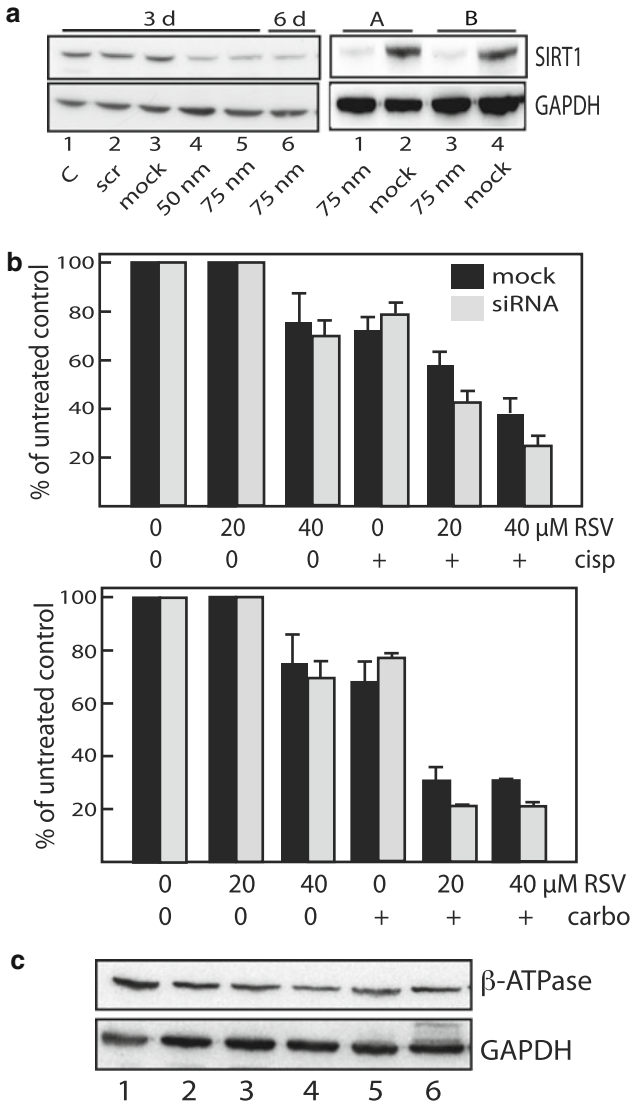


Fig. 4 Effects of SIRT1-siRNA transfection. **a** A2780 were transfected with SIRT1-siRNA as described and resulting SIRT1 expression was assessed by Western blot. *Left lane 1* non-transfected control (C), *lane 2* scrambled siRNA (scr), *lane 3* mock transfected, *lane 4* 50 nmol siRNA, 3 days post-transfection, *lane 5–6* 75 nmol siRNA, 3 and 6 days post-transfection. *Right* 3 days post-transfection 75 nmol, *lanes 1–2* transfection A, transfected and mock transfected, *lanes 3–4* transfection B, transfected and mock transfected. Transfections A and B both yielded results on IC_{50} (Table 1) and post-treatment regrowth (Fig. 4b). **b** Mock- and siRNA-transfected cells were subjected to regrowth assays as in Fig. 2. Shown here are results at 120 h, expressed as % of untreated control. *Top* regrowth after cisplatin (cisp, 2 µM), *bottom* regrowth after carboplatin (carbo; 16 µM). **c** Lysates from transfection A were analyzed for the expression of β-F_1-ATPase. *Lane 1* non-transfected, *lane 2* scrambled siRNA, *lane 3*, mock transfected, *lane 4* 50 nmol siRNA, 3 days post-transfection, *lane 5–6*: 75 nmol siRNA, 3 and 6 days post-transfection

treatments (Fig. 4b). SIRT1-siRNA did not affect β-F_1-ATPase expression (Fig. 4c). We conclude that the observed effects of RSV do not depend on SIRT1.

