ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 1 & 2, 2006
© Mary Ann Liebert, Inc.

Forum Original Research Communication

Effect of Polyphenolic Compounds on the Proteolytic Activities of Constitutive and Immuno-Proteasomes

ASSUNTINA PETTINARI, MANILA AMICI, MASSIMILIANO CUCCIOLONI, MAURO ANGELETTI, EVANDRO FIORETTI, and ANNA MARIA ELEUTERI

ABSTRACT

The effect of several polyphenols on the 20S proteasomes, both the constitutive and the LMP proteasomes, isolated from bovine tissues, has been investigated. Polyphenolic compounds show many biological activities such as antiviral, antibacterial, antifungal, anti-inflammatory, antimutagenic, and antiallergic activities. However, the molecular mechanism underlying these effects has not been identified. It is well established that polyphenols possess inhibitory activities on several enzymes and among them the 20S proteasome. In the present work, the ChT-L, BrAAP, PGPH, and T-L activities of the isolated constitutive and immuno-proteasomes were assayed in order to get an overall information on the polyphenols binding to the complexes. The effects of the polyphenols on the proteasomal activities were analyzed, taking into account the different subunits composition of the two complexes. Furthermore the same activities were measured on whole extracts from cancer cells exposed to EGCG and gallic acid, evaluating, also, their antioxidant action under oxidative stress. EGCG and gallic acid are able to affect the 20S proteasomes functionality, depending on the complex subunit composition and, in cell extracts, they behave both as antioxidants and proteasome effectors. *Antioxid. Redox Signal.* 8, 121–129.

INTRODUCTION

Polyphenols are a group of natural products found in fruits, vegetables, nuts, seeds, and flowers, as well as in teas and wines, and are important constituents of human diet. They possess many biological and pharmacological activities, such as antibacterial, antiviral, antifungal, antimutagenic, anti-inflammatory, and neuroprotective actions (4, 18, 31). It is now well established that they exert the anticarcinogenic, anti-inflammatory, and cardiovascular activities through their antioxidant ability and the modulation of endogenous metabolizing and antioxidant enzymes (13). For example, superoxide dismutase (SOD) and catalase are activated by epigallocatechin-3-gallate (EGCG) (16), whereas the mouse brain monoamine oxidase (MAO) is inhibited by tannic acid (TA) (15). Furthermore, several flavonoids and phenolic antioxidants have been shown to activate the expression of some stress-response genes, such as glutathione S-transferase and heme-oxygenase 1 (6).

Among the enzymes involved in the regulation of cellular homeostasis, a pivotal role is played by the proteasome. In fact, it is responsible for the degradation of misfolded, aggregated, and oxidized proteins being part of the cell defense against oxidative stress. In addition, it has been shown that it degrades important cell-cycle regulators having an essential function in cell cycle progression (3, 7, 8, 10–12, 19, 24, 29).

The 20S proteasome is a 700 KDa particle composed of four stacked rings each containing seven subunits. The two inner rings, formed by seven different β subunits, harbor the catalytic sites, whereas the outer rings, made up of seven different α

Postgraduate School in Clinical Biochemistry, Department of Molecular, Cellular and Animal Biology, University of Camerino, Camerino, Italy.

subunits, have substrate gating functions and provide an anchorage for the regulatory particles 19S and 11S, constituting the 26S proteasome. In eukaryotes, of the seven β subunits only three possess a catalytic N-terminal threonine necessary for hydrolysis of the substrates giving rise to the chymotrypsin-like (ChT-L), trypsin-like (T-L) and peptidylglutamyl-peptide hydrolizing (PGPH) activities (8). Upon exposure of cells to interferon-γ (IFN-γ) the three catalytic subunits X, Y, and Z are replaced by three other subunits called LMP7, LMP2, and MECL-1, respectively, generating the so-called immunoproteasome. It shows different specificities, caused by those replacements: depression of the PGPH activity due to the replacement of the Y subunit by LMP2, which expresses a ChT-L activity and the appearance of a very active catalytic component which cleaves bonds after branched chain and aromatic amino acids, most likely showed by LMP7, and could be responsible for the generation of class I antigenic peptides.

However extensive interactions between the 20S proteasome subunits, both active and inactive ones, affecting the catalytic properties of the enzyme should be taken into account.

Recently, several studies have shown the effect of green tea polyphenols on a recombinant 20S proteasome of *Methanosarcina thermophila*, an archea form of the complex, indicating an inhibitory action of those compounds on the ChT-L activity (27–28, 32). A similar result was also obtained measuring this activity on lysates from Jurkat T cells treated with the antioxidants (21).

We present here the effect of several polyphenols on the 20S proteasomes, both the constitutive and the LMP proteasomes, isolated from bovine tissues. Studying mammalian 20S proteasomes, the ChT-L, BrAAP, PGPH and T-L components have been assayed in order to get overall information on the polyphenols binding to the complexes. Furthermore the same activities have been measured on whole extracts from cancer cells exposed to EGCG and gallic acid, evaluating, also, their antioxidant action under oxidative stress.

MATERIALS AND METHODS

Materials

Bovine brain and thymus were obtained from the local slaughterhouse. They were rapidly frozen in liquid nitrogen and maintained at −70°C. The antisubunit X, Y, LMP7, and LMP2 antibodies were purchased from Affiniti Research Products Ltd. (Mamhead, Exeter, UK). The Oxidized Protein Detection Kit (OxyBlot) was purchased from Appligene-Oncor (Strasbourg, France).

Substrates for assaying the ChT-L, PGPH, and T-L activities were purchased from Sigma (Milano, Italy) (suc-LLVY-MCA and Z-GGL-pNA, Z-LLE-MCA and Z-LLE-2NA, Z-GGR-MCA and Z-GGR-2NA). The Z-GPALA-pAB substrate was the kind gift of Prof. M. Orlowski.

Highly purified polyphenols were purchased from Sigma and used directly without further purification: gallic acid and (-)-epigallocatechin gallate (EGCG) were dissolved in assay buffer (Tris-HCl 50 m*M*, pH 8.0); resveratrol, (-)-epicatechin, and rutin hydrate were solubilized in DMSO-assay buffer (1:1); quercetin, quercetin-3-D-galactoside (hyperosid), ellagic acid, and luteolin were dissolved in DMSO. Other reagents were obtained from Sigma-Aldrich Corp.

