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Rubus ulmifolius Leaf Extract Inhibits Proliferation of Murine Myeloma Cells

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ABSTRACT

In the present paper we report preliminary results on the antiproliferative activity of *Rubus ulmifolius* leaf extract against murine myeloma cells (P3X63-Ag8.653). Cytotoxic effects of plant extract (range 3-30 µL/mL of cell culture) were evaluated by microscopy analyses and viability assays, revealing a clear dose-response relationship for cytotoxicity in treated cells *vs.* controls. The number of viable cells was reduced to nearly 30% when plant extract was tested at 3 µL/mL, and to nearly 3% when plant extract was tested at 5 µL/mL. Higher concentrations of plant extract allowed the detection of a very limited number of viable cells. Furthermore, the effects of *R. ulmifolius* leaf extract at the molecular level were investigated through a comparative proteomic approach, which allowed us to highlight how it might modulate protein expression in murine myeloma cells. In particular, only one protein spot was found under-expressed, whereas 44 protein spots showed significantly higher levels in plant extract-treated cells when compared to control cells. Our results may thus lay the basis and open new perspectives for future investigations of the effects of plant extracts in mammalian cells.

Keywords: anticancer activity, cancer, proteome, viability
Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; **CHAPS**, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; **DMEM**, Dulbecco's modified Eagle's medium; **DTE**, dithioerythritol; **FBS**, foetal bovine serum; **IAA**, iodoacetamide; **IEF**, isoelectric focusing; **Prdx1**, Peroxiredoxin 1; **ROS**, reactive oxygen species; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

Epidemiological studies correlate a diet rich in berry fruits, such as blackberry (*Rubus* sp.), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbousm*), cranberry (*Vaccinium macrocarpon*), red raspberry (*Rubus idaeus*) and strawberry (*Fragaria ananassa*) with a reduced risk to develop certain cancer types (Steinmetz and Potter 1991; Meyskens and Szabo 2005). Though unequivocal proof linking berry consumption with lower cancer risk is still lacking (Duthie 2007), berries are known to contain compounds believed to reduce malignant transformation. Laboratory and animal studies have been produced to corroborate such an hypothesis (reviewed in Seeram 2006), showing that complementary and overlapping mechanisms exist to explain the chemoprotective activity, including protection from oxidative stress and inflammation (Seeram 2008; Kaume *et al.* 2011), modulation of gene expression, induction of metabolizing enzymes, effects on cell proliferation, apoptosis and cell signalling (Huang *et al.* 2002; Seeram *et al.* 2006). Recently, several studies have highlighted the antioxidant activity of polyphenolic components of berry extracts as well as an interesting antiproliferative activity against cancer cells (Meyer *et al.* 1998; Aherne and O'Brien 2002; Hannum 2004).

The phenolic composition and antioxidant properties of a number of medicinal plants have been evaluated, but much still remains to be done for many plants (or their parts) that are, to some extent, still unexplored. In this context, blackberry leaves represent a good example (Komes *et al.* 2011), since, if compared to blackberry fruits, leaves are far less characterized. Nevertheless, a growing body of evidence indicates that blackberry leaves possess interesting activities as well. *Rubus ulmifolius* is a perennial plant growing in Italy from sea level up to 1100 m (Panizzi *et al.* 2002). Its leaves, like those from other *Rubus spp*., are used in traditional medicine for their anti-inflammatory, antimicrobial and antiviral properties. For instance, in Italian folk medicine, fresh bruised leaves are used to treat abscesses, furuncles and ulcers, whereas decoctions may be useful for topical applications against redden eyes, or internally against intestinal inflammations (Uncini Manganelli and Tomei 1999). Crude extracts and purified fractions obtained from *R. ulmifolius* leaves have been reported to have antimicrobial properties on several microorganisms, including: *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans* and *Helicobacter pylori* (McCutcheon *et al.* 1994; Panizzi *et al.* 2002; Martini *et al.* 2009). Furthermore, extracts obtained from *R. ulmifolius* roots could inhibit *S. aureus* biofilm formation to a degree that can be correlated with increased antibiotic susceptibility and without toxic relapses for normal mammalian cells (Quave *et al.* 2012).

