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Research paper

Wheat sprout extract-induced apoptosis in human cancer cells by proteasomes modulation

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ABSTRACT

Natural occurring modulators of proteasome functionality are extensively investigated for their implication in cancer therapy. On the basis of our previous evidences both on proteasomal inhibition by monomeric polyphenols, and on the characterization of wheat sprout hydroalcoholic extract, herein we thoroughly report on a comparative study of the effect of wheat sprout extract on both normal and tumour cells. Treatment of isolated 20S proteasomes with wheat sprout extracts induced a gradual inhibition of all proteasome activities. Next, two wheat sprout extract components were separated: a polyphenol and a protein fraction. Both components exerted an *in vitro* inhibitory effect on proteasome activity. HeLa tumour cells and FHs 74 Int normal cells were exposed to both fractions, resulting in different rates of proteasome inhibition, with tumour cells showing a significantly higher degree of proteasome impairment and apoptosis induction. Furthermore, a decrease in proteasome activities and in cell survival of the human plasmacytoma RPMI 8226 cell line, upon the same treatments, was observed. Collectively, our results provide additional evidences supporting the possible use of natural extracts as coadjuvants in cancer treatments.

1. Introduction

An increasing number of studies is presently assessing the efficacy of polyphenolic antioxidants from fruits and vegetables in lowering the risk of occurrence of several human pathologies, such as coronary heart diseases, inflammation, cancer and neurodegenerative diseases [1–3]. Specifically, green tea extracts, grapes and wine derived polyphenols can protect neurons from amyloid-β-induced damages in Alzheimer's disease [4] and down-regulate pro-apoptotic genes like *Bad* and *Bax* in SH-SY5Y cells [5]. Additionally, several papers reported on the chemopreventative effects of various natural plant extracts [6–11].

Due to the high content in organic phosphates, enzymes, reducing glycosides and polyphenols, wheat sprouts possess radical scavenging activity. In fact, the antioxidant molecules level in sprouts is higher than that of non-sprouted seeds, wheat germ or young wheat plants [8,12]. Antioxidant molecules isolated from wheat sprouts can also protect DNA against oxidative stress induced by reactive oxygen species [7] and the aqueous wheat sprout extract has antimutagenic properties [10,13].

Many natural occurring compounds are modulators of the proteasome, a large multi-subunit threonine–protease complex responsible for the extralysosomal proteolysis in eukaryotic and prokaryotic cells. It recognizes poly-ubiquitinated substrates, such as $p27^{kip-1}$ [14], IkBα [15], p53 [16], Bax [17], cyclins A, B, D and E, involved in cell cycle progression, proliferation and apoptosis [18,19]. The 20S proteasome represents the catalytic core of the 26S complex and it can degrade non-ubiquitinated oxidized, mutated, damaged, misfolded and unfolded proteins [20]. It is composed by four heptameric rings ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$) stacked to form a cylinder shaped structure. Seven distinct α subunits constitute the outer rings and regulate the access of extended polypeptides, whereas β subunits, assembled in the inner rings, are associated with the proteolytic activities: chymotrypsin-like (ChT-L), trypsin-like (T-L), peptidylglutamyl-peptide hydrolyzing (PGPH), branched-chain

Abbreviations: AMC, 7-amino-4-methyl-coumarin; AP-N, Aminopeptidase-N; BrAAP, branched-chain amino acid preferring; ChT-L, chymotrypsin-like; ECL, Enhanced ChemiLuminescence; EGCG, epigallocatechin-3-gallate; EGF, epidermal growth factor; FBS, fetal bovine serum; GAE, gallic acid equivalents; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-gamma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pAB, 4-aminobenzoate; PABA, 4-aminobenzoic acid; PARP, poly ADP ribose polymerase; PGPH, peptidyl-glutamyl-peptide hydrolyzing; pNA, p-aminobenzoate; ROS, reactive oxygen species; TPC, Total Phenolic Content.

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amino acid preferring (BrAAP) and small neutral amino acid preferring (SNAAP) components [21–24]. In the presence of the interferon-γ, the 20S proteasome is converted in an immune-complex involved in the generation of antigenic peptides, namely the immunoproteasome, in which the constitutive β5, β1, and β2 are replaced by the inducible subunits β5i, β1i, and β2i [25].

Previous data demonstrated that proteasome inhibition leads to growth arrest and to apoptosis in tumour cells [17,26–29], whereas other studies indicate that tumour and transformed cells are significantly more sensitive to proteasome inhibition than normal, non-transformed cells [30,31].

Among the natural modulators of the proteasome functionality, green tea catechins, apigenin, quercetin, kaempferol, myricetin, genistein, curcumin, resveratrol deserve particular attention [30,32–35].

In the present study, the effects exerted by wheat sprout extract on tumour cells and on a normal cell line have been investigated. Moreover, the extract composition has been further analyzed, testing the influence of its single components on the proteasome functionality *in vitro* and in cell cultures.

2. Materials and methods

2.1. Materials

Wheat sprout hydroalcoholic extract was kindly provided by GerminalLife S.r.l. (Perugia, Italy) [36]. Methanol, acetonitrile, phosphoric acid and gallic acid were purchased from J.T Baker (Italy). Folin–Ciocalteau reagent, (+)-catechin, (−)-epicatechin, epigallocatechin-3-gallate, (−)-epigallocatechin, ellagic acid, quercetin, luteolin, EGF, substrates for assaying the ChT-L, T-L, PGPH activities (Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC, respectively), lactacystin, caspase 3 substrate (Ac-Asp-Glu-Val-Asp-AMC) and 6.5–205 kDa molecular weight markers were all purchased from Sigma–Aldrich (Italy). The substrate Z-Gly-Pro-Ala-Phe-Gly-pAB and the inhibitor Z-Gly-Pro-Phe-Leu-CHO to test the BrAAP activity were the kind gift of Prof. Orlowski (Department of Pharmacology, Mount Sinai School of Medicine, New York). All chemicals and solvents were of the highest analytical grade available.

Aminopeptidase-N, used for the coupled assay utilized for detecting the BrAAP activity [37] was purified from pig kidney, as reported elsewhere [38,39]. The cervical carcinoma (HeLa), the human small intestine (FHs 74 Int) and the plasmacytoma (RPMI 8226) cell lines were acquired from ATCC (USA). All the media and reagents for cell cultures were purchased from Gibco Invitrogen Cell Culture (Italy).

PVDF membranes were obtained from Millipore (Italy). The anti-$p27^{kip-1}$ human and mouse monoclonal antibody was obtained from Oncogene Research products (Germany). The anti-PARP antibody was purchased from Calbiochem (Germany), while the anti-glyceraldehyde-3-phosphate and the anti-ubiquitin antibodies were purchased from Santa Cruz Biotecnology (Spain). The western blot stripping buffer was bought from Pierce (USA). Chromatographic analyses were performed on an AKTA Basic 100 FPLC system (GE Healthcare, USA) and on a Beckman Gold HPLC system (Beckman-Coulter, Italy), both equipped with a UV–vis detector. The Amberlite XAD7HP resin, used for the separation of the extract components, was obtained by Rohm and Haas (Italy). The Luna C18 column (2.5 μm porosity, 250 × 4 mm) the UltraSep ES RP18 pre-column, used for the qualitative analysis of polyphenols, were obtained from Phenomenex (Italy). Fluorimetric determinations were performed on an RF5301 Shimadzu spectrofluorimeter (Shimadzu Italia, Italy). Spectrophotometric assays were carried out on a Cary 100Bio device (Varian, USA). Enhanced Chem-iLuminescence Western blotting analysis system was obtained from Amersham-Pharmacia-Biotech (Sweden).

2.2. Chromatographic separation of protein and polyphenol components of wheat sprout extract

The separation of wheat sprout extract fractions was performed on an AKTA basic 100 FPLC using an Amberlite XAD7HP resin. The mobile phase composition was a mixture of water and ethanol. The protocol was optimized as follows: after equilibrating the column in water, wheat sprout components were loaded and separated through a step-gradient of ethanol (10%, 20%, 30%, 40% and 50%) at a flow rate of 0.3 ml/min. The elution pattern was monitored at 278 nm.

Eluates at distinct ethanol percentage were collected in ice, dried and dissolved in water, upon sonication. Fractions were pooled, centrifuged to remove the insoluble residue, and finally resuspended in a minimal volume of water. Successively, Total Phenolic Content (TPC) was estimated by Folin–Ciocalteau assay [40]. A gallic acid solution was used as standard and results were expressed as μg of gallic acid equivalents (GAE) *per* ml of extract. Protein content was evaluated through Bradford method [41], followed by gel electrophoresis on 15% polyacrylamide gel, stained with Coomassie Brilliant Blue [42]. Qualitative analysis of polyphenols was performed on a Beckmann Gold HPLC system equipped with a C18 Phenomenex Luna column as previously described [34].

2.3. Effect of wheat sprout components on 20S proteasomes functionality

In vitro assays for 20S constitutive and immunoproteasome activities were carried out with fluorogenic peptides. Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC, Z-Gly-Pro-Ala-Phe-Gly-pAB substrates were respectively used to test ChT-L, T-L, PGPH and BrAAP activities [23,43,44]. Isolation and purification of the 20S proteasomes from bovine brain and thymus were performed as previously reported [44,45].

The incubation mixture contained the wheat extract components at different concentrations (the protein and the polyphenol fractions ranging from 0.1 to 30 μg/ml and from 0.1 to 30 μg GAE/ml respectively), 5 μg of the isolated constitutive or immune 20S proteasomes, the appropriate substrate and 50 mM Tris–HCl pH 8.0 up to a final volume of 500 μl. Incubation was carried out at 37 °C and, after 60 min, the measurements of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (PABA) were detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA: $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm) on a spectrofluorimeter Shimadzu RF5301.

Control assays were performed to evaluate the effect of the extract on the proteolytic activity of AP-N used in the determination of BrAAP component: incubation mixtures containing the extract, 8 μg of AP-N, the substrate L-Leu-pNA and 50 mM Tris–HCl pH 8.0 up to a final volume of 500 μl were incubated at 37 °C. After 10 min of incubation the activity was measured ($\lambda = 405$ nm) on a Cary 100Bio spectrophotometer.

2.4. Cells treatment with wheat sprout extract components

HeLa cell line was cultured in DMEM plus 10% FBS, 1% non-essential amino acids, 0.4% L-glutamine, penicillin and streptomycin. FHs 74 Int cells were cultured in Hybri-Care medium plus 10% FBS and 30 ng/ml EGF. RPMI 8226 cell line was cultured in RPMI medium supplemented with 10% FBS, penicillin and streptomycin. Cells were grown in 100 mm tissue culture dishes and maintained in a 5% CO_2 atmosphere at 37 °C.