Isolation of 20S proteasomes

Isolation and purification of 20S proteasomes were carried out following experimental protocols very similar to that previously utilized for the MPC isolation from other bovine organs (9, 22) and essentially based on a fractionation from 40% to 60% in ammonium sulfate, an ionic exchange chromatography, and two gel filtration columns that favor the removal of lower molecular weight contaminants. A higher degree of purification was obtained by adding a hydrophobic interaction chromatography step that seems to improve the separation of proteasome from the copurifying chaperonine Hsp90.

Immunoblot analyses using anti-X, Y, Z, and anti-LMP2, LMP7, and MECL1 antibodies revealed that the constitutive 20S proteasome was predominantly expressed in the bovine brain, whereas the immunoproteasome was detectable in the bovine thymus (2, 9).

Measurement of 20S proteasomes activity

The ChT-L, T-L, PGPH, and BrAAP activities of isolated 20S proteasomes were determined spectrophotometrically as reported previously (9, 23, 33), using Z-GGL-pNA, Z-GGR-2NA, Z-LLE-2NA, and Z-GPALA-pAB, respectively, as substrates. Fluorogenic substrates were also used: Suc-LLVY-AMC 5 μ*M* for the ChT-L activity, Z-LLE-AMC 5 μ*M* for the PGPH activity, Z-GGR-AMC 20 μ*M* for the T-L activity, and Z-GPALA-pAB 0.5 m*M* for the BrAAP activity. Cell lysates or isolated proteasomes were incubated with the synthetic substrates in the assay buffer. After 1 hour of incubation at 37°C, the measurement of the hydrolyzed 7-amino-4-methylcoumarin (AMC) or 4-aminobenzoic acid (PABA) were detected (AMC: λ_{exc} = 365 nm, λ_{em} = 449 nm; PABA: λ_{exc} = 304 nm, λ_{em} = 664 nm).

20S proteasomes activities were tested in the presence of increasing amounts of antioxidants: control assays were performed in order to evaluate DMSO-buffer interference.

Aminopeptidase N (EC 3.4.11.2), used for coupled assay utilized for detecting the BrAAP activity (23), was purified from pig kidney, as reported elsewhere (1, 26). Control experiments were done in the presence of specific inhibitors: Z-GPFL-CHO and lactacystin (5 μ*M* in the assay).

Cell culture and cell extract preparation

Human colon adenocarcinoma (Caco 2, Istituto Zooprofilattico Sperimentale – Lombardia ed Emilia Romagna, Italy) cell line was cultured in D-MEM medium (Gibco Invitrogen Cell Culture, Italy) supplemented with 15% fetal calf serum, 0.4% L-glutamine, 1% non-essential amino acids, penicillin, and streptomycin. Cells were maintained in a 5% CO_2 atmosphere at 37°C.

After removing the medium and washing with cold PBS, cells were harvested in 3 ml PBS, centrifuged at 1600 rpm for 5 minutes and the pellet was homogenized in a lysis buffer (20 m*M* Tris, pH 7.4, 250 m*M* sucrose, 1 m*M* EDTA and 5 m*M* β-mercaptoethanol) and passed through a 25-gauge

needle for at least ten times. Lysates were centrifuged at 12000 $\times$ *g* for 15 minutes and the supernatants were stocked at −80°C.

Exposure of cell culture to antioxidants

In order to verify the effect of antioxidants on proteasome activity and viability of living tumor cells, Caco cells were cultured for 12 hours and 24 hours with various concentrations of EGCG and gallic acid (from 0 to 300 μ*M*). Cell viability was evaluated immediately after exposure and cell lysates were used for activities measurements with fluorogenic substrates. Treated and untreated cells were harvested in PBS. Cell counting was performed following trypan blue staining. The cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution (Sigma-Aldrich Corp.) and subsequently evaluated under the light microscope. The membrane of dead cells is permeable to trypan blue (blue stained cells), whereas living cells remain unstained. Cell viability was expressed as percentage of surviving cells compared to the total number of cells. Significance was tested by using the Student's *t* test.

The antioxidant action of EGCG and gallic acid was tested incubating the cells for 12 hours or 24 hours in the presence or absence of polyphenolic compounds (150 μ*M* EGCG or 200 μ*M* gallic acid). After discarding the medium, cells were stressed with increasing amounts of H_2O_2 (0.0, 0.1, 0.5, and 1.0 m*M*). H_2O_2-medium was removed after 30 minutes incubation and cells were washed twice with PBS before harvesting.

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion as described previously (14, 20). Briefly, following experimental treatment, cells were washed in phosphate-buffered saline (PBS, pH 7.5) and then MTT (final concentration 0.5 mg/ml) was added to culture medium without fetal calf serum and incubated for 2 hours at 37°C. The medium was then removed and replaced with 100 μl DMSO. The optical density was measured at 550 nm in a microtiter plate reader. At least six cultures were utilized for each time point.

Polyacrylamide gel electrophoresis and Western blot analysis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (30) was done in 12% gels. Immunoblot experiments using anti-X and Y subunits or anti-LMP2 and LMP7 subunit antibodies (Affiniti Research Products Limited) were performed by electroblotting lysate samples, previously separated on a 12% SDS gel, onto a PVDF membrane (Millipore Corp., Bedford, MA) according to Towbin and Burnette (5, 30).

Immunoblot detection of carbonyl groups was performed with the OxyBlot oxidized protein detection kit (Appligene-Oncor), according to the manufacturer. Briefly, 15 μg of cell lysates were incubated for 15 minutes at room temperature with 2,4-dinitrophenylhydrazine (DNPH) to form the dinitrophenylhydrazone carbonyl derivate and separated on a 12% SDS/PAGE. The modified proteins, blotted on a PVDF membrane, were revealed by anti-DNP antibodies. The immunoblot detection was carried out with ECL (Enhanced ChemiLuminescence) western blotting analysis system (Amersham-Pharmacia-Biotech, Cologno Monzese, Italy) using peroxidase conjugated anti-rabbit or anti-mouse secondary antibodies.