Data on phenolic composition of plants is difficult to compare due to differences in extraction and analysis methods (Rusak *et al.* 2008). Nevertheless, herbal infusions are generally prepared by steeping leaves in hot water; for this reason, Komes *at al.* (2008) presented recently a comprehensive and thorough survey of the composition of water extracts of some traditionally used plants (including blackberry leaves) providing a deeper knowledge of their bioactive composition. This will likely allow, in turn, to develop new standardized and effect-optimized preparations fulfilling the standards for quality, safety and efficiency (Wagner and Ulrich-Merzenich 2009).

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Original Research Paper

Our aim was to investigate the presence in *R. ulmifolius* leaves of molecules inhibiting cell proliferation. For this reason, we tested the cytotoxic activity of *R. ulmifolius* leaf extract against a murine myeloma cell line (P3X63-Ag8.653) showing a clear dose-response relationship for cytotoxicity. Additionally, a comparative proteomic approach was undertaken in order to evaluate the effects at the proteome level. To the best of our knowledge, this is the first study providing a comparative proteomic analysis of the effects of *R. ulmifolius* leaf extract on murine myeloma cells. Such an analysis allowed us to highlight that levels of specific proteins may be modulated.

MATERIALS AND METHODS

Plant material and chemicals

Blackberry leaves (*R. ulmifolius* Schott, 10 years old plant) were harvested during June in a farm located in Campagnano di Roma (Italy) where they grew spontaneously. Unless otherwise indicated, solvents and reagents were from Sigma (St. Louis, MO, USA).

Plant extract

Ten grams of fresh leaves were weighted and homogenized in 20 ml absolute ethanol at room temperature. After 50 min, the ethanol extract was centrifuged (2000 rpm for 5 min) and the supernatant concentrated three times in an evaporator. The resulting residue was diluted with deionized water to obtain a watery solution. The sample was then centrifuged (10,000 rpm for 3 min), filtered through a 0.22 µm cellulose syringe filter (Minisart 25 mm, Sartorius Stedim, Florence, Italy) and stored at -20°C until testing.

Cell cultures

Biological activity of *R. ulmifolius* leaf extract was tested on a murine myeloma cell line (P3X63-Ag8.653, abbreviated as P3X in the text) purchased from American Type Culture Collection (ATCC CRL-1580). This is a non-secretor; 8-azaguanine resistant cell line derived from a Balb/c mice strain. For the experiments, P3X cells were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker - Lonza, Milan, Italy) supplemented with 10% foetal bovine serum (FBS, BioWhittaker), penicillin, streptomycin and 2 mM glutamine (BioWhittaker) in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air.

Cytotoxicity assay

The cytotoxic effects of hydrolyzed leaf extract were determined by incubating cells (10^5/mL) with different concentrations of extract (range 3-30 µL/mL of culture) for 24 h in standard 24-well plates (Cellstar, Greiner bio-one, International PBI SA, Milan, Italy).

For each treatment, 10^5 cells were sown in 1 ml of DMEM and the appropriate volumes of the extract sample were added directly to the cell suspensions After 20 h, the number of cells was assessed by cell counts performed manually by using a Thoma-Zeiss chamber (10 fold magnification). The occurrence of dead cells was recorded by a vitality test that utilized a 0.4% solution of Trypan blue (Cook and Mitchell 1989). The assay was replicated six times in order to ensure reliability of our results.

Inverted microscope digital imaging

Cells morphology was also investigated using a Nikon microscope (Elipse TS100) and objectives of 40 fold magnification. Photo images from videotapes were obtained using the software Studio9 (Pinnacle v9.0.4.170, Avid Technology Inc., USA).

