[Frontiers in Bioscience 17, 498-508, January 1, 2012]

Resveratrol and diallyl disulfide enhance curcumin-induced sarcoma cell apoptosis

Laura Masuelli[1], Laura Marzocchella[2], Chiara Focaccetti[3], Ilaria Tresoldi[2], Camilla Palumbo[2], Valerio Izzi[2], Monica Benvenuto[2], Massimo Fantini[2], Florigio Lista[4], Umberto Tarantino[5], Andrea Modesti[2], Fabio Galvano[6], Roberto Bei[2]

[1]Department of Experimental Medicine, University of Rome, Sapienza, Rome, Italy, [2]Department of Experimental Medicine and Biochemical Sciences, University of Rome, Tor Vergata, Rome, Italy, [3]Department of Biology, STA, Rome, Italy, [4]Army Medical and Veterinary Research Center, Rome, Italy, [5]Department of Surgery, University of Rome, Tor Vergata, Rome, Italy, [6]Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania, Italy

TABLE OF CONTENTS

1.ABSTRACT

Malignant tumors of mesenchimal origin such as rhabdomyosarcoma and osteosarcoma are highly aggressive pedriatic malignancies with a poor prognosis. Indeed, the initial response to chemotherapy is followed by chemoresistance. Diallyl disulfide (DADS), resveratrol (RES) and curcumin (CUR) are dietary chemopreventive phytochemicals which have been reported to have antineoplastic activity on rhabdomyosarcoma and osteosarcoma cells as single drugs. In this study we evaluated whether, as compared to the single compounds, the combination of DADS+RES, DADS+CUR and RES+CUR resulted in an enhancement of their antitumor potential on malignant rhabdoid (SJ-RH4, RD/18) or osteosarcoma (Saos-2) cell lines. Through FACS analysis and activated caspase-3 labeling we demonstrate that CUR induces apoptosis of rabdomyosarcoma and osteosarcoma cells and that this effect is potentiated when CUR is combined with RES or DADS. Further, we explored the effects of the compounds, alone or in combination, on signal transduction pathways involved in apoptosis and growth of cancer cells and show that in rhabdomyosarcoma cells the apoptotic effect of CUR, either alone or in combination, is independent of p53 activity. Our findings suggest that CUR and CUR-based combinations may have relevance for the treatment of p53-deficient cancers, which are often unaffected by conventional chemotherapies or radiotherapy.

2. INTRODUCTION

Malignant tumors of mesenchimal origin are in most of the cases highly aggressive pedriatic malignancies and although the prognosis of these tumors has improved, it still remains poor (1-3). Current treatment modalities are based on the combination of chemotherapy and radiotherapy (1-3). However, the initial response to chemotherapy is followed by chemoresistance (1-3). Thus, discovery and development of innovative drugs can supplement the pharmaceutical armamentarium presently used for the treatment of rhabdomyosarcoma and osteosarcoma (4-5). Diallyl disulfide (DADS), an organo-sulfur compound derived from garlic (*Allium sativum*) has been demonstrated to inhibit proliferation of various types of tumors (6-9). Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) (RES), a polyphenol compound isolated from grapes, berries, plums, peanuts and pines, has several biological properties, including antioxidant, anti-inflammatory, anticancer and anti-aging activities (10-12). Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (CUR), a polyphenol compound found in the spice turmeric, a product of the plant *Curcuma longa,* has been revealed to have anti-tumor, anti-inflammatory, antioxidant, immunomodulatory and antimicrobial activities both in rodents and in humans (13-15).

It has been reported that DADS, RES and CUR inhibit the growth of rhabdomyosarcoma and osteosarcoma cell lines when employed as single drugs (16-23). In

particular, RES has been found to inhibit the growth of osteosarcoma cells through the activation of the ERKs/p53 signaling pathway (17) and to induce apoptosis with minor effects on normal osteoblasts (16). Further, it exerts a strong inhibition of rhabdomyosarcoma cell proliferation (18). CUR has been demonstrated to induce cell cycle arrest and apoptosis in human osteosarcoma cells (19-20) and to inhibit growth of Rh1 and Rh30 rhabdomyosarcoma cells by inhibiting phosphorylation of the mammalian target of rapamycin (mTOR) (21). In addition, CUR has been reported to induce apoptosis and to regulate radiosensitivity in Ewing's sarcoma cells (22, 23). Finally, garlic extract and Diallyl trisulfide inhibited the growth of rat sarcoma cells and the osteosarcoma cell line Saos-2, respectively (24, 25).

The aim of this study was to determine whether the combination of two of these dietary phytochemicals (DADS+RES, DADS+CUR and RES+CUR) resulted in an enhancement of their antitumor activities on malignant rhabdoid (SJ-RH4, RD/18) or osteosarcoma (Saos-2) cell lines, as compared to the single compounds. In addition we explored their effect and interaction on signal transduction pathways involved in apoptosis and growth of cancer cells.

3. MATERIALS AND METHODS

3.1. Reagents

DMSO, diallyl disulphide (DADS), trans-resveratrol (RES), curcumin from *Curcuma Longa* (CUR), Sulforhodamine B (SRB), staurosporine and Hoechst 33342 were purchased from Sigma-Aldrich (Milan, Italy). Rabbit polyclonal anti-Bax and mouse monoclonal anti-Bcl-2 antibodies were obtained from BD Pharmingen (BD Biosciences). Antibodies against ERK1/2 (C-14), phospho-ERK (E-4), AKT and p53 (DO-1) were obtained from Santa Cruz Biotechnology (CA, USA). The anti-activated caspase 3 antibody was purchased from Cell Signaling Technology (MA, USA). The goat anti-rabbit IgG Alexa fluor-594-conjugated secondary antibody was from Invitrogen (Milan, Italy) and the goat anti-mouse or -rabbit IgG peroxidase-conjugated secondary antibodies were from Sigma-Aldrich.

3.2. Cell lines and treatments

Malignant rhabdoid (SJ-RH4, RD/18) and osteosarcoma (Saos-2) cell lines were obtained from Prof. P.L. Lollini (University of Bologna, Italy) and Prof. C. Dominici (Sapienza University, Rome, Italy) and maintained in RPMI containing 10% fetal bovine serum, 100 u/ml penicillin, and 100 µg/ml streptomycin (complete medium). Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO_2. SJ-RH4 and RD/18 cells are of alveolar and embryonal histotype respectively (26). For treatments, cells were incubated for the indicated times in the presence of DADS, RES and CUR alone or in combination of two compounds (dose range 6-50 µM) or vehicle control (DMSO ≤0.1%).

3.3. Sulforhodamine B (SRB) assay

Cells were seeded at 4 x 10^3/well in 96-well plates and incubated at 37°C to allow cell attachment. After 24 hours, the medium was changed and the cells were treated with DADS, RES and CUR alone or in combination or with DMSO and incubated for 48 hours. Cells were then fixed with cold trichloroacetic acid (final concentration 10%) for 1 hour at 4°C. After 4 washes with distilled water, the plates were air-dried and stained for 30 min with 0.4% (wt/vol) SRB in 1% acetic acid. After 4 washes with 1% acetic acid to remove the unbound dye, the plates were air-dried and cell-bound SRB was dissolved with 200 µl/well of 10 mM unbuffered Tris base solution, pH 10. The optical density (O.D.) of the samples was determined at 540 nm using a spectrophotometric plate-reader. The percentage survival of the cultures treated with the compounds or DMSO was calculated by normalization of their O.D. values to those of untreated control cultures (27, 28). The experiments were performed in triplicate and repeated three times.