RESULTS

Inhibition of purified 20S proteasome activities by polyphenolic compounds

It has been reported that several tea polyphenols containing ester bonds inhibit the ChT-L activity of isolated 20S proteasome (*Methanosarcina thermophila*, recombinant, *Escherichia coli*). In the present study we have investigated the effect of several polyphenols on the proteolytic activities of constitutive and interferon-γ inducible 20S proteasomes isolated from bovine brain and thymus, respectively. Table 1 summarizes the IC_{50} values of tested polyphenols calculated for the ChT-L (Z-GGL-pNA), BrAAP (Z-GPALA-pAB), PGPH (Z-LLE-2NA), and T-L (Z-GGR-2NA) activities. Together these data show that polyphenolic compounds selectively alter individual proteasome activities. In fact we have found that EGCG strongly inhibits the ChT-L activity of both proteasomes, whereas it seems to be a specific inhibitor of the immunoproteasome BrAAP component. It is also effective on the T-L activity of the two enzymes but with a lower IC_{50} for the inducible complex. Gallic acid affects the ChT-L activity of both complexes at the same extent, while its inhibitory effect on the T-L activity is higher for the constitutive proteasome. The other tested polyphenols seem to exert none or a weaker inhibition on all the assayed activities for the two enzymes.

Moreover, in order to compare our results with those previously showed by other authors (21, 27, 28) which utilized Suc-LLVY-AMC for measuring the ChT-L component of a recombinant 20S proteasome from *Methanosarcina thermophila,* activity assays were performed with fluorogenic peptides in the presence of increasing amounts of EGCG. To check whether the inhibitory effect was higher in the activated enzyme compared to the basal form, the assays were executed in the presence or absence of SDS. Table 2 reports the IC_{50} values calculated for the two 20S proteasomes.

From these data it is evident that the ChT-L, measured with Suc-LLVY-AMC, shows a higher inhibition which does not seem to increase for the SDS-activated immunoproteasome, whereas the inhibitory action improves significantly in the SDS-activated XYZ-proteasome. However, the IC_{50} obtained for the eukaryotic 20S proteasomes are always higher than those reported for the *Methanosarcina thermophila* 20S complex.

Effect of antioxidants on proteasome activities in Caco cell lysates

We then verified if the more effective polyphenols (EGCG and gallic acid) could inhibit the 20S proteasome activities in tumor cell lysates. In order to do that, we first analyzed the proteasome subunits expression in cultured Caco cell line through immunoblot experiments using anti-X and Y subunits or anti-LMP2 and LMP7 subunits antibodies. Both constitutive and interferon-γ inducible subunits were detected in cell lysates, even if a prevalence of a constitutive type of complex seems to be expressed by the cells (Fig. 1).

In addition, we determined the effects of increasing amounts of EGCG and gallic acid (from 0 to 0.3 m*M*) on the cellular viability in order to establish the toxic doses for the two polyphenols. After 12 hours and 24 hours incubation with 150

TABLE 1. POLYPHENOLS IC_{50} VALUES OBTAINED FOR THE CHT-L, BRAAP, PGPH, AND T-L ACTIVITIES OF THE XYZ- AND LMP- PROTEASOMES

		*IC₅₀ (m*M*)*			
		ChT-L	*BrAAP*	*PGPH*	*T-L*
Gallic acid	XYZ	0.72 ± 0.10	13.55 ± 4.34	4.90 ± 1.09	0.253 ± 0.151
	LMP	0.71 ± 0.09	6.63 ± 0.39	5.78 ± 0.64	4.45 ± 0.44
EGCG	XYZ	0.22 ± 0.06	4.3 ± 0.89	3.4 ± 0.24	0.321 ± 0.075
	LMP	0.46 ± 0.10	0.24 ± 0.07	2.07 ± 0.31	0.126 ± 0.015
(-)-Epicatechin	XYZ	14.29 ±1.98	5.37 ± 1.66	NI*	NI*
	LMP	12.61 ± 2.14	11.56 ± 4.20	NI*	13.69 ± 2.12
Luteolin	XYZ	17.88 ± 8.67	2.87 ± 0.41	15.67 ± 12.00	13.62 ± 3.35
	LMP	7.26 ± 1.54	4.41 ± 0.26	7.93 ± 1.05	NI*
Quercetin	XYZ	3.11 ± 0.32	5.38 ± 0.84	NI*	1.58 ± 0.50
	LMP	2.42 ± 0.28	1.17 ± 0.72	2.41 ± 0.60	4.62 ± 1.30
Hyperosid	XYZ	9.57 ± 0.88	24.97 ± 11.73	NI*	14.64 ± 1.89
	LMP	6.18 ± 0.36	15.19 ± 6.91	NI*	NI*
Rutin	XYZ	7.16 ±1.42	14.60 ± 0.43	NI*	6.58 ± 0.97
	LMP	7.25 ± 0.63	35.81 ± 21.99	NI*	4.09 ± 0.74
Resveratrol	XYZ	1.61 ± 0.49	9.24 ± 2.15	NI*	10.07 ± 1.65
	LMP	1.88 ± 0.57	9.07 ± 0.83	45.32 ± 7.04	15.79 ± 2.51
Ellagic acid	XYZ	2.32 ± 0.67	14.55 ± 12.49	2.54 ± 0.30	3.26 ± 0.2
	LMP	2.63 ± 0.24	7.20 ± 4.69	1.34 ± 0.45	NI*

*N.I., no inhibition.

μ*M* EGCG or 200 μ*M* gallic acid was assured a viability higher than 80% of the controls, allowing us to work with essentially viable cell cultures.

Therefore, activity assays were performed on cell lysates after 12 hours and 24 hours antioxidant incubation. Figure 2 summarizes the effects on the ChT-L, BrAAP, PGPH, and T-L activities. To investigate whether the proteolytic components of the 20S proteasome were responsible for the substrates degradation, the activities were also examined in the presence of specific proteasome inhibitors (see Materials and Methods); the activities dramatically decreased (data not shown). From Figure 2, an evident inhibitory effect of EGCG on the ChT-L, at 12 and 24 hours treatment, and on the BrAAP activity, after 24 hours, can be noticed, whereas the other components do not seem to decrease, with the PGPH activity being activated after 24 hours treatment. Gallic acid inhibits only the ChT-L (12 hours exposure) and the BrAAP (24 hours) activities.