After 24 h starvation period, performed to increase the G1 phase population at the time of the treatment, cells were treated for 6, 24, 48 h either with the polyphenol fraction (5 μg GAE/ml dissolved in the culture medium), or with 15 μg/ml of the protein component, or with both components (5 μg GAE/ml polyphenols + 15 μg/ml

peptides). Cells were also treated with 28 μg GAE/ml of the whole extract. Preliminary studies performed exposing cells to increasing concentration of wheat sprout extract showed 28 μg GAE/ml produced the best results in terms of proteasome inhibition (data not shown). The stability of wheat sprout fractions in different culture media was checked and no significant variations were detected.

Cells were harvested in PBS, centrifuged and the pellet was resuspended in a lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12 000 × *g* for 15 min and the supernatants were stocked at −80 °C. Protein concentration in cell lysates was determined by the Bradford method [41].

2.5. Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [46]. After experimental treatment, MTT was added to the culture medium at a final concentration of 0.5 mg/ml and incubated for 2 h at 37 °C. The medium was replaced with 100 μl of DMSO and the optical density was measured at 550 nm in a microtiter plate reader. At least six cultures were utilized for each time point.

2.6. Proteasome activities on cell lysates

Activity assays with cell lysates (5 μg of total proteins in the mixture) were performed using the substrates listed above; control experiments were done in the presence of specific inhibitors: Z-Gly-Pro-Phe-Leu-CHO and lactacystin (5 μM in the reaction mixture) [47]. The BrAAP activity specific inhibitor Z-GPFL-CHO was used to verify the effective contribution to the proteolysis of this component. The ability of this compound to interact with the proteasome has been already demonstrated, suggesting a strong inhibitory effect of Z-GPFL-CHO specifically towards the BrAAP component [48,49]. A 80% decrease in BrAAP activity was obtained in the presence of the inhibitor (data not shown). Tests for the BrAAP activity were performed in the presence of AP-N [23]. Incubation was carried out at 37 °C for 60 min in 50 mM Tris–HCl, pH 8.

26S ChT-L activity was performed including in the reaction mixture 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP. Incubation was carried out at 37 °C and, after 60 min, the hydrolyzed 7-amino-4-methyl-coumarin (AMC) was detected.

2.7. Western blotting analyses

Ubiquitinated proteins, p27 and poly (ADP)-ribose polymerase (PARP) expression levels were analyzed through Western blotting assays. Cell lysates (30 μg total proteins) were loaded on a 12% SDS-PAGE, then electroblotted onto PVDF membranes. After incubation with anti-ubiquitin, anti p27, and anti-PARP antibodies, the immunoblot detections were carried out on an Enhanced Chem-iLuminescence Western blotting analysis system.

Each gel was loaded with 6.5–205 kDa molecular weight markers. As a control for equal protein loading glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized: membranes were stripped and reprobed for GAPDH using a monoclonal antibody diluted 1:500. A densitometric algorithm was developed to quantitate Western blot results [50].

2.8. Caspase 3

Caspase 3 activity assays in cell lysates (20 μg of total proteins in the mixture) were performed using the Ac-Asp-Glu-Val-Asp-AMC substrate in 50 mM Tris–HCl, 50 mM NaCl, 5 mM $CaCl_2$, 1 mM EDTA, 0.1% CHAPS, 5 mM β-mercaptoethanol, pH 7.5. Incubation was carried out at 37 °C for 60 min, then the fluorophore hydrolysis product was detected (AMC: λ_{exc} = 365 nm, λ_{em} = 449 nm) on a Shimadzu RF5301 spectrofluorimeter.

2.9. DNA fragmentation assay

The assay was conducted as described by Buonanno et al. [51]. Briefly, 1 × 10^6 cells were grown in microtiter plates and, upon treatments, were collected and suspended in lysis buffer (50 mM Tris–HCl, pH8, 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K). After 1 h incubation at 50 °C, 10 μg/ml RNase was added to the lysates and incubated for 1 h at 50 °C and for 10 min at 70 °C. DNA was precipitated adding sodium acetate (pH 5.2) and ice-cold 100% ethanol, incubated on ice for 10 min and centrifuged at 10 000 × *g* for 10 min. Pellets were suspended in sterile water. Samples were resolved on a 1.8% agarose gel stained with ethidium bromide.

2.10. Hoechst nuclear staining

Upon the abovementioned treatments, cells (1 × 10^6 cells/ml) were fixed in methanol/acetic acid (3:1), incubated with 0.05 μg/ml Hoechst 33258 nuclear stain for 15 min, and observed under an inverted fluorescence microscope.

2.11. Statistical analysis

Values are expressed as mean values and standard error of results obtained from independent experiments. Student's *t* test was used to compare differences of means between control and treated groups in the densitometry analyses of Western blotting.

Analysis of variance (ANOVA), followed by the Holm-Sidak test for multiple comparisons, was used to assess differences among multiple datasets obtained with cells untreated and treated with the whole extract and with its single components. Statistical tests were performed with Sigma-Stat 3.1 software (SPSS, Chicago, IL). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Qualitative analysis of wheat sprout extract components

Polyphenol and protein fractions were characterized as described in Material and methods section. Fig. 1 reports the protein

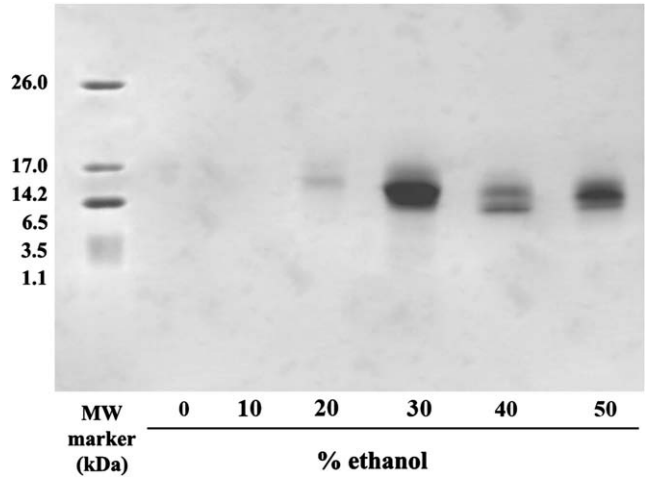

Fig. 1. Protein distribution on wheat sprout extract fractions. FPLC eluates at distinct ethanol percentages were separated on 15% acrylamide gel electrophoresis, and stained with Comassie brilliant blue.

distribution in the eluates at distinct ethanol concentrations. 14–17 kDa peptides were eluted at 30–50% ethanol, whereas polyphenols were mostly eluted at 10% ethanol.

Qualitative HPLC-analysis of the polyphenols eluted at 10% ethanol revealed the presence of high content of catechin, epicatechin and epigallocatechin gallate (data not shown). From the quantitative determinations wheat sprout extract contains polyphenols (10% ethanol eluate) and peptides (30–50% ethanol eluates) at a concentration ratio of approximately 1:2.5.

3.2. 20S proteasomes in vitro inhibition by wheat sprout components

ChT-L, T-L, PGPH and BrAAP activities of isolated 20S proteasomes were assayed in the presence of increasing amount of the polyphenol (0–30 μg GAE/ml) and the protein components (0–30 μg/ml) to disclose the role of each fraction in 20S proteasome inhibition.

Fig. 2 shows that both wheat sprout fractions affected the isolated proteasomes. The protein fraction exerted a more rapid and potent inhibitory effect on the ChT-L and T-L activities of the constitutive enzyme, as compared to the polyphenol component. ChT-L and BrAAP were the most influenced activities by both wheat sprout fractions.

T-L and BrAAP activities of the immunoproteasome decreased of the 60% in the presence of 30 μg GAE/ml polyphenol fraction. Moreover, the polyphenol component exerted a stronger inhibitory effect towards the immunoproteasome T-L activity, inhibiting the activity already at physiological levels (1 μg GAE/ml) (Fig. 2). Previous works showed that plasma concentration after the ingestion of pure polyphenols or vegetable extracts is in the range of 1–5 μM, corresponding approximately to 1 μg GAE/ml [52].

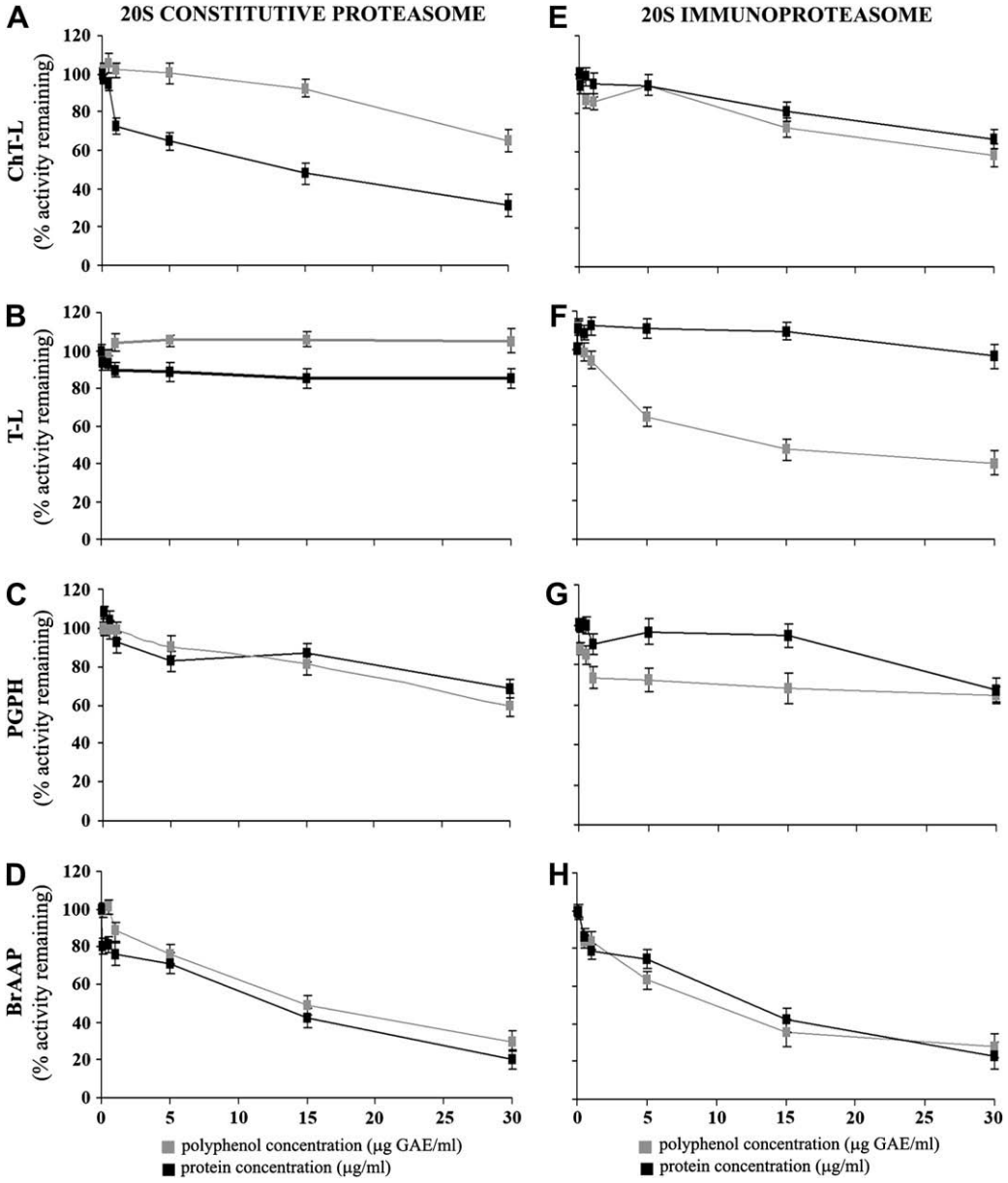

Fig. 2. Effect of wheat sprout extract separated components on the activity of isolated 20S proteasomes. Effect of increasing amounts of polyphenol fraction (■ grey) and protein fraction (■ black) on the ChT-L (panels A–E), T-L (panels B–F), PGPH (panels C–G) and BrAAP (panels D–H) proteolytic components of constitutive and immuno- 20S proteasomes (left and right panels respectively), isolated from bovine brain and thymus. The data shown are expressed as mean values ± SE obtained from six distinct determinations.