3.4. Ultrastructural analysis

Ultrastructural analyses were performed on SJ-RH4 cells untreated or treated with DADS, RES and CUR, alone or in combination. Cells were fixed in 2.5% glutaraldehyde in PBS pH 7.4 and the samples were processed for transmission electron microscopy following routine procedures (29, 30).

3.5. FACS analysis

Asynchronized log-phase growing cells (60% confluent, about 2.5 x 10^5/well in 6-well plates) were treated with DADS, RES and CUR alone or in combination or with DMSO in complete culture medium. After 48 hours, adherent as well as suspended cells were harvested, centrifuged at 1,500 rpm for 10 min and washed twice with cold phosphate buffered saline (PBS) as previously described (28, 31). Cell pellets were resuspended in 70% ethanol for 1 hour at −20°C. Cells were then washed twice with cold PBS, centrifuged at 1,500 rpm for 10 min, incubated for 1 hour in the dark with propidium iodide (25 µg/ml final concentration in 0.1% citrate and 0.1% Triton X-100) and analyzed by flow cytometry using a FACSCalibur cytometer running CellQuest software.

3.6. *In situ* detection of apoptosis

For *in situ* detection of programmed cell death, SJ-RH4 and Saos-2 cells were seeded at 5×10^3 cells/well in 8-well chamber-slides and, after 24 hours, treated with 25 µM of DADS, RES and CUR alone or in combination of two compounds, or with vehicle control. After 48 hours, cells were fixed in 4% formaldehyde for 15 min, washed and incubated with an anti-activated caspase-3 polyclonal antibody for 1 hour. After additional washings the cells were labeled with a goat anti-rabbit IgG Alexa fluor-594-conjugated antibody for 30 min (32). After a third washing the cells were incubated with 0.1 µg/ml Hoechst 33342 and mounted under a coverslip with glycerol. Cells treated for 24 hours with staurosporine 1 µM were used as positive control. The percentage of apoptotic cells was calculated by determining the ratio between the cells positive for activated caspase-3 and the total number of cells present in

five randomly chosen microscopic fields. Cell counts were done in a blinded fashion.

3.7. Preparation of cell lysates and Western Blotting

About 1 x 10^6 cells were seeded in 100 mm tissue culture dishes 24 hours prior to the addition of 25 μM of compounds alone or in combination or vehicle control. For determining Erk phosphorylation levels upon serum stimulation, SJ-RH4 cells were treated for 32 hours with 25 μM of compounds or DMSO, starved for 16 hours in serum-free medium containing 0.2% bovine serum albumin in the presence of compounds or DMSO and then either left unstimulated or stimulated for 5 minutes with 20% of serum. At the endpoint of the experiments, the cells were harvested, washed twice with cold PBS and lysed in RIPA buffer (Triton X-100 1%, SDS 0.1%, NaCl 200 mM, Tris HCl 50 mM pH 7.5, PMSF 1 mM, NaOV 1 mM). After 30 minutes at 4°C, the mixtures were centrifuged at 12,000 g for 15 minutes and the supernatants were analyzed by western blotting (32, 33).

For immunoblot analysis, 50 μg of cell lysates were resolved in 10% SDS-PAGE and then transferred to nitrocellulose membranes. Equal loading and transfer of proteins was verified by Ponceau red staining of the filters. After blocking, the membranes were incubated with specific primary antibodies at the concentration of 1-2 μg/ml overnight at 4˚C. After washing, the filters were incubated with goat anti-mouse or -rabbit IgG peroxidase-conjugated antibodies and developed by chemiluminescence as previously described (32-34).

3.8. Statistical analysis

Data distribution of cell survival and FACS analyses were preliminarily verified by the Kolmogorov-Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were regarded as significant when p value was less than 0.05. Differences in number of apoptotic cells were evaluated by a two-tailed t-test.

4. RESULTS

4.1. Inhibition of malignant rhabdoid (SJ-RH4, RD/18) and osteosarcoma (Saos-2) cell survival by DADS, RES and CUR alone or in combination

Survival of malignant rhabdoid (SJ-RH4, RD/18) or osteosarcoma (Saos-2) cells was evaluated by the SRB assay after exposure to increasing doses of DADS, RES and CUR (6 to 50 μM), alone or in combination of two compounds (DADS+RES, DADS+CUR, RES+CUR) or vehicle control for 48 hours. CUR was the most effective compound inhibiting cell survival (Figure 1). The effect of CUR was dose-dependent and gained statistical significance at all doses tested as compared to vehicle control treatments. RES significantly decreased cell survival in all three cell lines at high doses (25-50 μM), whereas at 12 μM it was effective only in rhabdomyosarcoma cells. On the other hand, DADS significantly inhibited cell growth of the SJ-RH4 cell line only and only at the highest concentration (Figure 1). Besides, the effects obtained with equimolar combinations of two compounds (DADS+RES, DADS+CUR, RES+CUR) were not significantly different from those obtained using the more potent compound present in each combination (Figure 1).

4.2. Morphological features of SJ-RH4 cells after treatment with DADS, RES and CUR alone or in combination

Morphological features of SJ-RH4 cells after treatment with DADS, RES and CUR, alone or in combination at the concentration of 25 μM were examined by transmission electron microscopy. Untreated and DMSO-treated cells were used for comparison. Treatment of cells with DMSO did not cause significant changes as compared to untreated cells (not shown). DMSO-treated SJ-RH4 cells (Figure 2, a) showed heterogeneous forms with a predominance of stretched over round cells. The nuclei appeared large, mainly formed of euchromatin with low dense heterochromatin in the periphery. In the cytoplasm numerous mitochondria in the condensed phase, cisterns of rough endoplasmic reticulum, glycogen, rare vacuoles, and not organized filaments were detected. Conversely, treatment with DADS induced the presence of numerous cytoplasmic vacuoles, surrounded by single or double membrane, the latter being likely of autophagic origin (Figure 2, b). In addition, some mitochondria were swollen and the rough endoplasmic reticulum decreased. Treatment with RES induced the presence of cracks in the cytoplasm, while major alterations were found in the mitochondria which appeared greatly shrunken and condensed (Figure 2, c). Among all the treatments, CUR was confirmed to be the most harmful to the cells. In fact, most of the cells showed alterations related to apoptosis, with condensation of cytoplasm, presence of vacuoles of various sizes, intact mitochondria and nuclear pyknosis. Rare features of necrosis were also observed (Figure 2, d). Cells simultaneously treated with the combination DADS+RES, appeared swollen with rarefaction of the cytoplasm in which mitochondria appeared swollen as well and with abnormal cristae. In addition several vacuoles were observed (Figure 2, e). DADS+CUR and RES+CUR combined treatments produced morphological effects similar to those observed in the samples treated with CUR only. In fact, apoptotic features such as cell shrinkage, nuclear condensation and presence of numerous cytoplasmic vacuoles were prevalent (Figure 2, f-g).