Antioxidant action of polyphenolic compounds in tumor cell line

In order to verify the protective effect of EGCG and gallic acid under oxidative conditions, induced by different H_2O_2 concentrations, immunoblot detection of carbonyl groups was performed in cell lysates.

Cells were first analyzed for cellular viability by quantification of MTT reduction, following exposure to increasing concentrations of H_2O_2 in the presence and in absence of EGCG (150 μ*M*) or gallic acid (200 μ*M*) (Fig. 3). Treating the cells with polyphenols induces a general slight decrease of cell survival. This effect is more evident with EGCG than with gallic acid.

The results obtained from the Oxyblot procedure (Figs. 4 and 5) indicate that increasing amounts of hydrogen peroxide induce a gradual increase of carbonyl groups and that cell preincubation with 150 μ*M* EGCG or 200 μ*M* gallic acid clearly prevent protein oxidation documented by the fact that the amounts in carbonyl groups do not increase upon H_2O_2 exposure. From the autoradiographies it seems that EGCG has a higher antioxidant effect with respect to gallic acid.

Furthermore, with the aim to test whether the antioxidant effect of the polyphenolic compounds was reflected on the proteasome functionality, the ChT-L, PGPH, BrAAP, and T-L activities were measured on cell lysates obtained from Caco cells treated with EGCG and gallic acid for 12 hours or 24 hours before exposure to H_2O_2 (Fig. 6). From the graphics an

TABLE 2. POLYPHENOLS IC_{50} VALUES OBTAINED FOR THE CHT-L, BRAAP, PGPH, AND T-L ACTIVITIES OF THE XYZ- AND LMP- PROTEASOMES IN THE PRESENCE AND ABSENCE OF 0.02% SDS

		*IC₅₀ (m*M*)*			
		ChT-L	*BrAAP*	*PGPH*	*T-L*
EGCG + SDS (0.02%)	XYZ	0.038 ± 0.004	0.017 ± 0.003	NI*	0.645 ± 0.179
	LMP	0.09 ± 0.03	0.031 ± 0.006	0.105 ± 0.008	0.702 ± 0.181
EGCG − SDS	XYZ	0.14 ± 0.03	0.06 ± 0.01	0.75 ± 0.35	0.608 ± 0.173
	LMP	0.06 ± 0.01	0.018 ± 0.003	0.033 ± 0.002	0.242 ± 0.032

*N.I., no inhibition.

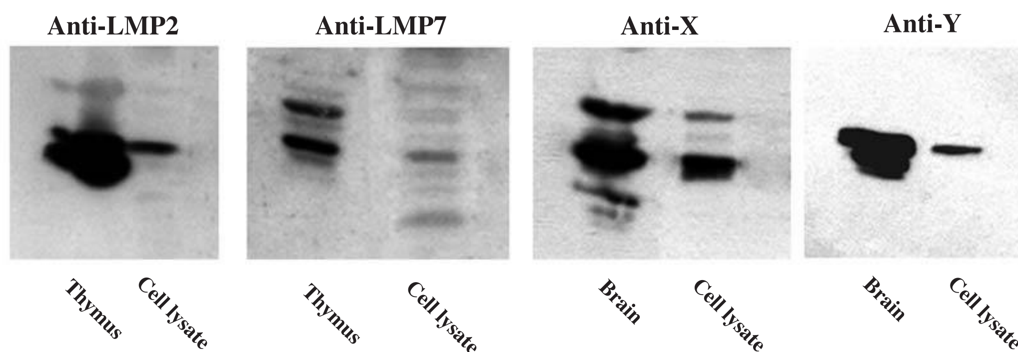

FIG. 1. Autoradiographs of the constitutive and the interferon-γ inducible subunits of Caco cell lysates. Comparison with purified 20S proteasomes isolated from bovine thymus, as a control for the immunoproteasome, and from bovine brain, as a control for the constitutive proteasome.

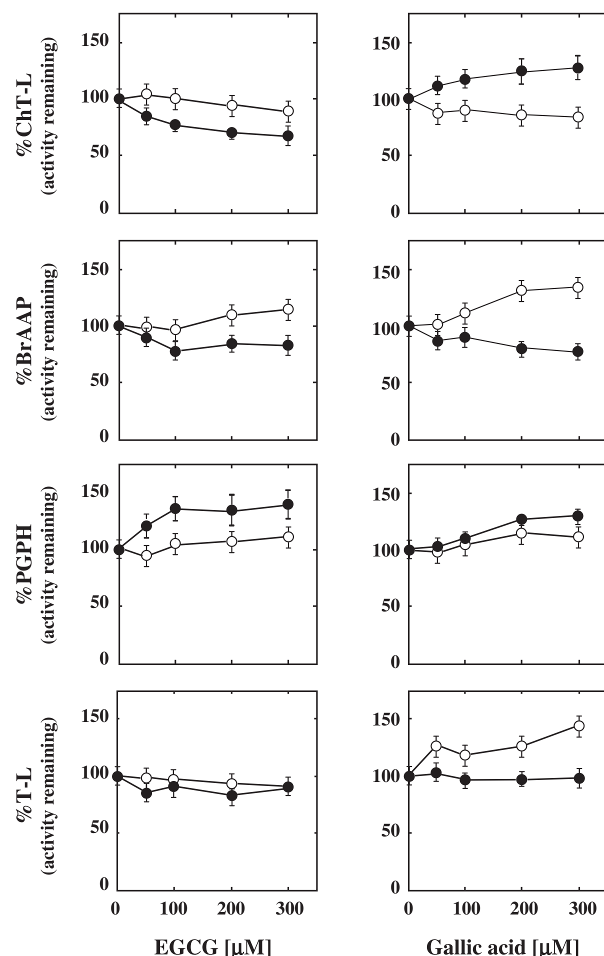

FIG. 2. Effect of EGCG and gallic acid in Caco cells lysates. Caco cells were treated for 12 hours (○) or 24 hours (●) with increasing amounts of EGCG and gallic acid. Cells were then harvested, and ChT-L, BrAAP, PGPH, and T-L activity assays were executed (see Materials and Methods for details). Data are representative of at least three independent experiments.

activation of the proteasomal components upon EGCG and gallic acid exposure, with the former inducing a more marked effect than the latter, is present.