3.3. Different susceptibility of HeLa and FHs 74 Int cells to wheat sprout extract components treatment

Green tea polyphenols were reported to inhibit human tumour cells growth and proteasome functionality [35,53]. Several natural occurring compounds selectively affect proteasome functionality in tumour cells. HeLa tumour cell line and the normal FHs 74 Int cells were treated for 6, 24 and 48 h with the whole wheat sprout extract (28 μg GAE/ml), the polyphenol (5 μg GAE/ml) and the protein (15 μg/ml) fractions separately and with the two fractions simultaneously, to investigate the effect of each component on different cell types. The MTT assay outlined that, while FHs 74 Int cell survival was not influenced by the treatments, HeLa cells survival significantly diminished in the presence of the protein fraction. The loss of vitality is particularly marked after 48 h treatment with protein (15 μg/ml) and polyphenol (5 μg GAE/ml) fractions together (Fig. 3, panel A).

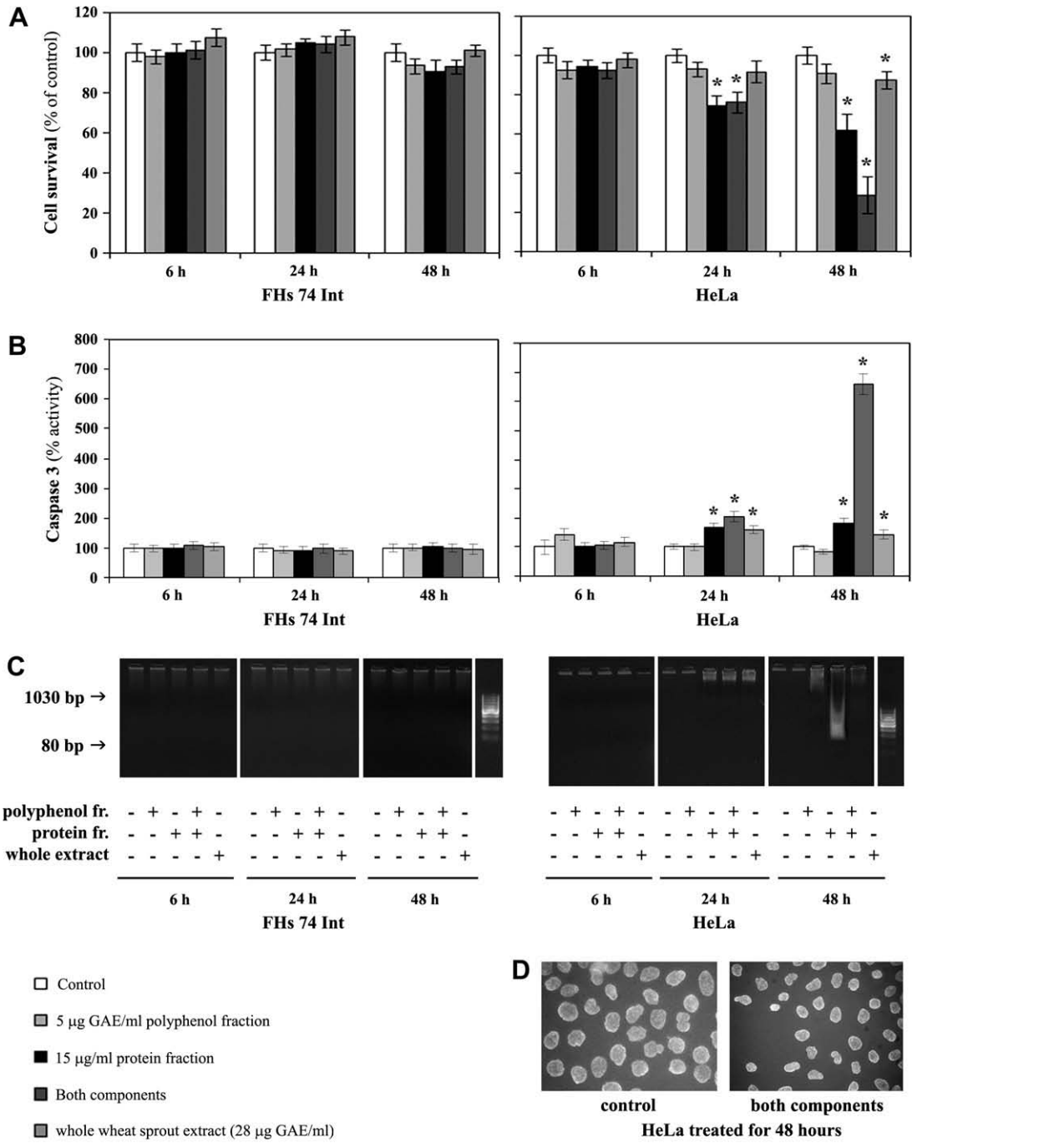

Fig. 3. Apoptotic events. FHs 74 Int and Hela cells were treated with 5 μg GAE/ml polyphenol fraction (▒); 15 μg/ml protein fraction (■); both components (■); whole wheat sprout extract (28 μg GAE/ml) (■) for 6, 24 and 48 h as described in Materials and methods. Panel A: Cell survival is reported as % of cell viability referred to control. Panel B: Caspase 3 assay. The specific activity percentage of the respective control non-treated cells are reported. Results are representative of six distinct experiments. Data points marked with an asterisk are statistically significant compared to their respective non-treated control cells ($^*p < 0.05$). Panel C: DNA fragmentation assay. Samples were resolved on a 1.8% agarose gel electrophoresis, stained with ethidium bromide. Panel D: HeLa morphological alterations after 48 h treatment with both components as revealed by Hoechst fluorescence staining.

3.4. Induction of apoptosis in tumour HeLa cells treated with extract components

Caspase 3 is a crucial enzyme in apoptosis, since it catalyzes the proteolysis of many cellular regulatory proteins, finally resulting in DNA fragmentation [54]. Caspase 3 activity was measured to elucidate the basis for the observed cellular death. Its activity was significantly enhanced in HeLa cells at 24 and 48 h in the presence of the protein component of the extract (Fig. 3, panel B). The activation became stronger at 48h in the presence of both fractions, suggesting that apoptosis was the involved mechanism. No variations were detected in FHs 74 Int cells.

Moreover, to better explore the apoptotic pathway, DNA fragmentation was determined as described in Materials and methods section. The assay revealed the presence of DNA ladder in HeLa cells treated for 24 and 48 h with protein fraction, with both components and with the whole extract (Fig. 3 panel C).

To evaluate cell morphology and further confirm the occurrence of apoptotic events, a Hoechst staining assay was performed. Hoechst 33342 is a fluorescent dye which permeates the plasma

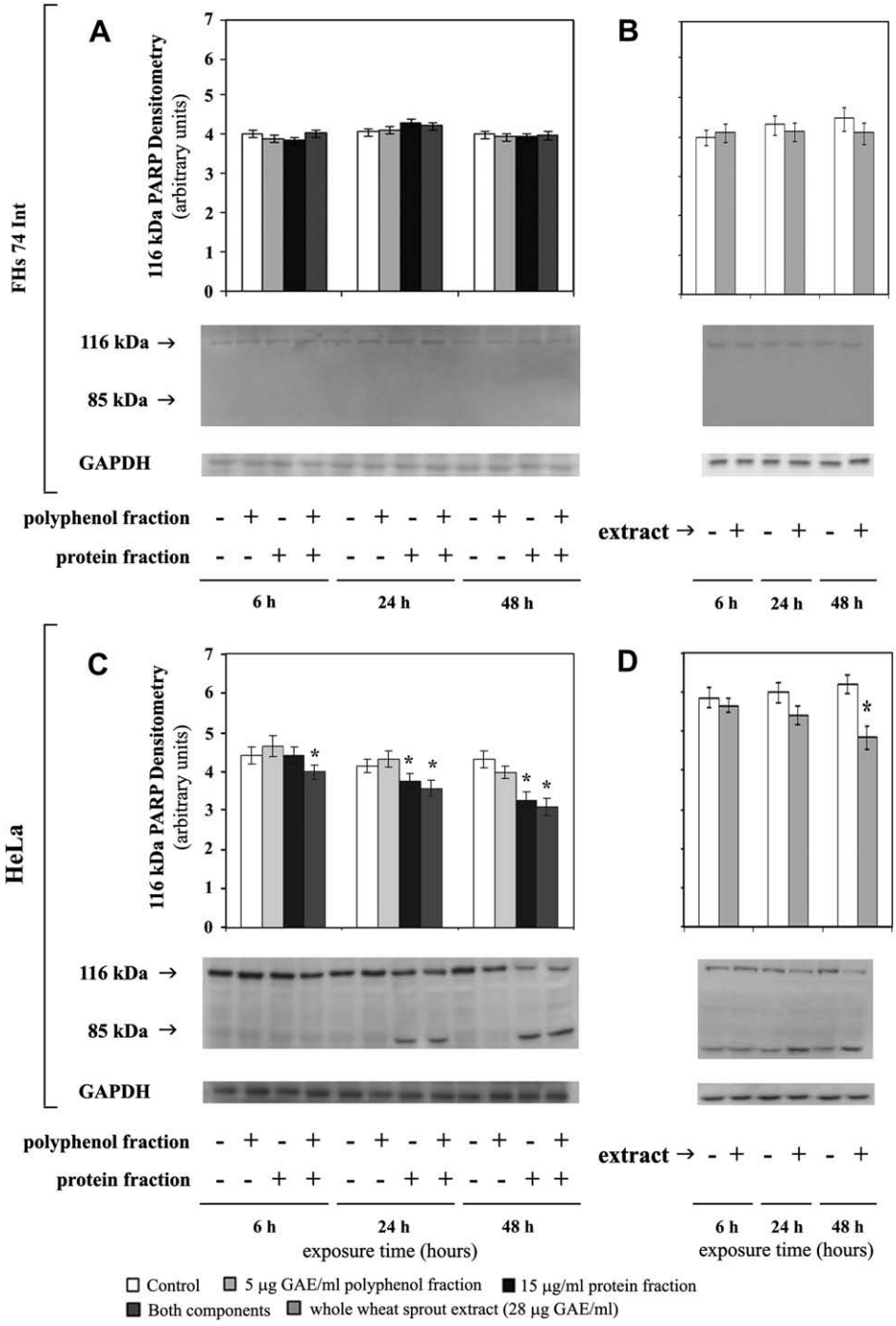

Fig. 4. PARP levels in treated cells. Autoradiographs of PARP expression in FHs 74 Int (panels A–B) and HeLa (panels C–D) cells treated with wheat sprout extract components for 6, 24, 48 h as described in Material and methods section. Representative Western blots are shown. The densitometric analyses of the 116 kDa full-length PARP protein from six separate blots provided for quantitative analysis are presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells (*$p < 0.05$).