4.3. RES and DADS potentiate the apoptotic effect of CUR on SJ-RH4 and Saos-2 cell lines

In order to determine the effect of the compounds alone or in combination on apoptosis and cell cycle distribution of malignant rhabdoid (SJ-RH4, RD/18) or osteosarcoma (Saos-2) cells, a FACS analysis of DNA content was performed. Figure 3 shows a representative experiment in which the effects of increasing doses of compounds administered alone or in combination are compared with each other as well as with those obtained with DMSO vehicle only. As compared to DMSO, DADS treatment scarcely affected cell cycle in the different cell lines, except that RD/18 cells treated with 6-50 μM DADS accumulated in the G2/M phase ($p<0.05$). Treatment with RES—resulted in a dose-dependent decrease in the

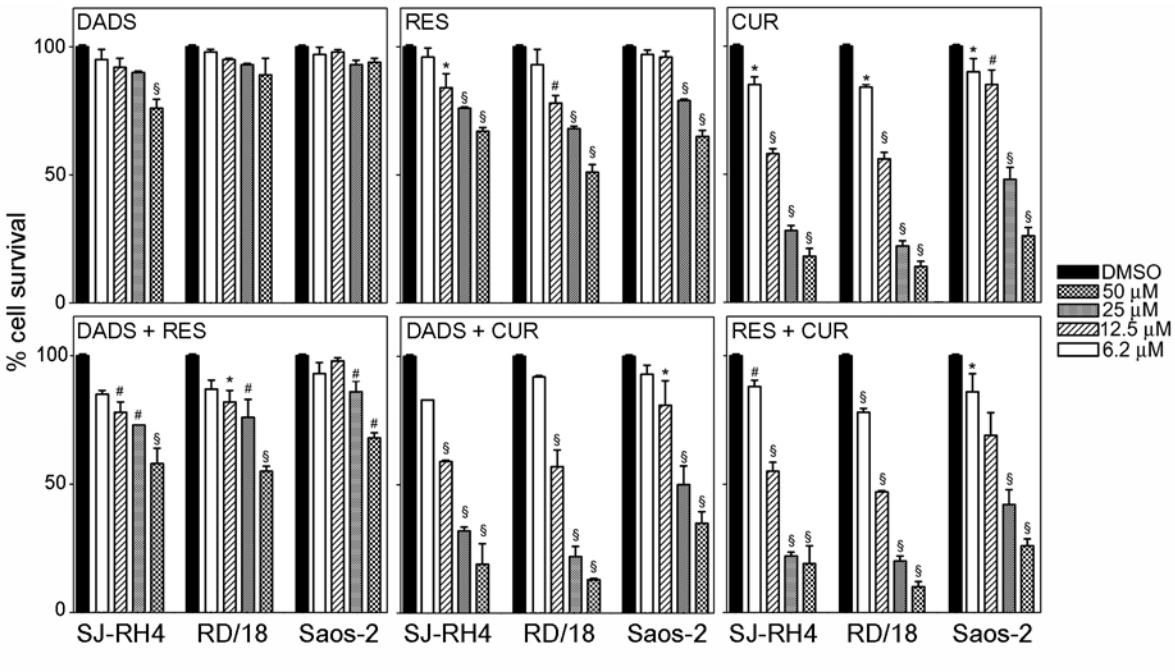

Figure 1. Effect of DADS, RES and CUR alone or in combination on rhabdomyosacoma or osteosarcoma cell survival. Survival of rhabdomyosacoma (SJ-RH4, RD/18) and osteosarcoma (Saos-2) cells was assessed by the SRB assay after 48 hours of treatment with DMSO or DADS, RES and CUR alone or in equimolar combinations of two compounds (DADS+RES, DADS+CUR, RES+CUR). The percentage survival of the cultures treated with compounds or DMSO as vehicle control was calculated by normalization of their O.D. values to those of untreated control cultures. Results reported are mean ± SD values from three experiments performed in triplicates. #: $p \leq 0.0001$; §: $p \leq 0.001$; *: $p \leq 0.05$ *vs.* cultures treated with DMSO.

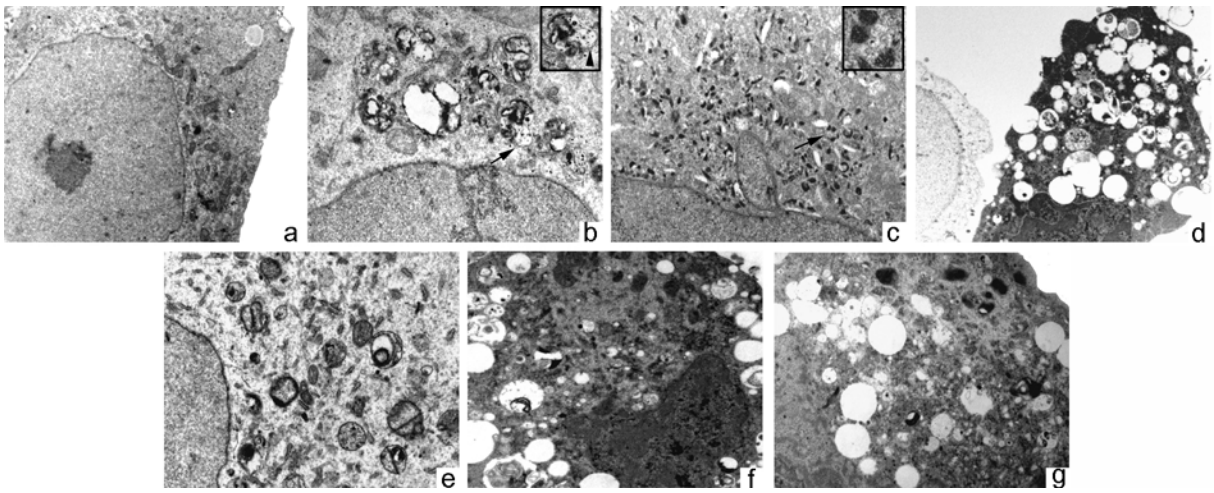

Figure 2. Morphological features of SJ-RH4 cells after treatment with DADS, RES and CUR alone or in combination. Ultrastructural analysis performed on SJ-RH4 cells treated for 48 hours with (a) DMSO as vehicle control (original magnification (o.m.) x3700), (b) DADS (o.m. x6500), (c) RES (o.m. x6500), (d) CUR (o.m. x3700), (e) DADS+RES (o.m. x6500), (f) DADS+CUR (o.m. x6500), and (g) RES+CUR (o.m. x5900), at 25 µM. The arrow in (b) indicates a double membrane vacuole (high magnification in the square); the arrow in (c) indicates mitochondrial alterations (high magnification in the square).

percentage of cells in G0/G1 and G2/M phases ($p<0.05$) and an increased percentage of cells in the S phase ($p<0.05$). In addition, RES treatment induced a modest to moderate increase in the apoptotic sub-G0 population at high doses (Figure 3 and Table 1). On the other hand, in all cell lines treatment with CUR resulted in a marked, dose-dependent increase of the percentage cells in the sub-G0 phase and a decrease in the number of cells in G0/G1,

Table 1. Effects of DADS, RES and CUR alone or in combination on the percentage of rhabdomyosarcoma and osteosarcoma cells in the sub G0 phase