DISCUSSION

It has been demonstrated that polyphenolic compounds show many biological activities such as antiviral, antibacterial, antifungal, anti-inflammatory, antimutagenic, and antiallergic activities; however, the molecular mechanism underlying these effects has not been characterized. It is now clear that polyphenols possess inhibitory activities on several enzymes, among them the 20S proteasome.

In fact, according to a number of recent studies, the naturally occurring ester bond containing green tea polyphenols, such as EGCG, GCG, and ECG, selectively inhibit the proteasome ChT-L activity *in vitro* and *in vivo,* whereas they do not affect the T-L component activity (21, 27–28, 32).

In the present work the effect of polyphenols on the constitutive and immuno-proteasomes isolated from bovine tissues was examined. The ChT-L, PGPH, BrAAP, and T-L activities were measured in both enzymes in order to evaluate a different susceptibility to polyphenolic compounds. Our data clearly show that, according to previous studies, EGCG is the most potent inhibitor of the ChT-L activity, measured with the substrate Z-GGL-pNA, with an IC_{50} of 0.22 m*M* for the XYZ-proteasome and 0.46 m*M* for the immunoproteasome. Those values are three orders lower than the values obtained by S. Nam *et al*. (21) who assayed the ChT-L component of a recombinant 20S proteasome from an archeabacterium, *Methanosarcina thermophila*, using the substrate Suc-LLVY-AMC. In order to address the different inhibitory effect of EGCG on these complexes we measured the bovine 20S proteasome ChT-L activity with the substrate Suc-LLVY-AMC obtaining a better inhibition (especially when the complexes were activated by SDS). The T-L activity also behaves differently in the mammalian 20S proteasomes with respect to the archea proteasome: in fact in the constitutive 20S proteasome it is inhibited by EGCG with an IC_{50} of 0.321 m*M* and in the immunoproteasome with an IC_{50} of 0.126 m*M*. Since those values were obtained, spectrophotometrically, with the substrate Z-GGR-2NA, the measurements were performed using Z-GGR-AMC

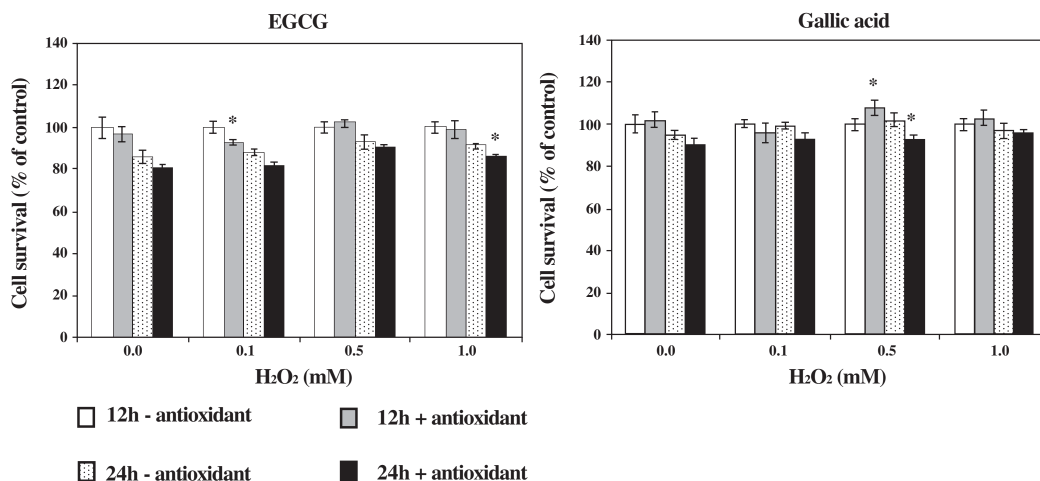

FIG. 3. Cell survival analyzed with the MTT assay. Caco cells, treated with 150 µ*M* EGCG and 200 µ*M* gallic acid for 12 and 24 hours, were exposed to 0, 0.1, 0.5 and 1.0 m*M* H_2O_2 for 30 minutes. Data are expressed as the percent of control values. Data are the mean and SEM of results from at least eight cultures from three separate experiments. *$p < 0.05$ compared to the respective control cultures without polyphenol.

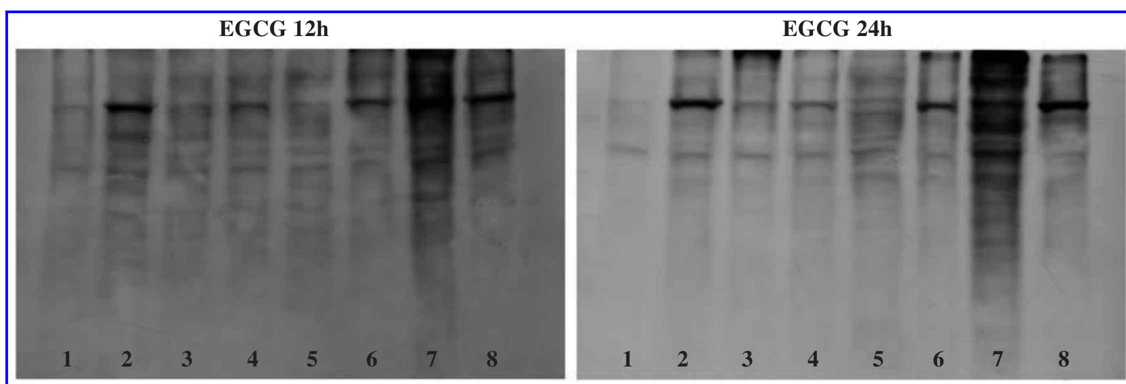

FIG. 4. Protein oxidation analyzed with the Oxyblot procedure (see Materials and Methods for details). Caco cells were treated with 150 µ*M* EGCG (*lanes 2, 4, 6, 8*) for 12 (*left panel*) and 24 hours (*right panel*) and then exposed to 0 (*lanes 1, 2*), 0.1 (*lanes 3, 4*), 0.5 (*lanes 5, 6*), and 1 m*M* H_2O_2 (*lanes 7, 8*) for 30 minutes.