RSV does not induce energy–stress

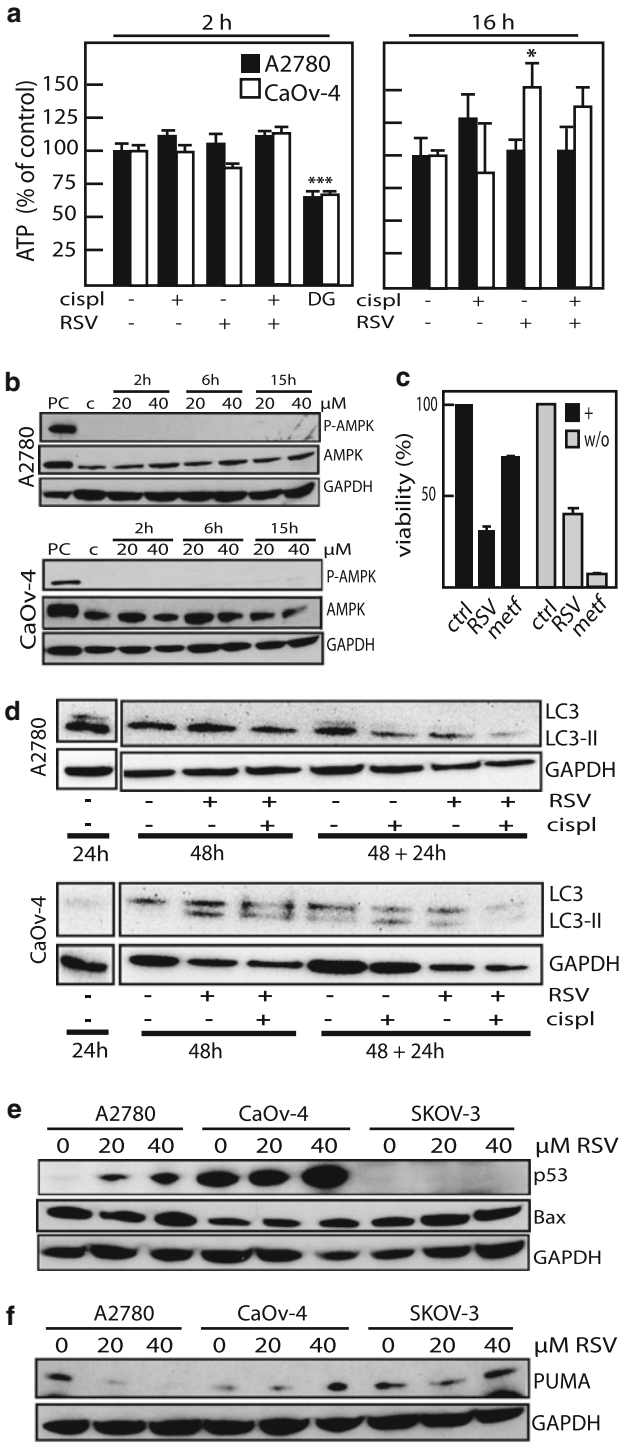
RSV is reported to inhibit glycolysis [15]. Using the same protocol as in Fig. 2, we have reported that the glycolysis inhibitor 2-deoxyglucose (2-DG) prevents post-platinum regrowth in CaOv-4 cells [12]. Here, we observed a major net loss of CaOv-4 cells treated with RSV + cisplatin (Fig. 2) that was not seen with 2-DG + cisplatin [12]. That the two drugs differ with respect to energy metabolism was confirmed by assessment of intracellular ATP: while 2-DG led to decreased ATP, RSV increased it in both A2780 and CaOv-4 cells (Fig. 5a). In line with no ATP deficit in RSV-treated cells, RSV did not induce AMPK phosphorylation (Fig. 5b). Supported by observations that RSV did not affect expression of the glucose transporter GLUT1 nor extracellular acidification (not shown), the results demonstrate that the effect and mechanism of RSV are highly dissimilar to those of 2-DG and do not involve glycolysis inhibition and/or decreased ATP or AMPK activation.

Metformin is a diabetes drug which, similar to RSV and 2-DG, is a CR mimetic and has anti-tumoral effects believed to involve AMPK activation [21]. Unlike RSV, metformin was highly cytotoxic to CaOv-4 cells in the absence of glucose (Fig. 5c). We conclude that the effect and mechanism of RSV are dissimilar also to those of metformin.