	SJ-RH4		RD/18		Saos-2	
	mean[1]	p	mean	p	mean	p
DMS0	1,09		0,98		1,10	
DADS 6.2	0,82		0,63		0,82	
DADS 12.5	1,18		0,58		1,10	
DADS 25	1,06		0,49		1,43	
DADS 50	1,88		0,89		1,34	
RES 6.2	1,04		0,99		1,80	
RES 12.5	1,21		1,49		1,81	
RES 25	2,46		8,21	2	3,97	2
RES 50	3,29	2	19,84	2	6,46	2
CUR 6.2	0,78		1,18		1,03	
CUR 12.5	7,69	2	2,70		1,53	
CUR 25	54,26	2	73,37	2	17,6	2
CUR 50	72,18	2	80,37	2	66,3	2
DADS+RES 6.2	0,68		2,96		1,60	
DADS+RES 12.5	1,24		1,34		3,10	
DADS+RES 25	2,36		7,16		10,42	
DADS+RES 50	5,33		28,54	4	14,48	
DADS+CUR 6.2	0,88		0,82		1,10	
DADS+CUR 12.5	10,42		2,74		1,57	
DADS+CUR 25	64,82	3	75,13		25,40	
DADS+CUR 50	81,98	3	83,55		66,80	
RES+CUR 6.2	1,19		1,35		2,29	
RES+CUR 12.5	18,70	3	6,67		6,72	3
RES+CUR 25	71,30	3	81,43		39,70	3
RES+CUR 50	84,60	3	90,44		82,23	3

[1]Percentage of cells in the sub G0 phase were calculated using CellQuest software. The data are representative of three experiments. DADS, RES and CUR were used in the range 6.2-50 μM. Statistical significance of the effects obtained with single-compound treatments was calculated *vs.* those obtained with DMSO ([2]$p<0.05$ *vs.* DMSO); statistical significance of the effects obtained with combined treatments was calculated *vs.* those obtained with the more potent compound present in each combination ([3]$p<0.001$ *vs.* CUR; [4]$p<0.001$ *vs.* RES)

($p<0.001$), S ($p<0.001$) and G2/M ($p<0.001$) (Figure 3 and Table 1).

As for the effects of combined treatments, the DADS+RES combination induced a dose-dependent decrease in the percentage of SJ-RH4 and RD/18 cells in G0/G1 ($p<0.05$) and G2/M ($p<0.01$) phases and an increase of those in S phase ($p<0.01$), similar to those obtained with RES alone. Thus RES counteracted the increase of cells in G2/M induced by DADS in the RD/18 cell line. In addition, in RD/18 cells this combination treatment at the higher dose induced an increase in the percentage of cells in sub-G0 as compared to treatment with RES alone ($p<0.001$) (Figure 3 and Table 1). In particular, the apoptotic rate obtained with the combined treatment was 1.4 times higher than that obtained with RES alone, *i.e.* the more potent compound present in the combination. Conversely, in the Saos-2 cell line the treatment with DADS+RES significantly decreased the percentage of cells in G0/G1 as compared to DADS and RES alone ($p<0.05$), while it did not affect the G2/M phase. Based on these results, DADS and RES appeared to interact in a cell-context dependent manner.

Although the effects of DADS+CUR were on the whole similar to those obtained with CUR alone, at high concentrations this combination induced a significant increase in the percentage of SJ-RH4 cells in sub-G0 as compared to DADS and CUR alone ($p<0.001$) (Figure 3 and Table 1). Similarly, treatment with RES+CUR induced in SJ-RH4 and Saos-2 a significant, dose-dependent increase in the percentage of apoptotic, sub-G0 cells as compared to either compound administered alone ($p<0.001$), accompanied by a decrease in G2/M, G0/G1 and S phases ($p<0.001$) (Figure 3 and Table 1). From the comparison of the apoptotic rates obtained with the RES+CUR combination and those obtained with CUR alone, it emerged that in SJ-RH4 cells the combined treatment allowed to reduce the dose of CUR required to achieve an apoptotic rate of 30%, 50% or 70% by 1.2, 1.2 and 1.9 times, respectively. Similarly, in Saos-2 cells the dose of CUR required to achieve an apoptotic rate of 30% or 50% could be reduced 1.5 and 1.3 times, respectively, through the combination of CUR with RES.

It is of note that, according to the results of the SRB assays, the reduction of cell survival obtained with DADS+CUR and RES+CUR was not significantly different from that obtained using CUR alone, the more potent compound present in the combinations, while the results of FACS analysis indicate that CUR-induced apoptosis was potentiated by the combination with DADS in SJ-RH4 cells and by the combination with RES in SJ-RH4 and Saos-2 cells. However, this apparent discrepancy is reconciled when considering that SRB has been demonstrated to stain live as well as recently died cells (35). Still, to further corroborate the data on apoptotic cell death obtained by FACS analysis, SJ-RH4 and Saos-2 cells were labeled with an anti-activated caspase-3 polyclonal antibody after treatment with DADS, RES and CUR alone or in combination at 25 μM for 48 hours. Cells treated with DMSO vehicle were used as negative control, while cells