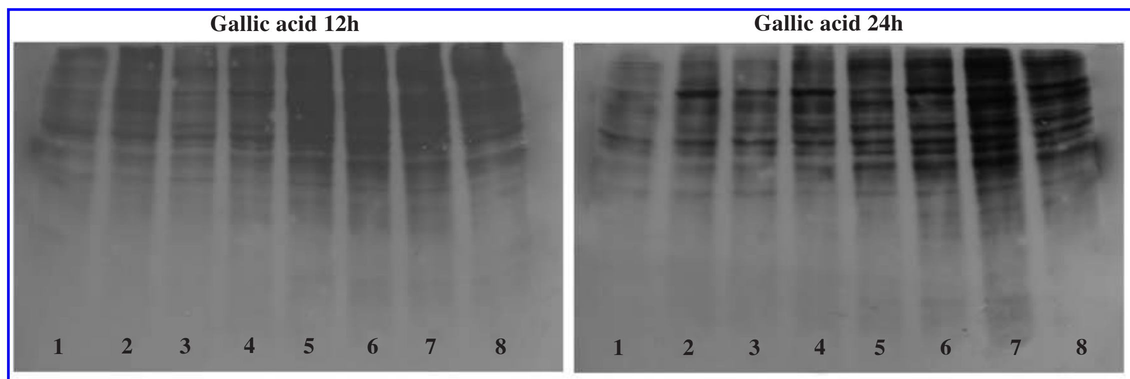

FIG. 5. Protein oxidation analyzed with the Oxyblot procedure (see Materials and Methods for details). Caco cells were treated with 200 µ*M* gallic acid (*lanes 2, 4, 6, 8*) for 12 (*left panel*) and 24 hours (*right panel*) and then exposed to 0 (*lanes 1, 2*), 0.1 (*lanes 3, 4*), 0.5 (*lanes 5, 6*) and 1 m*M* H_2O_2 (*lanes 7, 8*) for 30 minutes.

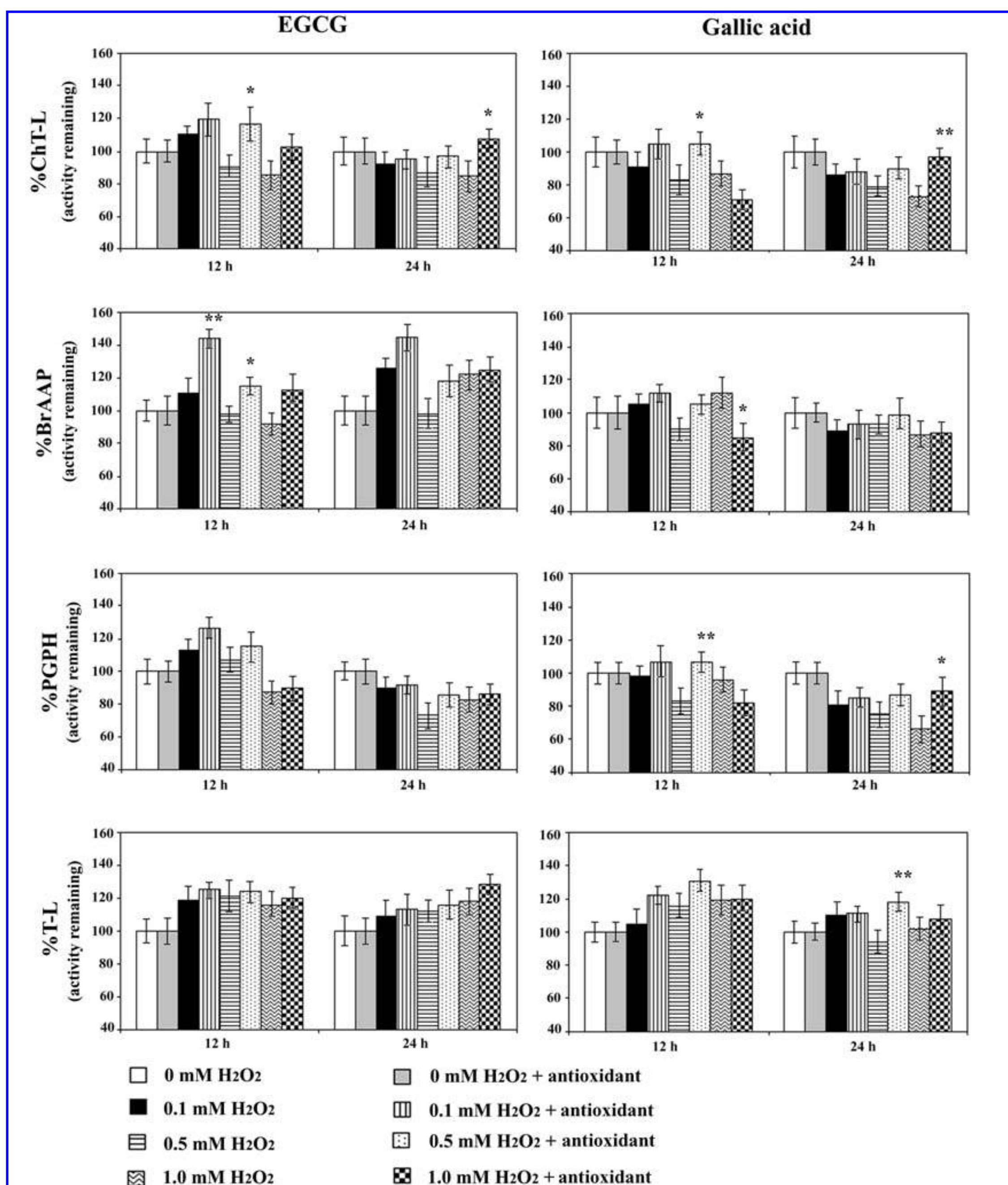

FIG. 6. Proteasomal activities of Caco cells lysates after exposure to H_2O_2 in the presence and absence of EGCG and gallic acid. Caco cells were treated for 12 hours or 24 hours with 150 μ*M* EGCG and 200 μ*M* gallic acid. Cells were then exposed to increasing amounts of H_2O_2 for 30 minutes. They were then harvested and lysated as described in Materials and Methods. In the extracts ChT-L, BrAAP, PGPH, and T-L activity assays were executed (see text for details). Data are representative of at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to the respective control cultures without polyphenol.

in a fluorimetric assay. Again the T-L component was inhibited by EGCG with an IC_{50} of 0.608 m*M* and 0.242 m*M* for the constitutive and the immuno-proteasome, respectively. The presence of SDS did not affect the XYZ- T-L activity, whereas in the LMP- T-L activity SDS seems to decrease the inhibitory effect of EGCG.