To assess autophagy in the setting of regrowth assays, lysates were prepared after 48 h of treatment and after 48 + 24 h in drug-free medium. Autophagy was then assessed as induction of the LC3 protein and its processed form, the autophagosome marker LC3-II (Fig. 5d). Untreated A2780 cells had high-level background autophagy compared with CaOv-4, and RSV or combination treatment did not further increase it. In CaOv-4 cells, RSV induced LC3-II; interestingly, cisplatin also led to post-treatment induction of LC3-II. In sum, an autophagic response was induced by cisplatin, RSV, and the combination in CaOv-4 but not in A2780 cells.

RSV induces p53 and PUMA, but not Bax, in sensitive cells

Having excluded several metabolism-related targets of RSV to explain the observed effects, we then examined apoptosis regulation via p53, Bax, and PUMA. While Bcl-2 levels were not altered by RSV in any cell line (not shown), p53 was induced in A2780 and CaOv-4 (Fig. 5e). p53 is mutated in CaOv-4, albeit not at a "hot spot site" [36], while SKOV-3 is p53-deficient. Bax was high in A2780 cells (Fig. 5e), despite low baseline apoptosis (Fig. 1a), suggesting they are resistant to Bax-mediated apoptosis. Moreover, RSV treatment led to loss of PUMA protein in A2780 (Fig. 5f), which likely contributes to lack of acute



◀ **Fig. 5** Effects on energy metabolism and apoptosis. **a** Intracellular ATP in A2780 (*black*) and CaOv-4 (*white*) cells was assessed in quadruplicates at 2 and 16 h of treatments with cisplatin (cispl; 2 μM) and RSV (40 μM). 2-deoxyglucose (DG; 5 mM) was used as a control for glycolysis inhibition ($P < 0.001$). The RSV-induced increase at 16 h was significant ($P < 0.05$). Similar results were obtained in another experiment. **b** Phospho- and total AMPK in A2780 and CaOv-4 cells after indicated treatments with RSV. As with all lysates in this work, they were made in the presence of phosphatase inhibitors. 30 μg protein was loaded per lane. The experiment was repeated three times. *PC* positive control lysate. **c** Viability of CaOv-4 after 72 h in RSV (40 μM) or metformin (metf; 20 mM) in the presence (*black*) or absence of glucose (*gray*). **d** Western blots of LC3 and the autophagy marker LC3-II in A2780 and CaOv-4 cells after treatments with RSV (40 μM) ± cisplatin (2 μM). Controls at 24 h are from the same membrane as the subsequent samples; the intervening sample was irrelevant. The experiment was repeated with similar results. **e** p53 and Bax expression after 24 h in 20–40 μM RSV. **f** PUMA expression after 24 h in 20–40 μM RSV

apoptosis in these cells. In sensitive CaOv-4 cells, PUMA but not Bax was induced (Fig. 5e, f).

Discussion

Whether due to resistant subpopulations or insufficient concentrations of chemotherapeutic drugs, post-treatment regrowth of tumor cells remains a major clinical problem. Nevertheless, such regrowth is an aspect that is frequently overlooked in experimental studies.

We show here that combining resveratrol (RSV) and low-dose platinum (Pt; cisplatin or carboplatin) prevented drug-free regrowth and indeed induced irreversible growth inhibition and loss of clonogenicity. By contrast, cells treated with Pt or RSV per se readily resumed growth in drug-free medium. Combination-induced growth ablation was seen both in A2780 cells that did not undergo acute apoptosis, and in platinum-resistant, metastatic, and glycolytic CaOv-4 cells in which RSV did induce apoptosis. It is of general interest for the planning and interpretation of similar in vitro studies that the total antiproliferative effect takes longer than 48 h to become manifest, and had we performed only apoptosis assays only at this timepoint, we would not have noted the potential of platinum–RSV combinations. Importantly, the clinical implications are interesting not only because of the long-term single-dose potentiation but because platinum could be used at such low doses that they per se were not antiproliferative.

Initially, we hypothesized that the altered energy metabolism of cancer cells—notably increased glycolysis and mitochondrial alterations—provides a basis for the observed anticancer effects of RSV. We focused on three target areas pertaining to metabolism: the deacetylase SIRT1 reported to mediate RSV effects on life-span, metabolism, and mitochondrial biogenesis [8, 16]; mitochondrial markers and β-F_1-ATPase expression as indicators of metabolic phenotype and progression [9, 13, 18, 19, 28], and of the autophagic response known as mitophagy, and also on β-F_1-ATPase as a known target of RSV, at least in vitro [11]; thirdly, on glycolysis, ATP levels and AMPK as readouts of energy–stress such as induced by CR mimetics.