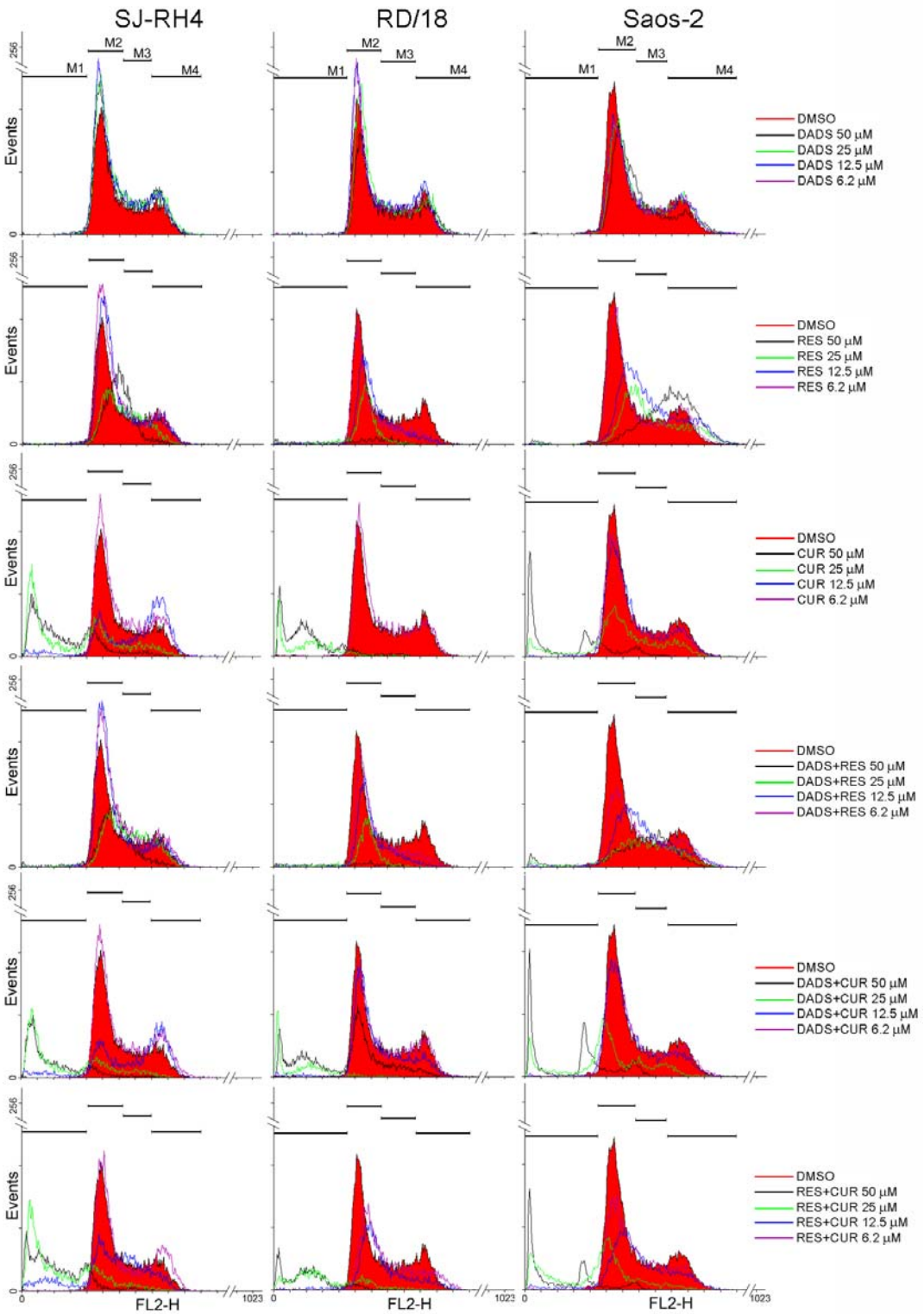

Figure 3. Effects of DADS, RES and CUR alone or in combination on apoptosis and cell cycle distribution of rhabdomyosacoma and osteosarcoma cells. FACS analysis of DNA content performed on SJ-RH4, RD/18 and Saos-2 cells treated for 48 hours with DMSO or DADS, RES and CUR alone or in combination. In all cell lines CUR induced an increase of the percentage of cells in sub G0 (M1) and a decrease of the percentage of cells in G0/G1 (M2), S (M3) and G2/M (M4) in a dose-dependent manner. DADS increased the sub G0 peak induced by CUR in SJ-RH4 cells when the compounds were used at the highest concentration. RES increased the sub G0 peaks induced by CUR in SJ-RH4 and Saos-2 cells in a dose-dependent manner.

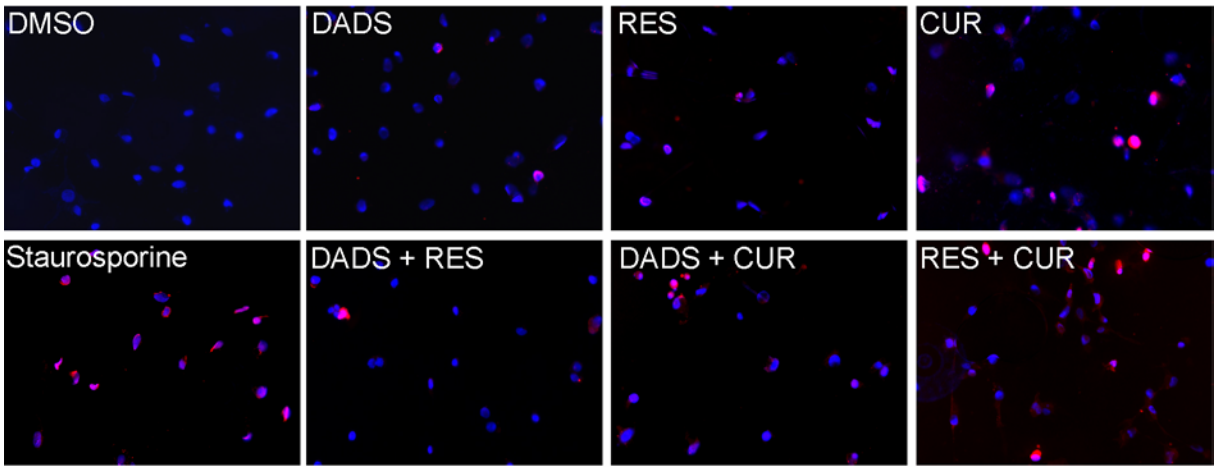

Figure 4. *In situ* detection of apoptosis. Induction of apoptosis in SJ-RH4 cells by DADS, RES and CUR alone or in combination, DMSO as vehicle control and staurosporine as positive control, as assessed by immunolabeling with an anti-activated-caspase 3 polyclonal antibody. DADS, RES and CUR alone or in combination were used at 25 μM for 48 hours; staurosporine was used at 1 μM for 24 hours. Original magnification x200. Nuclei were counterstained with Hoechst.

treated with staurosporine at 1 μM for 24 hours were used as positive control. Representative pictures of immunolabeled SJ-RH4 cells are reported in Figure 4. According to activated caspase 3 positivity, the treatment with staurosporine resulted in apoptotic rates of about 95-96% in either cell line, whereas treatment with DMSO, DADS and RES had no relevant effect on the induction of apoptosis in SJ-RH4 and Saos-2. On the other hand, CUR induced a significant increase of apoptosis in both cell lines. Furthermore, the apoptotic rate of SJ-RH4 cells treated with DADS+CUR was significantly higher than that obtained with CUR alone (46.4% *vs.* 36.4%, $p<0.001$). Similarly, the apoptotic rates of SJ-RH4 and Saos-2 cells treated with the RES+CUR combination were higher than those observed after treatment with CUR alone (50.8% *vs.* 36.4% for SJ-RH4 cells, $p<0.001$; 30% *vs.* 18.4% for Saos-2 cells, $p<0.001$), consistent with the results obtained by FACS analysis.

4.4. Effect of DADS, RES and CUR alone or in combination on apoptosis and pro-survival signaling proteins

The expression levels of apoptosis and pro-survival signaling proteins were investigated by western blotting in SJ-RH4 and RD/18 cells treated for 48 hours with the three compounds alone or in combination at 25 μM. A representative experiment is illustrated in Figure 5, upper panels. CUR either alone or in combination with DADS or RES induced in both cell lines the appearance of the 18 kDa Bax isoform (p18 Bax), which is known as a more potent inducer of apoptotic cell death than the full-length p21 Bax (36). The appearance of p18 Bax was concomitant with an overall decrease of Bcl-2 expression in both cell lines. Conversely, p18 Bax was not observed nor the ratio between Bax and Bcl-2 significantly modified after treatment with DADS and RES alone or in combination. In addition, DADS and RES alone or in combination did not change or slightly decreased the expression of the pro-survival signaling protein AKT. Conversely, CUR and CUR combinations strongly reduced AKT expression levels. Collectively, these results are in agreement with the reported apoptotic activity of the compounds at the employed concentration.

Next, the expression of p53 was analyzed in order to determine whether apoptosis induced by CUR in SJ-RH4 and RD/18 cells was p53-dependent. Unlike RD/18 cells, where p53 expression was decreased by CUR treatment, SJ-RH4 cells did not express p53, indicating that CUR can trigger p53-independent apoptosis in rhabdomyosarcoma cells.