These differences in the extent of inhibition could be due to a different subunit composition between the archea and the mammalian proteasomes. Interestingly, the immunoproteasome shows a higher susceptibility to the inhibitory action of the polyphenolic compounds, with the BrAAP activity having the lowest IC_{50} with EGCG (0.24 m*M*). Data obtained in this study clearly indicate that proteasome functionality is strictly related to its subunit composition and that polyphenols influence the proteolytic activities depending on the different subunit assemblies in the two complexes.

To determine whether EGCG and gallic acid could also affect the living cell proteasomal activities, Caco cells were exposed to the action of antioxidants increasing concentrations (0, 50, 100, 200 and 300 μ*M*). The 20S proteasome activities were measured in the whole cell lysates and an evident inhibition of the ChT-L (70% residual activity with 200 μ*M* EGCG) after 24 hours EGCG exposure was noticed. The T-L component does not seem to be affected by EGCG, whereas in the *in vitro* assays, a decrease of the activity was observed. Gallic acid treatment of the Caco cells produced an overall activation effect with the exception of a slight inhibition of the ChT-L, after 12 hours exposure, and BrAAP activities, after 24 hours exposure. In order to verify if EGCG and gallic acid, at the most effective concentrations but also less detrimental for the cells, could prevent oxidation, the carbonyl content of the lysates was measured after 12 and 24 hours treatment with the antioxidant compounds followed by an additional incubation with various concentrations of H_2O_2. It appears that both molecules protect the cells against oxidative stress (especially evident at 1 m*M* hydrogen peroxide). The proteasomal activities were also tested in those samples: an activation in the presence of the polyphenols, significant at 0.1 and 0.5 m*M* H_2O_2 upon 12 hours treatment and at 1 m*M* H_2O_2 upon 24 hours, can be observed. We did expect the antioxidant effect of the polyphenols but it was rather surprising to see that the protection promoted the proteasomal functionality especially at 0.1 and 0.5 m*M* H_2O_2.

Taken together, the results obtained in this study indicate that EGCG is the most effective polyphenol, especially toward the ChT-L activity of the constitutive and the immuno-proteasomes and the BrAAP component in the LMP-complex. Therefore it is reasonable to suggest that EGCG is able to bind not only the N-terminal threonine in the X subunit (21, 27–28), but also in the LMP subunit, confirming that the polyphenol–proteasome interaction is directly related to the complexes subunit composition. The EGCG inhibitory action in the cell extracts is evident for the ChT-L activity, whereas the other components are slightly affected. EGCG has a clear antioxidant effect when the cells are exposed to oxidative stress, preventing oxidation and deterioration of the proteasome functionality. These findings could be important if the implication of the 20S proteasome functionality in oxidative stress associated pathologies, including normal aging, is taken into account. The antioxidant effect of EGCG and gallic acid, in parallel to their promoting action on the 20S proteasome functionality, could support the cellular defense both preventing the oxidative injuries and favouring the proteasomal degradation of damaged proteins.

Perspectives

Based on our current work, we demonstrate that EGCG and gallic acid are polyphenol compounds able to affect the 20S proteasomes functionality, depending on the complex subunit composition and that, in cell extracts, they behave both as antioxidants and proteasome effectors. Further studies will be needed to understand whether the proteasome–polyphenol interaction could influence the proteasome function in degrading cell cycle and cell death regulators, such as the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (25) and the proapoptotic protein Bax (17).

ACKNOWLEDGMENTS

This work was supported by a grant from Fondazione Cassa di Risparmio di Fabriano e Cupramontana Italy, and a grant from MURST Biophenolix.

ABBREVIATIONS

AMC, 7-methylcoumaryl amide; BrAAP, branched chain amino acid preferring; ChT-L, chymotrypsin-like; DMSO, dimethylsulfoxide; IFN-γ, interferon-γ; LMP, low molecular weight protein; 2NA, 2-naphthylamide; pAB, *p*-aminobenzoate; PABA, 4-aminobenzoic acid; PGPH, peptidyl-glutamyl peptide hydrolyzing; pNA, *p*-nitroaniline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; Suc, succinyl; T-L, trypsin-like; Z, benzyloxycarbonyl; conventional one letter abbreviations are used for amino acids.

REFERENCES

1. Almenoff J and Orlowski M. Membrane-bound kidney neutral metalloendopeptidase: interaction with synthetic substrates, natural peptides and inhibitors. *Biochemistry* 22: 590–599, 1983.
2. Amici M, Lupidi G, Angeletti M, Fioretti E, and Eleuteri AM. Peroxynitrite-induced oxidation and its effects on isolated proteasomal systems. *Free Radic Biol Med* 34: 987–996, 2003.
3. Baumeister W, Walz J, Zühl F, and Seemüller E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 92: 367–380, 1998.
4. Bors W, Heller W, Michel C, and Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* 186: 343–355, 1990.
5. Burnette WN. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112: 195–203, 1991.
6. Chen C, Yu R, Owuor ED, and Kong AN. Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch Pharm Res* 23: 605–612, 2000.
7. Davies KJA. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 83: 301–310, 2001.
8. DeMartino GN and Slaughter CA. The proteasome. A novel protease regulated by multiple mechanisms. *J Biol Chem* 274: 22123–22126, 1999.
9. Eleuteri AM, Angeletti M, Lupidi G, Tacconi R, Bini L, and Fioretti E. Isolation and characterization of bovine thymus multicatalytic proteinase complex. *Protein Expr Purif* 18: 160–168, 2000.
10. Grune T, Reinheckel T, and Davies KJA. Degradation of oxidized proteins in mammalian cells. *FASEB J* 11: 526–534, 1997.
11. Grune T, Klotz LO, Gieche J, Rudeck M, and Sies H. Protein oxidation and proteolysis by the nonradical oxidants