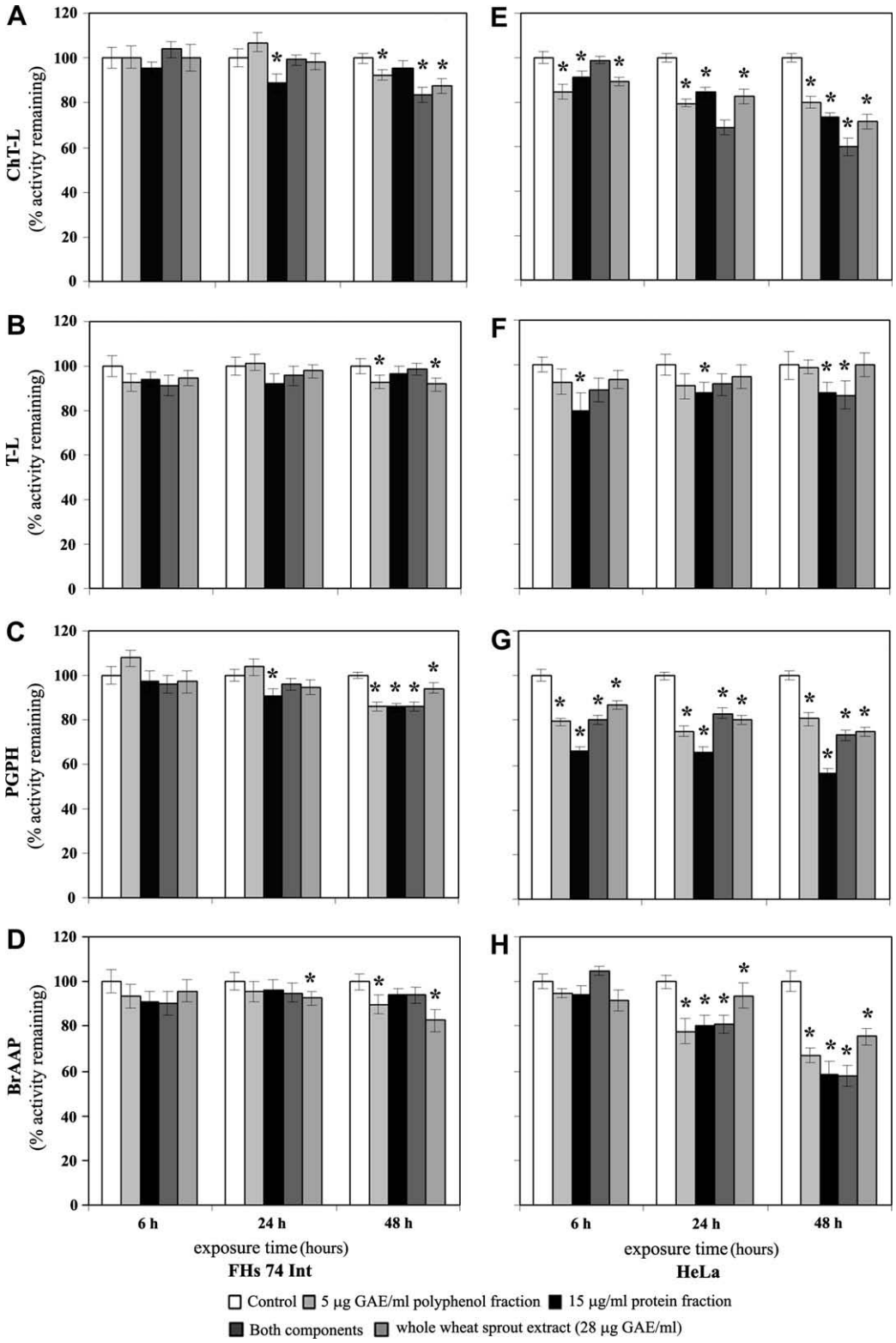

Fig. 5. Proteasomal activities measured in cell lysates upon treatment with wheat sprout extract components. FHs 74 Int and HeLa cell lysates were tested for the ChT-L (panels A–E), T-L (panels B–F), PGPH (panels C–G) and BrAAP (panels D–H) activities, using fluorescent substrates (suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC, Z-Gly-Pro-Ala-Leu-Ala-pAB respectively). Fluorescence units were subtracted of the values of control assays in the presence of specific inhibitors; results derived from six independent experiments are expressed as % activity remaining towards control (□). Values represent mean ± SD ($n = 6$). $p < 0.05$ by ANOVA with post-hoc Holm-Sidak test.

membrane and binds to the dsDNA resulting in a blue fluorescence. Apoptotic cells chromatin is condensed and nuclei are fragmented with respect to normal nuclear size. The assay revealed morphological changes of HeLa cells nuclei upon 48 h treatment with both components, such as reduction in the volume and nuclear chromatin condensation (Fig. 3, panel D).

To corroborate the hypothesis that wheat sprout extract fractions stimulate apoptosis in tumour HeLa cells, the intracellular level of the Poly (ADP-ribose) polymerase protein were measured. PARP is a nuclear enzyme involved in several processes such as cell death pathways, DNA damage detection and repair [55]. It has been also reported that PARP is cleaved by caspase 3 during apoptosis, generating the enzymatically active C-terminal fragment of 85 kDa [56,57]. In the present work, we found that PARP fragmentation is enhanced in HeLa lysates, particularly upon 48 h treatment with the protein component, thus matching the apoptotic events above described (Fig. 4, panels C–D). FHs 74 Int cells showed no variations in the 116 kDa full-length PARP protein levels (Fig. 4, panels A–B).

3.5. Wheat sprout extract components inhibit proteasome activities in HeLa tumour cells

ChT-L, T-L, PGPH and BrAAP activities, assayed in cell lysates, are presented in Fig. 5. Interestingly, HeLa tumour cell line showed a higher sensitivity to wheat sprout components treatments, with respect to normal FHs 74 Int cells, resulting in a clear global decrease of proteasome functionality. In detail, a significant inhibition of ChT-L and PGPH activities was present already upon short incubation time and it reached 30% and 40%, respectively, after 48 h treatment with 15 μg/ml peptides. Polyphenols and peptides, separately administered, induced a 30 and 40% decrease of BrAAP activity respectively. Co-exposing the cells to polyphenol and protein fractions caused a 40% inhibition of the ChT-L and the BrAAP components (Fig. 5, panels E–H). In contrast, FHs 74 Int cells 20S proteasome activities decreased of 10–20% upon long exposure time (Fig. 5, panels A–D). Moreover 28 μg GAE/ml wheat sprout whole extract was able to inhibit HeLa proteasome ChT-L, PGPH and BrAAP activities, even though the effect was more evident in the presence of the separated components.

The simultaneous treatment of HeLa cells with the two fractions caused a stronger inhibition of ChT-L and BrAAP activities compared to the whole extract. This is probably due to the complex composition of the extract, which contains polyphenols, peptides, phosphates and reducing glycosides. Such constituents may delay or alter proteasome inhibition.

In addition, 26S proteasome ChT-L activity was measured to investigate whether the ubiquitin–proteasome pathway was also affected by the treatments. The extract and its components significantly decreased HeLa 26S ChT-L activity after 6 h. The inhibition was particularly evident in HeLa cells after 48 h in the presence of the protein component (30% decrease) and with both fractions (60% decrease) (Fig. 6, panel B). A weak (10–15%) and delayed inhibition was observed in FHs 74 Int cells (Fig. 6, panel A), suggesting that the tumour cell line is more sensitive to the wheat sprout extract treatment with respect to the normal cell line.

Proteasome inhibition is followed by a significant accumulation of ubiquitinated proteins, particularly evident upon long exposure time with both components or with peptide fraction or with the whole extract (Fig. 7, panels C–D).

p27 is an established proteasome substrate and an increase in its intracellular expression levels leads to the activation of pro-apoptotic mechanisms [58]. An increase in p27 levels was detected in HeLa cells treated for 48 h with 5 μg GAE/ml of polyphenol fraction, with 15 μg/ml of the protein component and with the two fractions simultaneously (Fig. 8, panels C). The whole extract was also able to increase p27 expression in HeLa cells upon 24 and 48 h treatment (Fig. 8, panel D).

3.6. Proteasome inhibition by wheat sprout extract components in plasmacytoma RPMI 8226 cell line

Following our encouraging results obtained with HeLa cells we decided to confirm such data investigating cell viability and proteasome functionality in an additional cancer cell line. In particular, we chose the RPMI 8226 cells, a human plasmacytoma cell line, since myelomas are the system in which proteasome inhibition has given the most promising results as an alternative cure for this disease and that the molecular pathways affected were extensively characterized [29,59,60]. MTT assay revealed a 20% decrease in

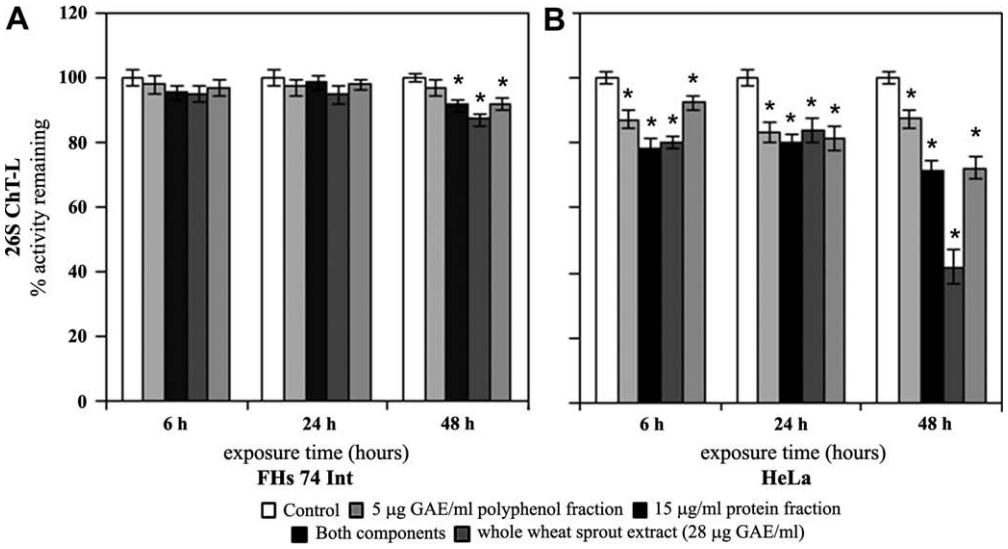

Fig. 6. 26S proteasome ChT-L activity. FHs 74 Int (panel A) and HeLa (panel B) cells were treated with wheat sprout extract components as described in Material and method section. 26S activity was measured in cell lysates including in the reaction mixture 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP. Data are expressed as % activity remaining towards control (□) in each time set ($^*p < 0.05$).