Finally, it was investigated the effect of the compounds on Erk phosphorylation (Figure 5, upper panels). In both SJ-RH4 and RD/18 cells DADS had no notable effects on phospho-Erk levels, RES increased Erk phosphorylation and a further increase was observed after treatment with DADS+RES. On the other hand, while CUR reduced phospho-Erk levels in SJ-RH4 cells, the same compound increased phospho-Erk in the RD/18 cell line. Of note, when CUR was administered with RES it was able to counteract the increased Erk phosphorylation induced by RES in SJ-RH4 cells. In order to substantiate the observed cell context-dependent modulation of Erk signaling, both cell lines were treated for 32 hours, starved for 16 hours in serum-free medium and then stimulated for 5 minutes with 20% of serum in the continuous presence of the three compounds alone or in combination (Figure 5, lower panels). In SJ-RH4 cells all compounds inhibited serum-mediated Erk phosphorylation as compared to DMSO, the maximal inhibitory activity being obtained with CUR either alone or in combination with DADS or RES. Conversely, in RD/18 cells CUR, either alone or in combination with DADS or RES, increased Erk posphorylation upon serum stimulation as compared to DMSO vehicle treatment, while no major effects were observed with DADS and RES.

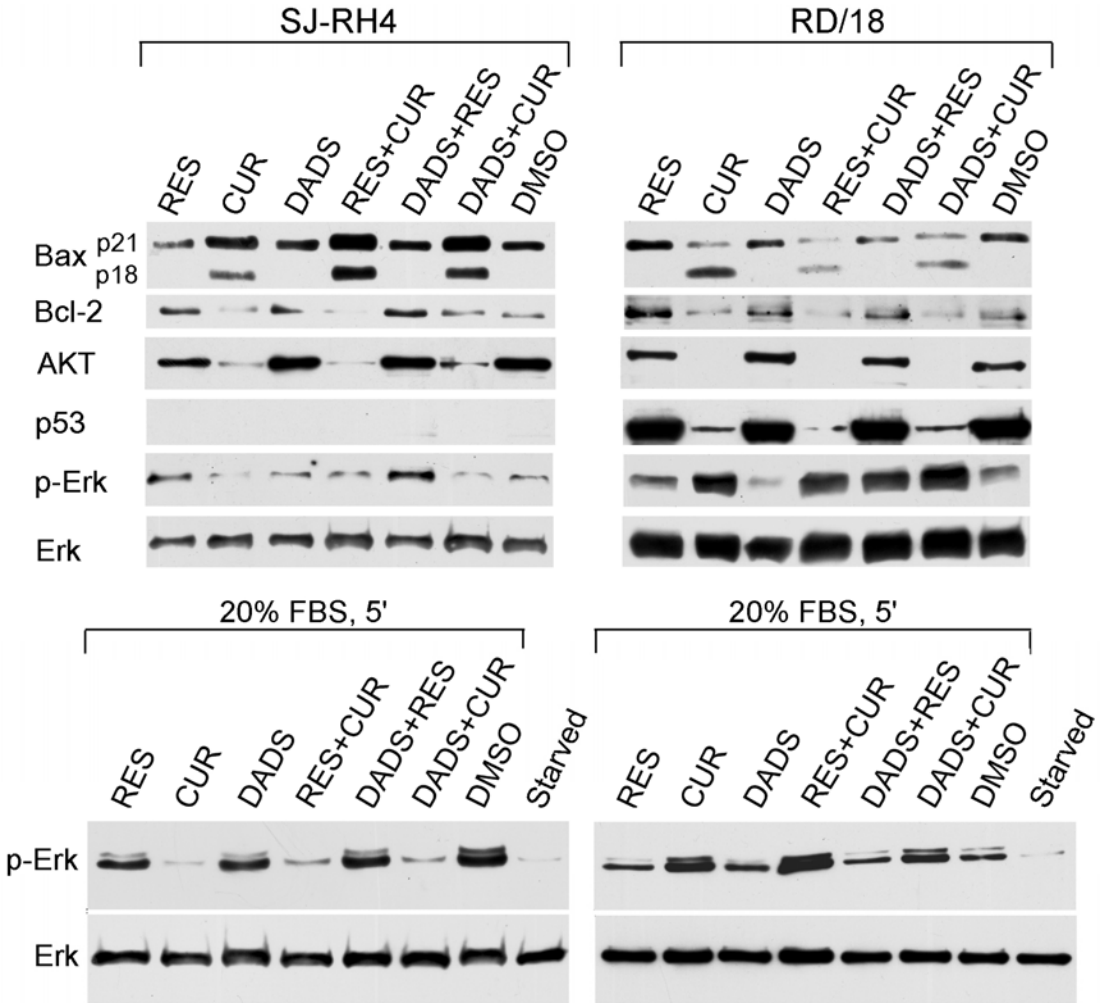

Figure 5. Effect of DADS, RES and CUR alone or in combination on apoptosis and pro-survival signaling proteins. Upper panels: assessment of Bax, Bcl-2, AKT, p53, phosphorylated Erk (p-Erk) and Erk levels by western blotting in SJ-RH4 and RD/18 cells treated for 48 hours with DADS, RES and CUR alone or in combination at 25 μM or with DMSO as vehicle. Lower panels: assessment of p-Erk levels upon serum stimulation of SJ-RH4 cells treated with DMSO or with the three compounds alone or in combination: after 32 hours of treatment, the cells were starved for 16 hours and then stimulated for 5 minutes with 20% of serum in the continuous presence of compounds or DMSO. Shown are representative experiments.

5. DISCUSSION

Chemoprevention involves the use of natural or synthetic substances to prevent certain diseases such as cancer. Indeed, chemoprevention has been proven to be relatively successful in preventing cancer (37-42) and several chemopreventive agents are now being tested for their anti-cancer therapeutic activity in tumor bearing hosts (37, 41-43). Therapeutic approaches based on drug combinations aim at increasing clinical responses while lowering toxicity and the incidence of drug resistance. The advantage of combining multiple agents stems from the fact that each agent can have a single target or mechanism of action or different agents may share the same target or mechanism of action against cancer cells (44, 45). Therefore the combination treatment could either increase the number of targets and/or mechanisms of action or potentiate the effects on the same target, thus lowering the drug concentrations needed to exert a biological effect against cancer cells.

CUR, RES and DADS have been demonstrated to have anti-tumor activity both *in vitro* and *in vivo* using animal models (6-25). Furthermore, some clinical trials have highlighted the potential role of CUR as an antineoplastic agent (37, 46). However, pharmacokinetic studies have shown that this compound is poorly absorbed and rapidly eliminated from the human body (37, 46). In the first report of the joint FAO/WHO expert committee on food additives it was established that an acceptable daily intake (ADI) for CUR is of 0–0.1 mg/kg body weight (47). Thus it may be beneficial to find other chemopreventive agents that can potentiate the anti-cancer activity of CUR.

In this study we provide evidence that CUR induces apoptosis of rhabdomyosarcoma and osteosarcoma

cell lines and that the combination of RES or DADS plus CUR potentiates the apoptotic effect of CUR. In particular, in SJ-RH4 cells the RES+CUR combined treatment allowed to reduce the dose of CUR required to achieve an apoptotic rate of 30%, 50% or 70% by 1.2, 1.2 and 1.9 times, respectively. Similarly, in Saos-2 cells the dose of CUR required to achieve an apoptotic rate of 30% or 50% could be reduced 1.5 and 1.3 times, respectively, through the combination of CUR with RES.