singlet oxygen or peroxynitrite. *Free Radic Biol Med* 30: 1243–1253, 2001.
12. Grune T. Oxidative stress, aging and the proteasomal system. *Biogerontology* 1: 31–40, 2000.
13. Guglielmone HA, Agnese AM, Montoya SCN, and Cabrera JL. Anticoagulant effect and action mechanism of sulphated flavonoids from *Flaveria bidentis. Thromb Res* 105: 183–188, 2002.
14. Holtsberg FW, Steiner MR, Bruce-Keller AJ, Keller JN, Mattson MP, Moyers JC, and Steiner SM. Lysophosphatidic acid and apoptosis of nerve growth factor-differentiated PC12 cells. *J Neurosci Res* 53: 685–696, 1998.
15. Lee SJ, Chung HY, Lee IK, Oh SU, and Yoo ID Structure-activity relationship of dietary flavonoids for inhibitory activity of mouse brain monoamine oxidase (MAO) *in vitro*. *Food Sci Biotechnol* 9: 304–307, 2000.
16. Levites Y, Weinreb O, Youdim MBH, and Mandel S. Green tea polyphenol (-)-epigallocatechin-3-gallate prevents N-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine-induced dopaminergic neurodegeneration. *J Neurochem* 78: 143–148, 2000.
17. Li B and Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. *Proc Natl Acad Sci USA* 97: 3850–3855, 2000.
18. Mandel S and Youdim MBH. Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. *Free Radic Biol Med* 37: 304–317, 2004.
19. Merker K and Grune T. Proteolysis of oxidised proteins and cellular senescence. *Exp Gerontology* 35: 779–786, 2000.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
21. Nam S, Smith DM, and Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*. *J Biol Chem* 276: 13322–13330, 2001.
22. Orlowski M and Michaud C. Pituitary multicatalytic proteinase complex. Specificity of components and aspects of proteolytic activity. *Biochemistry* 28: 9270–9278, 1989.
23. Orlowski M, Cardozo C, and Michaud C. 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: 1563– 572, 1993.
24. Orlowski M and Wilk S. Catalytic activities of the 20S proteasome, a multicatalytic proteinase complex. *Arch Biochem Biophys* 383: 1–16, 2000.
25. Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, and Rolfe M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269: 682–685, 1995.
26. Pfleiderer G. Particle-bound aminopeptidase from pig kidney. *Meth Enzymol* 19: 514–521, 1970.
27. Smith DM, Wang Z, Kazi A, Li L, Chan TH, and Dou QP. Synthetic analogs of green tea polyphenols as proteasome inhibitors. *Mol Med* 8: 382–392, 2002.
28. Smith DM, Daniel KG, Wang Z, Guida WC, Chan TH, and Dou QP. Docking studies and model development of tea polyphenol proteasome inhibitors: applications to rational drug design. *Proteins* 54: 58–70, 2004.
29. Suoza J M, Choi I, Chen Q, Weisse M, Daikhin E, Yudkoff M, Obin M, Ara J, Horwitz J, and Ischiropoulos H. Proteolytic degradation of tyrosine nitrated proteins. *Arch Biochem Biophys* 380: 360–366, 2000.
30. Towbin H, Staehelin T, and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc Natl Acad Sci USA* 76: 4350–4354, 1979.
31. Vender Berghe DAR, Haermers A, and Vlietinck AJ. Chapter 17. In: *Bioactive natural products; detection and structural determination*, edited by Colegate SM and Molyneux RJ. London: CRC Press, 1993, pp. 405–440.
32. Wan SB, Chen D, Dou QP, and Chan HT. Study of green tea polyphenols catechin-3-gallate (CG) and epicatechin-3-gallate (ECG) as proteasome inhibitors. *Bioorg Med Chem* 12: 3521–3527, 2004.
33. Wilk S and Orlowski M. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *J Neurochem* 40: 842–849, 1983.

Address reprint requests to:
Dr. Anna Maria Eleuteri
Dept. of Molecular, Cellular and Animal Biology
University of Camerino
Via Scalzino, 7
62032 Camerino (MC), Italy

E-mail: annamaria.eleuteri@unicam.it

Received for publication July 26, 2005; accepted August 2, 2005.

This article has been cited by:

1. A. M. Eleuteri, M. Amici, L. Bonfili, V. Cecarini, M. Cuccioloni, S. Grimaldi, L. Giuliani, M. Angeletti, E. Fioretti. 2009. 50Hz Extremely Low Frequency Electromagnetic Fields Enhance Protein Carbonyl Groups Content in Cancer Cells: Effects on Proteasomal Systems. *Journal of Biomedicine and Biotechnology* **2009**, 1-11. [CrossRef]
2. Laura Bonfili, Valentina Cecarini, Manila Amici, Massimiliano Cuccioloni, Mauro Angeletti, Jeffrey N. Keller, Anna M. Eleuteri. 2008. Natural polyphenols as proteasome modulators and their role as anti-cancer compounds. *FEBS Journal* . [CrossRef]
3. Hee Moon, Hyun Ho Choi, Ji Yun Lee, Hyo Jin Moon, Sang Soo Sim, Chang Jong Kim. 2008. Quercetin inhalation inhibits the asthmatic responses by exposure to aerosolized-ovalbumin in conscious guinea-pigs. *Archives of Pharmacal Research* **31**:6, 771-778. [CrossRef]
4. Chan Hun Jung, Ji Yun Lee, Chul Hyung Cho, Chang Jong Kim. 2008. Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. *Archives of Pharmacal Research* **30**:12, 1599-1607. [CrossRef]
5. Dr. Jeffrey N. Keller . 2006. The Many Nuances of Oxidative Stress and ProteolysisThe Many Nuances of Oxidative Stress and Proteolysis. *Antioxidants & Redox Signaling* **8**:1-2, 119-120. [Citation] [PDF] [PDF Plus]