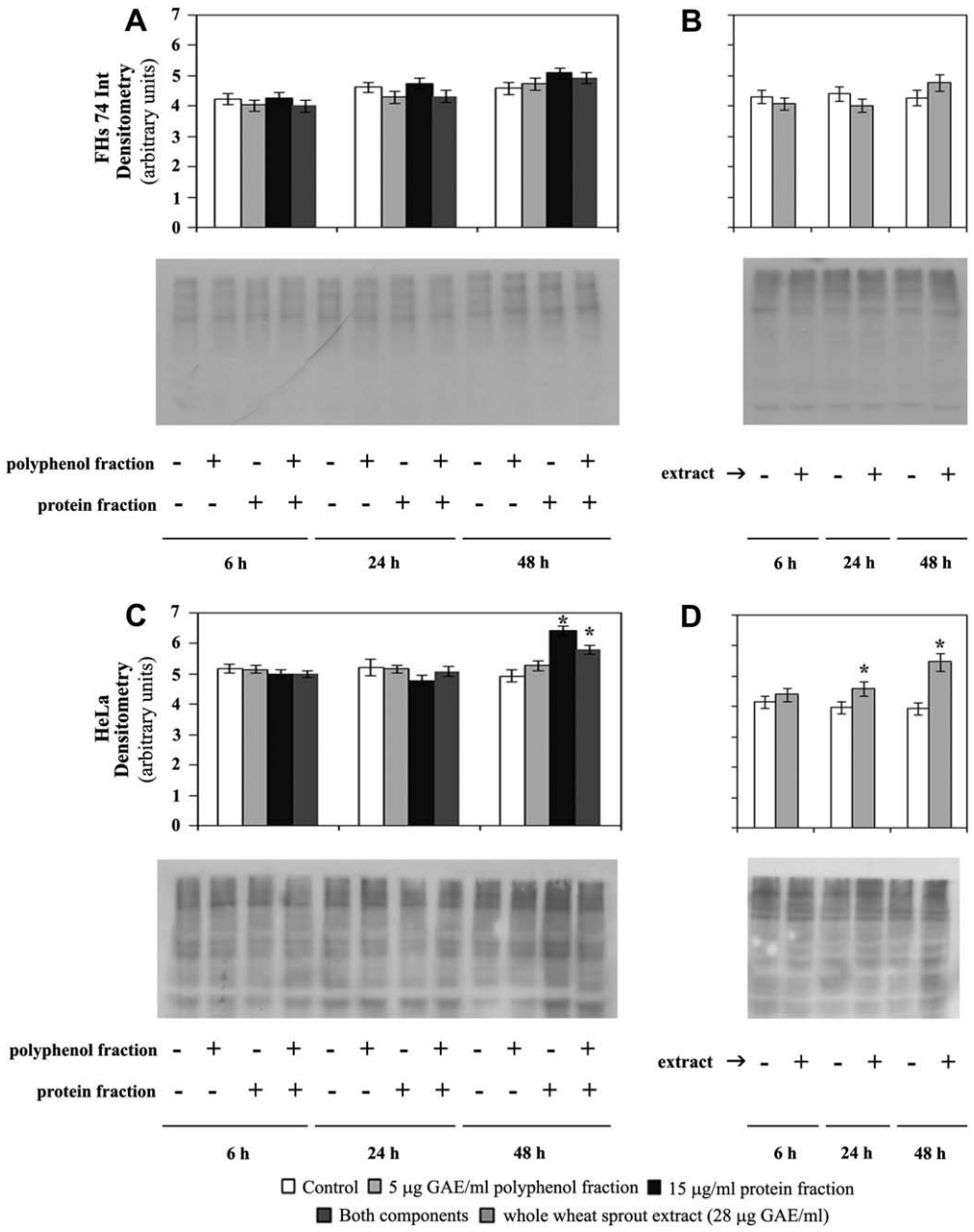

Fig. 7. Ubiquitinated proteins in treated cells. Autoradiographs of ubiquitinated proteins levels in FHs 74 Int (panels A–B) and HeLa (panels C–D) cells treated with wheat sprout extract components for 6, 24, 48 h. Representative Western blots are shown. The densitometric analyses from six separate blots provided for quantitative analysis are presented. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells ($^*p < 0.05$).

RPMI 8226 cell viability especially upon exposure to the protein component and to the two components together (Fig. 9, panel A). The same treatment caused a 20–40% increase in caspase 3 activity, particularly evident at 24 and 48 h (Fig. 9, panel B). Moreover, DNA fragmentation was induced by protein fraction and both fractions treatment at 24 and 48 h (Fig. 9, panel C). PARP cleavage, significantly enhanced upon 24 and 48 h treatment with the protein component and with both fractions (Fig. 9, panel D), supported the hypothesis of apoptosis induction.

The ubiquitin–proteasome pathway was affected as demonstrated by the 26S proteasome ChT-L activity inhibition at long exposure time (Fig. 10, panel E) and by a significant accumulation of ubiquitinated proteins, at 24 and 48 h treatment with protein fraction, with both components simultaneously administered (Fig. 11, panel A) and with whole wheat sprout extract (Fig. 11, panel B). 20S proteasome activities were also influenced: a time dependent inhibition of all proteasome components was observed in the presence of polyphenol and protein fractions. ChT-L activity showed the most consistent inhibition (40%) in cells treated for 48 h with isolated peptides. BrAAP activity significantly decreased upon 24 and 48 h treatment with wheat sprout components. No synergistic effect was observed, since polyphenols or peptides, separately administered, induced a higher inhibition with respect to the simultaneous treatment with both fractions (Fig. 10, panels A–D).

Proteasome inhibition was consistent also with intracellular p27 protein accumulation, which was significant when RPMI 8226 cells were treated with the peptide component or with both fractions (Fig. 11, panels C–D).

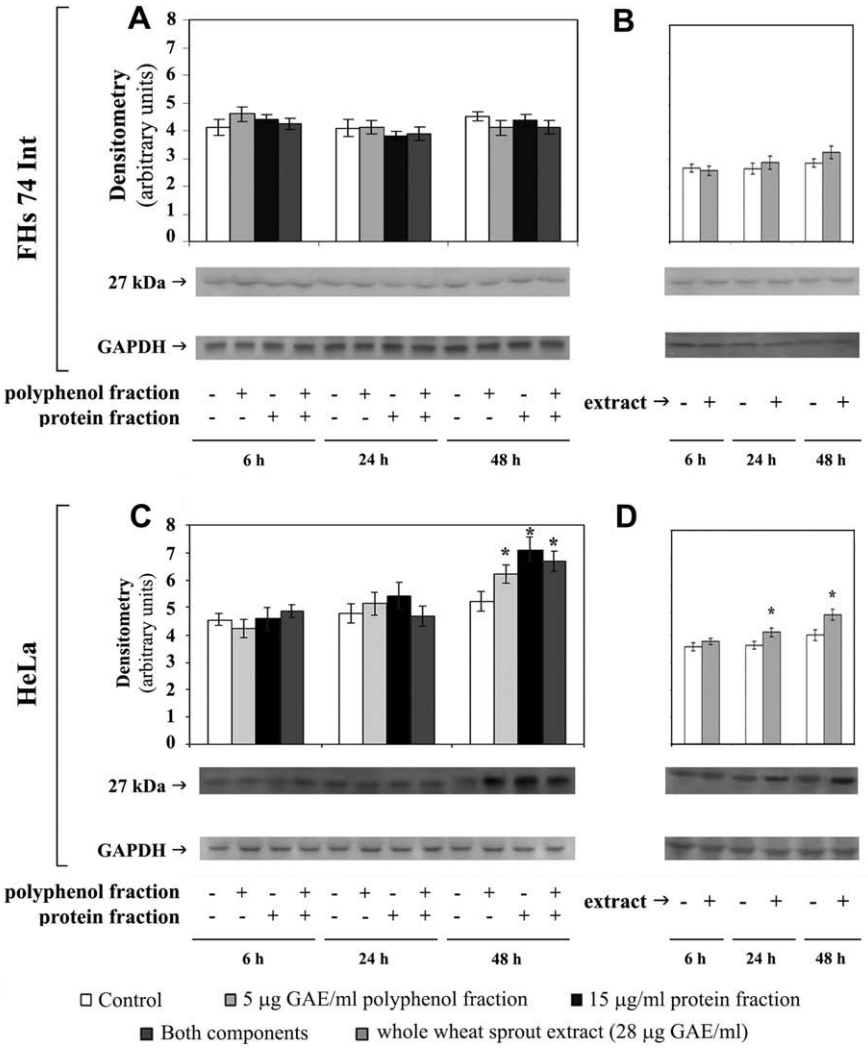

Fig. 8. p27 levels in treated cells. Autoradiographs of p27 expression in FHs 74 Int (panels A–B) and HeLa (panels C–D) cells treated with whole wheat sprout extract and its components for 6, 24, 48 h. Representative Western blots are shown and the densitometric analysis from six separate blots provided for quantitative analysis is presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells (*$p < 0.05$).

4. Discussion

Vast is the number of studies on health-promoting effects of natural occurring compounds and vegetable extracts, not only attributable to their antioxidant, anti-inflammatory, neuroprotective properties, but also to their antiproliferative and chemopreventative activities [2]. The anticarcinogenic effects of these molecules are often based on the induction of the apoptotic pathway [27,31,11,61].