We also demonstrate that CUR induced the expression of p18 Bax in both SJ-RH4 and RD/18 rhabdomyosarcoma cell lines. This truncated isoform of Bax is known as a more potent inducer of apoptotic cell death than the full-lenght p21 Bax (36). Conversely, CUR down-regulated the expression of the anti-apoptotic protein Bcl-2 and strongly reduced the expression of the pro-survival kinase AKT. Indeed, AKT promotes cell survival and resistance to apoptosis by sequestering different protein targets, including the FOXO family of forkhead transcription factor and the pro-apoptotic protein Bad, as well as by activating the pro-survival transcription factor NF-kB (48). Most notably, we showed that CUR alone or in combination induced apoptosis of rhabdomyosarcoma cells independently of p53 activity. This finding indicates that CUR may have relevance for the treatment of p53-deficient cancers, which are often unaffected by conventional chemotherapies or irradiation therapy.

Interestingly, the effects of CUR on the modulation of basal and serum-induced Erk activity appeared different in SJ-RH4 and RD/18 cells. Indeed, while CUR reduced phospho-Erk levels in both unstimulated and serum-stimulated SJ-RH4 cells, the opposite effect was observed in the RD/18 cell line. In this respect it has been demonstrated that, depending on the specific context, Erk activation can either protect or contribute to drug-induced cell death (49, 50). Indeed, despite the opposite and cell type-specific modulation of Erk activity induced by CUR in SJ-RH4 (alveolar histotype) and RD/18 (embryonal histotype) rhabdomyosarcoma cells, this compound was able to produce a marked increase of the apoptotic rate of both cell lines.

In conclusion, our study provides evidence that the treatment of rhabdomyosarcoma or osteosarcoma cells with combinations of CUR plus RES or DADS can be more effective in inducing apoptosis than the treatment with CUR as a single compound. Still, additional studies performed both *in vitro* and *in vivo* will be needed to fully define the therapeutic potential of these compounds.

6. ACKNOWLEDGEMENTS

The authors thank Prof. P.L. Lollini (University of Bologna, Italy) and Prof. C. Dominici (Sapienza University, Rome, Italy) for providing SJ-RH4, RD/18 and Saos-2 cell lines and Barbara Bulgarini for editorial assistance in the preparation of the manuscript. This study was supported by grant MIUR-PRIN 2007-2009 and RSA-University of Rome, Tor Vergata.

7. REFERENCES

1. D. Walterhouse and A. Watson: Optimal management strategies for rhabdomyosarcoma in children**.** *Paediatr Drugs* 9, 391-400 (2007)

2. R. S. Benjamin and S. R. Patel: Pediatric and adult osteosarcoma: comparisons and contrasts in presentation and therapy. *Cancer Treat Res* 152, 355-363 (2009)

3. H. Lünenbürger, C. Lanvers-Kaminsky, B. Lechtape and M. C. Frühwald: Systematic analysis of the antiproliferative effects of novel and standard anticancer agents in rhabdoid tumor cell lines. *Anticancer Drugs* 21, 514-522 (2010)

4. M. Wachtel and B. W. Schäfer: Targets for cancer therapy in childhood sarcomas. *Cancer Treat Rev* 36, 318-327 (2010)

5. S. Y. Kim and L.J. Helman: Strategies to explore new approaches in the investigation and treatment of osteosarcoma. *Cancer Treat Res* 152, 517-528 (2009)

6. F. Khanum, K. R. Anilakumar and K. R. Viswanathan: Anticarcinogenic properties of garlic: a review. *Crit Rev Food Sci Nutr* 44, 479-488 (2004)

7. A. Herman-Antosiewicz and S. V. Singh: Signal transduction pathways leading to cell cycle arrest and apoptosis induction in cancer cells by Allium vegetable-derived organosulfur compounds: a review. *Mutat Res* 555, 121-131 (2004)

8. Y. Shukla and N. Kalra: Cancer chemoprevention with garlic and its constituents. *Cancer Lett* 247, 167-181 (2007)

9. A. A. Powolny and S. V. Singh: Multitargeted prevention and therapy of cancer by diallyl trisulfide and related Allium vegetable-derived organosulfur compounds. *Cancer Lett* 269, 305-314 (2008)

10. A. Bishayee: Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. *Cancer Prev Res* (Phila) 2, 409-418 (2009)

11. S. K. Goswami and D. K. Das: Resveratrol and chemoprevention. *Cancer Lett* 284, 1-6 (2009)

12. J. K. Kundu and Y. J. Surh: Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. *Cancer Lett* 269, 243-261 (2008)

13. G. Bar-Sela, R. Epelbaum and M. Schaffer: Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. *Curr Med Chem* 17, 190-197 (2010)

14. J. Epstein, I. R. Sanderson and T. T. Macdonald: Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. *Br J Nutr* 103, 1545-1557 (2010)

15. P. Anand, C. Sundaram, S. Jhurani, A. B. Kunnumakkara and B. B. Aggarwal: Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 267, 133-164 (2008)

16. Y. Li, C. M. Bäckesjö, L. A. Haldosén and U. Lindgren: Resveratrol inhibits proliferation and promotes apoptosis of osteosarcoma cells. *Eur J Pharmacol* 609, 13-18 (2009)

17. M. Alkhalaf and S. Jaffal: Potent antiproliferative effects of resveratrol on human osteosarcoma SJSA1 cells: novel cellular mechanisms involving the ERKs/p53 cascade. *Free Radic Biol Med* 41, 318-325 (2006)

18. A. W. Chow, G. Murillo, C. Yu, R. B. van Breemen, A. W. Boddie, J. M. Pezzuto, T. K. Das Gupta and R. G. Mehta: Resveratrol inhibits rhabdomyosarcoma cell proliferation. *Eur J Cancer Prev* 14, 351-356 (2005)

19. D. S. Lee, M. K. Lee and J. H. Kim: Curcumin induces cell cycle arrest and apoptosis in human osteosarcoma (HOS) cells. *Anticancer Res* 29, 5039-5044 (2009)

20. D. K. Walters, R. Muff, B. Langsam, W. Born and B. Fuchs: Cytotoxic effects of curcumin on osteosarcoma cell lines. *Invest New Drugs* 26, 289-297 (2008)

21. C. S. Beevers, F. Li, L. Liu and S. Huang: Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. *Int J Cancer* 119, 757-764 (2006)

22. M. Singh, A. Pandey, C. A. Karikari, G. Singh and D. Rakheja: Cell cycle inhibition and apoptosis induced by curcumin in Ewing sarcoma cell line SK-NEP-1. *Med Oncol* 27, 1096-1101 (2010)

23. J. Veeraraghavan, M. Natarajan, T. S. Herman and N. Aravindan: Curcumin-altered p53-response genes regulate radiosensitivity in p53-mutant Ewing's sarcoma cells. *Anticancer Res* 30, 4007-4015 (2010)

24. X. Hu, B. N. Cao, G. Hu, J. He, D. Q. Yang and Y. S. Wan: Attenuation of cell migration and induction of cell death by aged garlic extract in rat sarcoma cells. *Int J Mol Med* 9, 641-643 (2002)

25. Y. K. Zhang, X. H. Zhang, J. M. Li, S. Sun de, Q. Yang and D. M. Diao: A proteomic study on a human osteosarcoma cell line Saos-2 treated with diallyl trisulfide. *Anticancer Drugs* 20, 702-712 (2009)

26. S. Croci, L. Landuzzi, A. Astolfi, G. Nicoletti, A. Rosolen, F. Sartori, M. Y. Follo, N. Oliver, C. De Giovanni, P. Nanni and P. L. Lollini: Inhibition of connective tissue growth factor (CTGF/CCN2) expression decreases the survival and myogenic differentiation of human rhabdomyosarcoma cells. *Cancer Res* 64, 1730-1736 (2004)