RSV is extensively used as a SIRT1 activator. Here, higher basal levels of SIRT1 in A2780 than in CaOv-4

immediately suggested that SIRT1 is not the target in our experimental system. This was confirmed by our siRNA studies that clearly show that the RSV effects did not depend on SIRT1. The putative RSV activation of SIRT1 may indeed be an in vitro artifact [2] and we therefore examined whether RSV instead upregulates SIRT1 levels. This was not the case. Moreover, it was recently shown that RSV at 100 μM actually decreased SIRT1 protein, and that neither this decrease nor apoptosis induction was prevented by SIRT1 inhibitors [25].

SIRT1 promotes mitobiogenesis [14], but based on unaltered expression of β-F_1-ATPase and Hsp60, we could exclude mitobiogenesis—as well as mitophagy—to explain increased platinum sensitivity. Estrogen signaling also promotes mitobiogenesis [14], but a phytoestrogenic effect of RSV could be excluded, since the cell lines do not express estrogen receptors ERα/β (PCR and immunochemistry results, courtesy of Dr. J.Hartman, Karolinska Institute).

RSV has been reported to block glucose uptake over 24 h in ovarian cancer cells [15]. However, this report did not take into account that RSV-treated cells have arrested to the same extent that glucose uptake was reduced, while controls proliferated. Here, we found that RSV increased rather than decreased ATP, and AMPK was not activated, suggesting that if RSV did block glucose uptake, this was compensated for by other fuels. RSV-induced autophagy has been reported [15, 23, 26]; we also observe induction of LC3 and the autophagosomal cleavage product LC3-II by RSV ± cisplatin.

RSV did induce p53, a possible response to nutrient stress, DNA damage, or DNA repair, which have all been reported for RSV [1]. However, while p53 induction is associated with G1 arrest, we observed massive S-phase arrest in line with RSV-mediated inhibition of Cdc25C/Cdk2 [32] and downregulation of cyclinB [3, 32]. p53-mediated apoptosis involves upregulation of PUMA and Bax. In sensitive CaOv-4 cells, RSV induced p53 and PUMA. In A2780 cells, baseline expression of PUMA and Bax was high, in line with PUMA-regulated autophagy via Bax [37]. For unknown reasons, RSV decreased PUMA levels in these cells, possibly explaining their lack of RSV-induced apoptosis.

In summary, we have demonstrated a major potentiating effect of RSV when combined with low-dose platinum. This effect is established within 24–48 h, and may involve, but does not require, acute apoptosis. The efficiency of the irreversible mechanism is manifested as the prevention of tumor cell regrowth. This longer-term aspect of drug effects is too often overlooked in preclinical studies that have tended to focus on acute apoptosis.

The underlying mechanism of RSV was independent of many effects reported to underlie in particular its cancer-preventive potential. Thus, SIRT1, estrogen receptor signaling, upregulation of mitobiogenesis, β-F_1-ATPase, and PTEN/PI3 K were not involved, and ATP levels and respiration were not decreased. However, the cancer-preventive mechanisms of RSV are not necessarily the same as the cancer therapeutic [6]. While the anti-oxidant, CR-mimetic pro-autophagic and lifespan-promoting effects of RSV are well-documented and dominate in the normal end of the spectrum, our results underscore that its cytotoxic effects on cancer cells need to be further investigated and that SIRT1 is not a relevant target. With regard to other reports, it may also be of significance that we use fairly low concentrations of RSV (20–40 μM, often 50–300 μM in other studies).

Importantly, we suggest that RSV is an excellent candidate for potentiation of platinum treatment, rather than a cancer therapeutic drug in its own right. Furthermore, we have used single-dose treatments, while clinical treatment involves continuous drug administration and thus allows for lower in vivo concentrations than commonly used in preclinical studies (for cisplatin, 50 μM or more is often used). Clinical co-treatment with RSV may therefore have remarkable potential.

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Conflict of interest None.

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