Wheat sprout is a major source of antioxidant molecules and is able to protect against age-related alterations [6,8,12]. Furthermore, we recently characterized the composition of wheat sprout hydro-alcoholic extract, reporting high levels of catechin, epicatechin and epigallocatechin gallate, and additionally demonstrating that this extract is able to inhibit the 20S proteasome functionality *in vitro* and in Caco cells [34]. It was proved that proteasome activities are higher in tumour cells, with respect to a counterpart of normal cells, and that survival of tumour cells is related to proteasome function [17,62]. Several studies also reported that proteasome inhibition is associated with tumour cell death through apoptosis [32,63,64]. Natural polyphenols, such as green tea, grapes and wine derived polyphenols were largely investigated as potential chemopreventative agents in combination with current cancer therapies for their ability to inhibit the proteasome [27,33].

In this work the extract was further characterized and its ability to modulate proteasome functionality was extensively studied with the aim of evaluating its potential to be used as a cancer preventative agent.

Wheat sprout extract contains both a polyphenol and a protein fraction at a concentration ratio of 1:2.5. With the purpose to specifically address the role of each separated extract component, isolated 20S proteasomes were assayed in the presence of increasing amounts of the two fractions, separately. *In vitro* assays here reported show that both fractions have a role in the inhibition of the two complexes. However, while the protein component is a more rapid and potent effector of the constitutive complex, the immunoproteasome activities significantly decreased in the presence of 1 μg GAE/ml polyphenols. This value falls within the 1–5 μM plasma concentration range, obtained after ingestion of pure polyphenols or vegetable extracts [52]. No information on the concentration of polyphenols in serum after wheat sprout powder ingestion is currently available. Our data suggest that wheat sprout fractions, when administered separately, can differently modulate

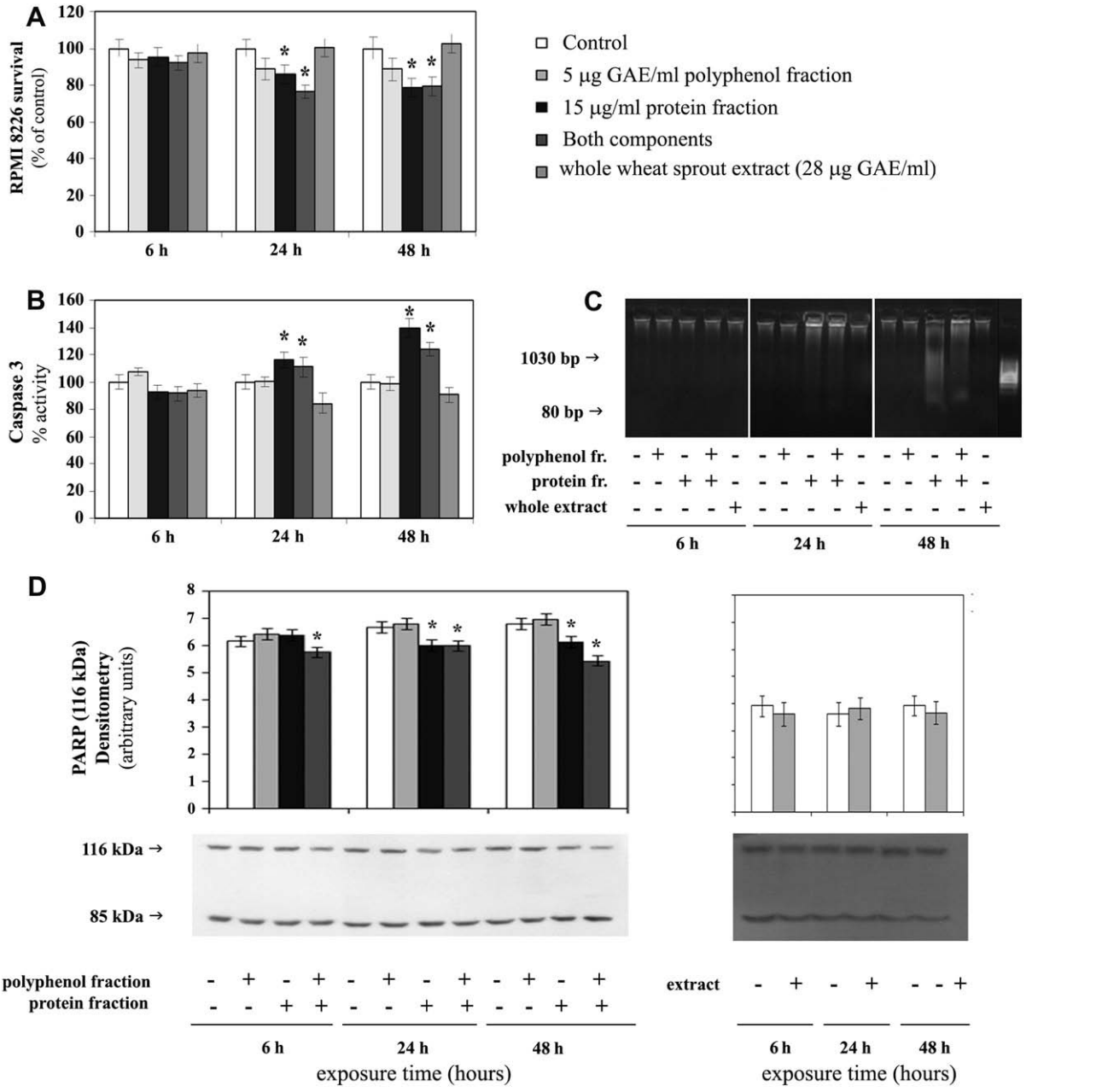

Fig. 9. Apoptotic events in RPMI 8226 cells upon wheat sprout extract components treatment. Panel A: Graphs related to survival of RPMI 8226 plasmacytoma cells; data are reported as % of cell viability referred to control; Panel B: Caspase 3 activity. The specific activity percentage of the respective control non-treated cells are reported: Panel C: DNA fragmentation assay. DNA samples, obtained from RPMI 8226 cells upon treatments, were resolved on a 1.8% agarose gel electrophoresis, stained with ethidium bromide. Panel D: Autoradiographs of PARP levels in RPMI 8226 cells. Representative Western blots are shown. The densitometric analyses of the 116 kDa full-length PARP protein from six separate blots provided for quantitative analysis are presented. Equal protein loading was verified by using an antibody directed against GAPDH (see Fig. 11). Data points marked with an asterisk are statistically significant compared to their respective not treated control cells ($^*p < 0.05$).

isolated 20S proteasomes depending on their subunits composition.

Furthermore, a possible different effect of the two fractions was investigated in HeLa tumour epithelial and in normal FHs 74 Int cell line. Interestingly, while FHs 74 Int cells survival was not altered upon treatments, a loss of vitality in HeLa cells treated with the protein fraction and with the two fractions simultaneously was observed, more evidently after 48 h treatment. Together, caspase 3 activation, p27 accumulation, PARP cleavage enhancement, DNA fragmentation induction and changes in nuclei morphology demonstrated that apoptosis was the involved mechanism in tumour cells selective death.

Previous works revealed that proteasome inhibitors are promising potent alternative drugs in myeloma treatment, through the induction of antiproliferative and pro-apoptotic mechanisms, [29,59,60]. In the present study the plasmacytoma cell line RPMI 8226 showed proteasome inhibition and p27 accumulation as already observed in HeLa cell lysates, upon the same treatments.

Caspase 3 activation, DNA fragmentation and PARP cleavage enhancement were significant, although less evident compared to HeLa cells.

However, our data are in agreement with previous works showing that compounds rich in polyphenols, such as curcumin, crisin, apigenin, quercetin, tannic acid, were able to induce

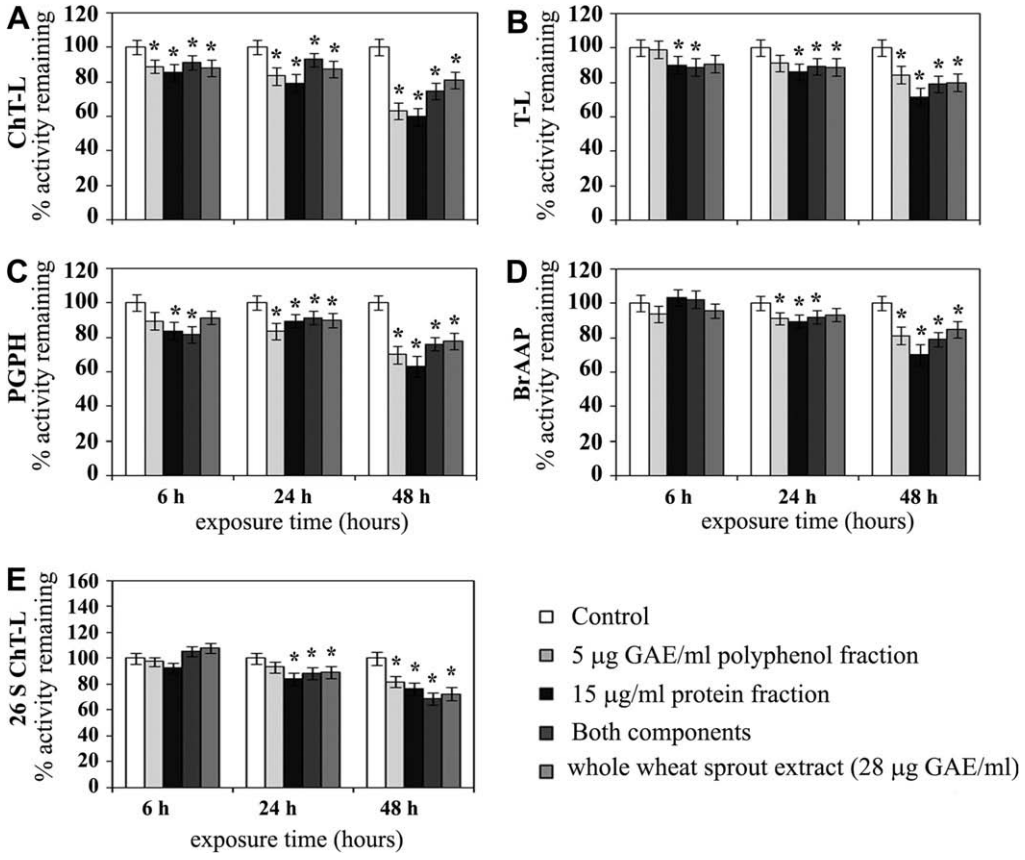

Fig. 10. Proteasome activities in RPMI 8226 lysates upon treatments with wheat sprout extract components. 20S proteasome ChT-L, T-L, PGPH and BrAAP activities are reported in panels A–D. 26S activity (panel E) was measured including in the reaction mixture 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP. Enzymatic activities were expressed as % activity remaining towards control (□) in each time set ($^*p < 0.05$ by ANOVA with post-hoc Holm-Sidak test). Fluorescence units were subtracted of the values of control assays in the presence of specific inhibitors; results derived from six independent experiments.

apoptosis in tumour cells, through the inhibition of the ubiquitin–proteasome pathway and the consequent accumulation of proteasome substrates [32,53,65,66]. The selective induction of the apoptotic pathway in the tumour cell line by the protein component of the extract is significant, but HeLa cells treated simultaneously with polyphenols and peptides show a higher degree of mortality and a stronger caspase 3 activation. These data suggested a possible synergistic effect of the two wheat sprout components. In order to verify this hypothesis and to gain insight into the activation of the apoptotic pathway, 20S and 26S proteasome functionalities were investigated and the expression levels of ubiquitinated proteins and of the pro-apoptotic proteasome substrate p27 were measured. HeLa cell proteasomes showed a higher degree of impairment with respect to normal cell complexes. In fact, the 26S ChT-L activity and the 20S ChT-L and BrAAP components were significantly depressed by the whole extract and its separated components, even at short incubation times. Only for the 26S and 20S ChT-L activities a synergistic effect of peptides and polyphenols was evident, with a 60% and a 40% decrease at 48 h respectively, compared to the inhibition induced by the two fractions separately. The protein fraction caused the strongest inhibition in PGPH and T-L. A similar trend was observed in RPMI 8226 plasmacytoma cells treated with wheat sprout polyphenols and protein components. Conversely, in the normal FHs 74 Int cell line, the 26S ChT-L activity was negligibly inhibited at 48 h treatments and all the assayed 20S activities showed a slight and delayed decrease.