27. L. Masuelli, C. Focaccetti, V. Cereda, F. Lista, D. Vitolo, P. Trono, P. Gallo, A. Amici, P. Monaci, M. Mattei, M. Modesti, G. Forni, M. H. Kraus, R. Muraro, A. Modesti and R. Bei: Gene-specific inhibition of breast carcinoma in BALB-neuT mice by active immunization with rat Neu or human ErbB receptors. *Int J Oncol* 30, 381-392 (2007)

28. L. Masuelli, L. Marzocchella, A. Quaranta, C. Palumbo, G. Pompa, V. Izzi, A. Canini, A. Modesti, F. Galvano and R. Bei: Apigenin induces apoptosis and impairs head and neck carcinomas EGFR/ErbB2 signaling. *Front Biosci* 16, 1060-1068 (2011)

29. L. Masuelli, P. Trono, L. Marzocchella, M. A. Mrozek, C. Palumbo, M. Minieri, F. Carotenuto, R. Fiaccavento, A. Nardi, F. Galvano, P. Di Nardo, A. Modesti and R. Bei: Intercalated disk remodeling in delta-sarcoglycan-deficient hamsters fed with an alpha-linolenic acid-enriched diet. *Int J Mol Med* 21, 41-48 (2008)

30. G. Tumino, L. Masuelli, R. Bei, L. Simonelli, A. Santoro and S. Francipane: Topical treatment of chronic venous ulcers with sucralfate: a placebo controlled randomized study. *Int J Mol Med* 22, 17-23 (2008)

31. R. Bei, L. Masuelli, P. Trono, P. L. Orvietani, S. Losito, L. Marzocchella, D. Vitolo, L. Albonici, M. A. Mrozek, E. Di Gennaro, F. Lista, G. Faggioni, F. Ionna, L. Binaglia, V. Manzari, A. Budillon and A. Modesti: The ribosomal P0 protein induces a spontaneous immune response in patients with head and neck advanced stage carcinoma that is not dependent on its overexpression in carcinomas. *Int J Oncol* 31, 1301-1308 (2007)

32. L. Masuelli, L. Marzocchella, C. Focaccetti, F. Lista, A. Nardi, A. Scardino, M. Mattei, M. Turriziani, M. Modesti, G. Forni, J. Schlom, A. Modesti and R. Bei: Local delivery of recombinant vaccinia virus encoding for neu counteracts growth of mammary tumors more efficiently than systemic delivery in neu transgenic mice. *Cancer Immunol Immunother* 59, 1247-1258 (2010)

33. R. Bei, L. Masuelli, E. Moriconi, V. Visco, A. Moretti, M. H. Kraus and R. Muraro: Immune responses to all ErbB family receptors detectable in serum of cancer patients. *Oncogene* 18, 1267-1275 (1999)

34. R. Bei, A. Budillon, L. Masuelli, V. Cereda, D. Vitolo, E. Di Gennaro, V. Ripavecchia, C. Palumbo, F. Ionna, S. Losito, A. Modesti, M. H. Kraus and R. Muraro: Frequent overexpression of multiple ErbB receptors by head and neck squamous cell carcinoma contrasts with rare antibody immunity in patients. *J Pathol* 204, 317-325 (2004)

35. Y. P. Keepers, P. E. Pizao, G. J. Peters, J. van Ark-Otte, B. Winograd and H. M. Pinedo: Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. Eur J Cancer 27, 897-900 (1991)

36. H. Toyota, N. Yanase, T. Yoshimoto, M. Moriyama, T. Sudo and J. Mizuguchi: Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. *Cancer Lett* 189, 221-230 (2003)

37. A. Shehzad, F. Wahid and Y. S. Lee: Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. *Arch Pharm* (Weinheim) 343, 489-499 (2010)

38. R. G. Mehta, G. Murillo, R. Naithani and X. Peng: Cancer chemoprevention by natural products: how far have we come? *Pharm Res* 27, 950-961 (2010)

39. P. H. Brown: Chemoprevention clinical trials: it is time to turn success into progress. *Cancer Epidemiol Biomarkers Prev* 16, 1531-1532 (2007)

40. E. N. Scott, A. J. Gescher, W. P. Steward and K. Brown: Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials. *Cancer Prev Res* (Phila) 2, 525-530 (2009)

41. J. J. Johnson and H. Mukhtar: Curcumin for chemoprevention of colon cancer. *Cancer Lett* 255, 170-181 (2007)

42. S. C. Thomasset, D. P. Berry, G. Garcea, T. Marczylo, W. P. Steward and A. J. Gescher: Dietary polyphenolic phytochemicals--promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int J Cancer* 120, 451-458 (2007)

43. K. R. Patel, V. A. Brown, D. J. Jones, R. G. Britton, D. Hemingway, A. S. Miller, K. P. West, T. D. Booth, M. Perloff, J. A. Crowell, D. E. Brenner, W. P. Steward, A. J. Gescher and K. Brown: Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. *Cancer Res* 70, 7392-7399 (2010)

44. A. R. Amin, D. Wang, H. Zhang, S. Peng, H. J. Shin, J. C. Brandes, M. Tighiouart, F. R. Khuri, Z. G. Chen and D. M. Shin: Enhanced anti-tumor activity by the combination of the natural compounds (-)-epigallocatechin-3-gallate and luteolin: potential role of p53. *J Biol Chem* 285, 34557-34565 (2010)

45. H. Jiang, X. Shang, H. Wu, G. Huang, Y. Wang, S. Al-Holou, S. C. Gautam and M. Chopp: Combination treatment with resveratrol and sulforaphane induces apoptosis in human U251 glioma cells. *Neurochem Res* 35, 152-161 (2010)

46. A. S. Strimpakos and R. A. Sharma: Curcumin: preventive and therapeutic properties in laboratory studies and clinical trials. *Antioxid Redox Signal* 10, 511-545 (2008)

47. Evaluation of certain food additives. Fifty-first report of the Joint/WHO expert Committee on food additives. *World Health Organ Tech Rep Ser* 891(i-viii), 1-168 (2000)

48. P. O-charoenrat, P. H. Rhys-Evans, H. Modjtahedi and S. A. Eccles: The role of c-erbB receptors and ligands in head and neck squamous cell carcinoma. *Oral Oncol* 38, 627-640 (2002)

49. S. Cagnol and J. C. Chambard: ERK and cell death: mechanisms of ERK-induced cell death-apoptosis, autophagy and senescence. *FEBS J* 277, 2-21 (2010)

50. J. A. McCubrey, L. S. Steelman, W. H. Chappell, S. L. Abrams, E. W. Wong, F. Chang, B. Lehmann, D. M. Terrian, M. Milella, A. Tafuri, F. Stivala, M. Libra, J. Basecke, C. Evangelisti, A. M. Martelli and R. A. Franklin: Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 1773, 1263-1284 (2007)

Key Words: Curcumin, Combined Treatments, Sarcoma, Apoptosis

Send correspondence to: Roberto Bei, Department of Experimental Medicine and Biochemical Sciences, University of Rome, Tor Vergata, Via Montpellier 1, 00133 Rome, Tel: 39-06-72596514, Fax: 39-06-72596506, E-mail: bei@med.uniroma2.it

http://www.bioscience.org/current/vol17.htm