These results are consistent with previous works indicating that natural polyphenolic compounds selectively inhibit proteasomes in cancer cells [31,53,67] and, additionally, evidence a relevant role for the extract protein fraction in proteasome inhibition.

Several studies reported that dietary flavonoids prevalently affect the ChT-L activity, due to the high binding affinity for the β5 subunit, both *in vitro* and in cell cultures [26,32,68]. Our results, besides confirming these data, show that the BrAAP activity was particularly influenced by both wheat sprout components, in agreement with previous works attributing this activity also to the β5 subunit [69]. The increase in the levels of p27, being it a substrate of the proteasome, further demonstrates the aforementioned inhibition.

Wheat sprout extract components, separately administered, exert a more rapid and potent effect with respect to the whole extract in tumour cells. Tumour cells are more sensitive to the treatments, compared to their counterpart of normal cells. In particular, the activation of the apoptotic pathway, due to the selective inhibition of tumour cell proteasomes, is responsible for the loss of HeLa and RPMI 8226 cells vitality. The lower effect induced by the whole extract can be due to its extremely heterogeneous composition, since it contains organic phosphates, enzymes, reducing glycosides and polyphenols, with a consequent matrix effect derived from interactions among the different constituents.

In addition, the whole wheat sprout extract affected only HeLa survival, whereas no significant effect on RPMI 8226 cells survival was observed. Similarly, the combined administration of polyphenol and protein fractions did not lead to a severe decrease in survival or in proteasome functionality, differently from HeLa cells, even though in both cases proteasome inhibition was observed. The most evident discrepancy between HeLa and RPMI 8226 cells was in the level of caspase 3 activation.

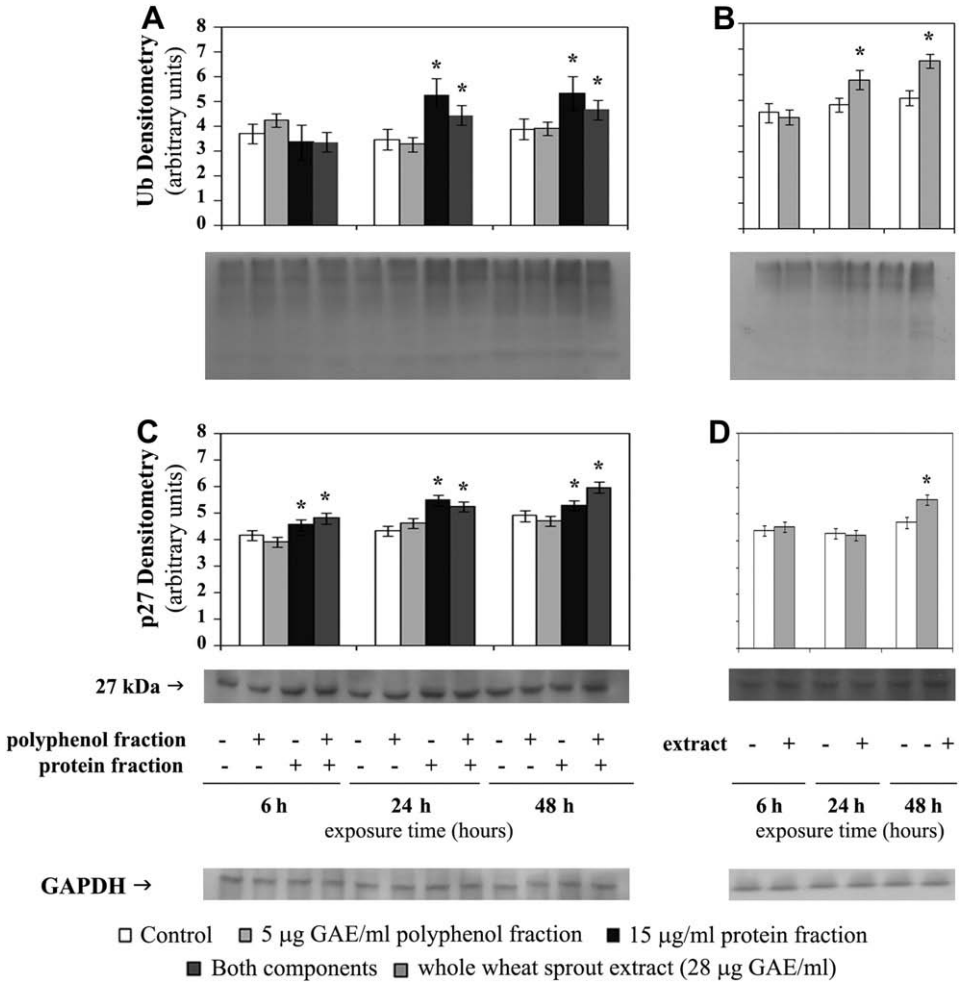

Fig. 11. Ubiquitinated proteins and p27 levels in RPMI 8226 cells. Autoradiographs of ubiquitinated proteins in RPMI 8226 cells treated with whole wheat sprout extract (panel B) and its components (panel A) for 6, 24, 48 h. p27 levels are shown in panels C and D. Representative Western blots are presented and the densitometric analysis from six separate blots provided for quantitative analysis is presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells ($^*p < 0.05$).

These observations indicate that tumour cells behave in a different manner in the presence of such fractions. Further experiments will clarify a potential significant role of wheat sprout extract as a therapeutic adjuvant in anticancer treatments.

References

[1] J.H. Weisburger, Lifestyle, health and disease prevention: the underlying mechanisms, Eur. J. Cancer Prev. 11 (Suppl. 2) (2002) S1–S7.

[2] M.D. dos Santos, M.C. Almeida, N.P. Lopes, G.E. de Souza, Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid, Biol. Pharm. Bull. 29 (2006) 2236–2240.

[3] L. Whyte, Y.Y. Huang, K. Torres, R.G. Mehta, Molecular mechanisms of resveratrol action in lung cancer cells using dual protein and microarray analyses, Cancer Res. 67 (2007) 12007–12017.

[4] S. Bastianetto, Z.X. Yao, V. Papadopoulos, R. Quirion, Neuroprotective effects of green and black teas and their catechin gallate esters against beta-amyloid-induced toxicity, Eur. J. Neurosci. 23 (2006) 55–64.

[5] Y. Levites, T. Amit, M.B. Youdim, S. Mandel, Involvement of protein kinase C activation and cell survival/ cell cycle genes in green tea polyphenol (−)-epigallocatechin 3-gallate neuroprotective action, J. Biol. Chem. 277 (2002) 30574–30580.

[6] A. Basso, G. Rossolini, A. Piantanelli, D. Amici, I. Calzuola, L. Mancinelli, V. Marsili, G.L. Gianfranceschi, Aging reversibility: from thymus graft to vegetable extract treatment – application to cure an age-associated pathology, Biogerontology 6 (2005) 245–253.

[7] G. Falcioni, D. Fedeli, L. Tiano, I. Calzuola, L. Mancinelli, V. Marsili, G.L. Gianfranceschi, Antioxidant activity of wheat sprouts extract in vitro: inhibition of DNA oxidative damage, J. Food Sci. 68 (2002) 2918–2922.

[8] V. Marsili, I. Calzuola, G.L. Gianfranceschi, Nutritional relevance of wheat sprouts containing high levels of organic phosphates and antioxidant compounds, J. Clin. Gastroenterol. 38 (2004) S123–S126.

[9] B. Peryt, T. Szymczyk, P. Lesca, Mechanism of antimutagenicity of wheat sprout extracts, Mutat. Res. 269 (1992) 201–215.

[10] B. Tudek, B. Peryt, J. Miloszewska, T. Szymczyk, M. Przybyszewska, P. Janik, The effect of wheat sprout extract on benzo(a)pyrene and 7,2-dimethylbenz(a)anthracene activity, Neoplasma 35 (1988) 515–523.

[11] K. Kang, H.J. Lee, C.Y. Kim, S.B. Lee, J. Tunsag, D. Batsuren, C.W. Nho, The chemopreventive effects of *Saussurea salicifolia* through induction of apoptosis and phase II detoxification enzyme, Biol. Pharm. Bull. 30 (2007) 2352–2359.

[12] I. Calzuola, V. Marsili, G.L. Gianfranceschi, Synthesis of antioxidants in wheat sprouts, J. Agric. Food Chem. 52 (2004) 5201–5206.

[13] B. Peryt, J. Miloszewska, B. Tudek, M. Zielenska, T. Szymczyk, Antimutagenic effects of several subfractions of extract from wheat sprout toward benzo[-a]pyrene-induced mutagenicity in strain TA98 of *Salmonella typhimurium*, Mutat. Res. 206 (1988) 221–225.

[14] M. Pagano, S.W. Tam, A.M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P.R. Yew, G.F. Draetta, M. Rolfe, Role of the ubiquitin–proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27, Science 269 (1995) 682–685.

[15] M. Roff, J. Thompson, M.S. Rodriguez, J.M. Jacque, F. Baleux, F. Arenzana-Seisdedos, R.T. Hay, Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo, J. Biol. Chem. 271 (1996) 7844–7850.

[16] C.G. Maki, J.M. Huibregtse, P.M. Howley, In vivo ubiquitination and proteasome-mediated degradation of p53(1), Cancer Res. 56 (1996) 2649–2654.

[17] B. Li, Q.P. Dou, Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 3850–3855.

[18] S.J. Thompson, L.T. Loftus, M.D. Ashley, R. Meller, Ubiquitin–proteasome system as a modulator of cell fate, Curr. Opin. Pharmacol. (2007) 90–95.

[19] R. Hjerpe, M.S. Rodriguez, Alternative UPS drug targets upstream the 26S proteasome, Int. J. Biochem. Cell Biol. (2007) 1126–1140.
[20] K.J. Davies, Degradation of oxidized proteins by the 20S proteasome, Biochimie 83 (2001) 301–310.
[21] C. Cardozo, Catalytic components of the bovine pituitary multicatalytic proteinase complex (proteasome), Enzyme Protein 47 (1993) 296–305.
[22] M. Orlowski, The multicatalytic proteinase complex, a major extralysosomal proteolytic system, Biochemistry 29 (1990) 10289–10297.
[23] M. Orlowski, C. Cardozo, C. Michaud, Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids, Biochemistry 32 (1993) 1563–1572.
[24] M. Orlowski, S. Wilk, Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex, Arch. Biochem. Biophys. 383 (2000) 1–16.
[25] P.M. Kloetzel, A. Soza, R. Stohwasser, The role of the proteasome system and the proteasome activator PA28 complex in the cellular immune response, Biol. Chem. 380 (1999) 293–297.
[26] A.F. Kisselev, A.L. Goldberg, Proteasome inhibitors: from research tools to drug candidates, Chem. Biol. 8 (2001) 739–758.
[27] M.S. Chen, D. Chen, Q.P. Dou, Inhibition of proteasome activity by various fruits and vegetables is associated with cancer cell death, In Vivo 18 (2004) 73–80.
[28] B. An, R.H. Goldfarb, R. Siman, Q.P. Dou, Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts, Cell Death Differ. 5 (1998) 1062–1075.
[29] I. Zavrski, C. Naujokat, K. Niemoller, C. Jakob, U. Heider, C. Langelotz, C. Fleissner, J. Eucker, K. Possinger, O. Sezer, Proteasome inhibitors induce growth inhibition and apoptosis in myeloma cell lines and in human bone marrow myeloma cells irrespective of chromosome 13 deletion, J. Cancer Res. Clin. Oncol. 129 (2003) 383–391.
[30] D. Soligo, F. Servida, D. Delia, E. Fontanella, G. Lamorte, L. Caneva, R. Fumiatti, G. Lambertenghi Deliliers, The apoptogenic response of human myeloid leukaemia cell lines and of normal and malignant haematopoietic progenitor cells to the proteasome inhibitor PSI, Br. J. Haematol. 113 (2001) 126–135.
[31] A. Kazi, D.A. Urbizu, D.J. Kuhn, A.L. Acebo, E.R. Jackson, G.P. Greenfelder, N.B. Kumar, Q.P. Dou, A natural musaceas plant extract inhibits proteasome activity and induces apoptosis selectively in human tumor and transformed, but not normal and non-transformed, cells, Int. J. Mol. Med. 12 (2003) 879–887.
[32] D. Chen, K.G. Daniel, M.S. Chen, D.J. Kuhn, K.R. Landis-Piwowar, Q.P. Dou, Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells, Biochem. Pharmacol. 69 (2005) 1421–1432.
[33] K.R. Landis-Piwowar, V. Milacic, D. Chen, H. Yang, Y. Zhao, T.H. Chan, B. Yan, Q.P. Dou, The proteasome as a potential target for novel anticancer drugs and chemosensitizers, Drug Resist. Updat. 9 (2006) 263–273.
[34] M. Amici, L. Bonfili, M. Spina, V. Cecarini, I. Calzuola, V. Marsili, M. Angeletti, E. Fioretti, R. Tacconi, G.L. Gianfranceschi, A.M. Eleuteri, Wheat sprout extract induces changes on 20S proteasomes functionality, Biochimie (2007) 790–801.
[35] A. Pettinari, M. Amici, M. Cuccioloni, M. Angeletti, E. Fioretti, A.M. Eleuteri, Effect of polyphenolic compounds on the proteolytic activities of constitutive and immuno-proteasomes, Antioxid. Redox Signal. 8 (2006) 121–129.
[36] I. Calzuola, G.L. Gianfranceschi, V. Marsili, Comparative activity of antioxidants from wheat sprouts, *Morinda citrofolia*, fermented papaya and white tea, Int. J. Food Sci. Nutr. 57 (2006) 168–177.
[37] M. Orlowski, C. Michaud, Pituitary multicatalytic proteinase complex. Specificity of components and aspects of proteolytic activity, Biochemistry 28 (1989) 9270–9278.
[38] G. Pfleiderer, Isolation of an aminopeptidase from kidney particles, Methods Enzymol. 19 (1970) 514–521.
[39] J. Almenoff, M. Orlowski, Membrane-bound kidney neutral metalloendopeptidase: interaction with synthetic substrates, natural peptides, and inhibitors, Biochemistry 22 (1983) 590–599.
[40] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventón, Analysis of total phenol and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent, Methods Enzymol. (1999) 152–178.
[41] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[42] C.M. Wilson, Studies and critique of Amido Black 10B, Coomassie Blue R, and Fast Green FCF as stains for proteins after polyacrylamide gel electrophoresis, Anal. Biochem. 96 (1979) 263–278.
[43] S. Wilk, M. Orlowski, Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex, J. Neurochem. 40 (1983) 842–849.
[44] A.M. Eleuteri, M. Angeletti, G. Lupidi, R. Tacconi, L. Bini, E. Fioretti, Isolation and characterization of bovine thymus multicatalytic proteinase complex, Protein Expr. Purif. 18 (2000) 160–168.
[45] M. Amici, G. Lupidi, M. Angeletti, E. Fioretti, A.M. Eleuteri, Peroxynitrite-induced oxidation and its effects on isolated proteasomal systems, Free Radic. Biol. Med. 34 (2003) 987–996.
[46] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[47] G. Fenteany, R.F. Standaert, W.S. Lane, S. Choi, E.J. Corey, S.L. Schreiber, Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin, Science 268 (1995) 726–731.
[48] A.M. Eleuteri, R.A. Kohanski, C. Cardozo, M. Orlowski, Bovine spleen multicatalytic proteinase complex (proteasome). Replacement of X, Y, and Z subunits by LMP7, LMP2, and MECL1 and changes in properties and specificity, J. Biol. Chem. 272 (1997) 11824–11831.
[49] A. Vinitsky, C. Cardozo, L. Sepp-Lorenzino, C. Michaud, M. Orlowski, Inhibition of the proteolytic activity of the multicatalytic proteinase complex (proteasome) by substrate-related peptidyl aldehydes, J. Biol. Chem. 269 (1994) 29860–29866.
[50] C. Marchini, M. Angeletti, A.M. Eleuteri, A. Fedeli, E. Fioretti, Aspirin modulates LPS-induced nitric oxide release in rat glial cells, Neurosci. Lett. 381 (2005) 86–91.
[51] F. Buonanno, L. Quassinti, M. Bramucci, C. Amantini, R. Lucciarini, G. Santoni, H. Iio, C. Ortenzi, The protozoan toxin climacostol inhibits growth and induces apoptosis of human tumor cell lines, Chem. Biol. Interact. 176 (2008) 151–164.
[52] C. Manach, G. Williamson, C. Morand, A. Scalbert, C. Remesy, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, Am. J. Clin. Nutr. 81 (2005) 230S–242S.
[53] S. Nam, D.M. Smith, Q.P. Dou, Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo, J. Biol. Chem. 276 (2001) 13322–13330.
[54] A.G. Porter, R.U. Janicke, Emerging roles of caspase-3 in apoptosis, Cell Death Differ. 6 (1999) 99–104.
[55] C. Soldani, A.I. Scovassi, Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update, Apoptosis 7 (2002) 321–328.
[56] E.B. Affar, M. Germain, E. Winstall, M. Vodenicharov, R.G. Shah, G.S. Salvesen, G.G. Poirier, Caspase-3-mediated processing of poly(ADP-ribose) glycohydrolase during apoptosis, J. Biol. Chem. 276 (2001) 2935–2942.
[57] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G.G. Poirier, W.C. Earnshaw, Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE, Nature 371 (1994) 346–347.
[58] H.C. Drexler, The role of p27Kip1 in proteasome inhibitor induced apoptosis, Cell Cycle 2 (2003) 438–441.
[59] R. LeBlanc, L.P. Catley, T. Hideshima, S. Lentzsch, C.S. Mitsiades, N. Mitsiades, D. Neuberg, O. Goloubeva, C.S. Pien, J. Adams, D. Gupta, P.G. Richardson, N.C. Munshi, K.C. Anderson, Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model, Cancer Res. 62 (2002) 4996–5000.
[60] D.J. Kuhn, Q. Chen, P.M. Voorhees, J.S. Strader, K.D. Shenk, C.M. Sun, S.D. Demo, M.K. Bennett, F.W. van Leeuwen, A.A. Chanan-Khan, R.Z. Orlowski, Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin–proteasome pathway, against preclinical models of multiple myeloma, Blood 110 (2007) 3281–3290.
[61] T. Katsuki, K. Hirata, H. Ishikawa, N. Matsuura, S. Sumi, H. Itoh, Aged garlic extract has chemopreventative effects on 1,2-dimethylhydrazine-induced colon tumors in rats, J. Nutr. 136 (2006) 847S–851S.
[62] A. Kumatori, K. Tanaka, N. Inamura, S. Sone, T. Ogura, T. Matsumoto, T. Tachikawa, S. Shin, A. Ichihara, Abnormally high expression of proteasomes in human leukemic cells, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 7071–7075.
[63] J. Adams, Potential for proteasome inhibition in the treatment of cancer, Drug Discov. Today 8 (2003) 307–315.
[64] D. Kuhn, W.H. Lam, A. Kazi, K.G. Daniel, S. Song, L.M. Chow, T.H. Chan, Q.P. Dou, Synthetic peracetate tea polyphenols as potent proteasome inhibitors and apoptosis inducers in human cancer cells, Front. Biosci. 10 (2005) 1010–1023.
[65] N.R. Jana, P. Dikshit, A. Goswami, N. Nukina, Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway, J. Biol. Chem. 279 (2004) 11680–11685.
[66] M.S. Weng, Y.S. Ho, J.K. Lin, Chrysin induces G1 phase cell cycle arrest in C6 glioma cells through inducing p21Waf1/Cip1 expression: involvement of p38 mitogen-activated protein kinase, Biochem. Pharmacol. 69 (2005) 1815–1827.
[67] S.B. Wan, D. Chen, Q.P. Dou, T.H. Chan, Study of the green tea polyphenols catechin-3-gallate (CG) and epicatechin-3-gallate (ECG) as proteasome inhibitors, Bioorg. Med. Chem. 12 (2004) 3521–3527.
[68] D.M. Smith, K.G. Daniel, Z. Wang, W.C. Guida, T.H. Chan, Q.P. Dou, Docking studies and model development of tea polyphenol proteasome inhibitors: applications to rational drug design, Proteins 54 (2004) 58–70.
[69] M. Groll, M. Bochtler, H. Brandstetter, T. Clausen, R. Huber, Molecular machines for protein degradation, Chembiochem 6 (2005) 222–256.