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cells were washed twice with sterile PBS and resuspended in 50 µL of a buffer containing 65 mM DTE, 65 mM CHAPS, 9 M urea, and 35 mM Tris-base. Cell disruption was achieved by sonicating briefly in an ice bath. Protein content in cell lysates was assessed according to Bradford (1976).

Cell lysates were first mixed with a buffer containing 8 M urea, 35 mM CHAPS, 10 mM DTE, and a trace of bromophenol blue. Fifty µg of proteins were adsorbed onto Immobiline Dry Strips (IPG 18 cm, non linear 3-10 pH range, Bio-Rad, Hercules, CA) for 10 h. Isoelectric focusing (IEF) was carried out with a Protean IEF cell (Bio-Rad). The voltage was linearly increased from 300 to 3500 V during the first three hours and then stabilised at 5000 V for 22 h (total 110 kV × hour). Prior to SDS-PAGE, IPG strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris-HCl pH 6.8 containing first 2% (w/v) DTE and later 2.5% (w/v) IAA. SDS-PAGE was carried out applying 40 V per gel until the dye front reached the bottom of gels.

Silver ammoniacal staining was carried out according to Switzer *et al.* (1979). Protein spots of interest were identified by gel matching with proteomic reference maps (Smales *et al.* 2004; Dinnis *et al.* 2006).

Image analysis

Images of gels were acquired (Image Scanner, Amersham Biosciences) and analysed with Image Master™ Platinum (Amersham Biosciences). For comparative proteomic analysis, spot % relative volume was adopted; significant thresholds for fold change values (ratio between extract treated/vehicle control cells) were set as ≥ 2.0 and ≤ 0.5 to highlight over-expressed and under-expressed proteins, respectively.

Statistical analysis

All of the experiments were carried out in triplicate; data are presented as mean values with standard deviation. Differences with at least a P-value ≤ 0.05 were considered significant. For comparative proteomic analyses, only representative gels are shown.

RESULTS AND DISCUSSION

In this work, we investigated the cytotoxic effects of *R. ulmifolius* leaf extract on a P3X murine myeloma cell line. After such a preliminary screening, we also investigated the effects of the tested extract at molecular level through a comparative proteomic approach, allowing us to highlight how *R. ulmifolius* leaf extract might modulate protein expression in P3X cells.

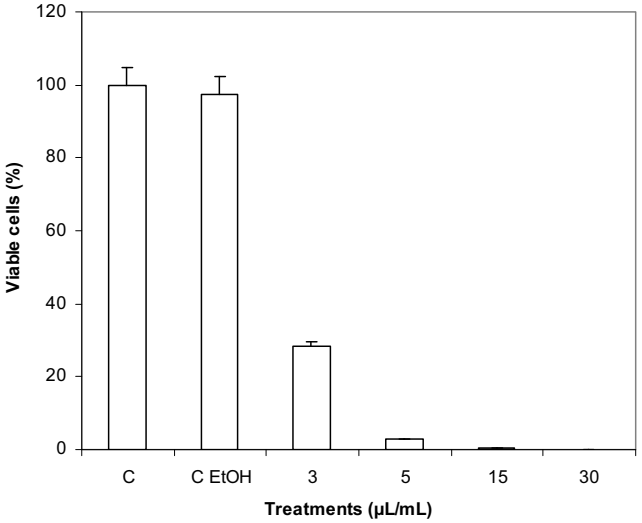

Fig. 1 Proliferation of murine myeloma P3X cells. Treatments were as follows: C, control (untreated) cells; C EtOH, vehicle treated cells; 3, cells treated with 3 µL/mL; 5, cells treated with 5 µL/mL; 15, cells treated with 15 µL/mL; 30, cells treated with 30 µL/mL of *R. ulmifolius* leaf extract. Mean values ± standard deviation are reported.

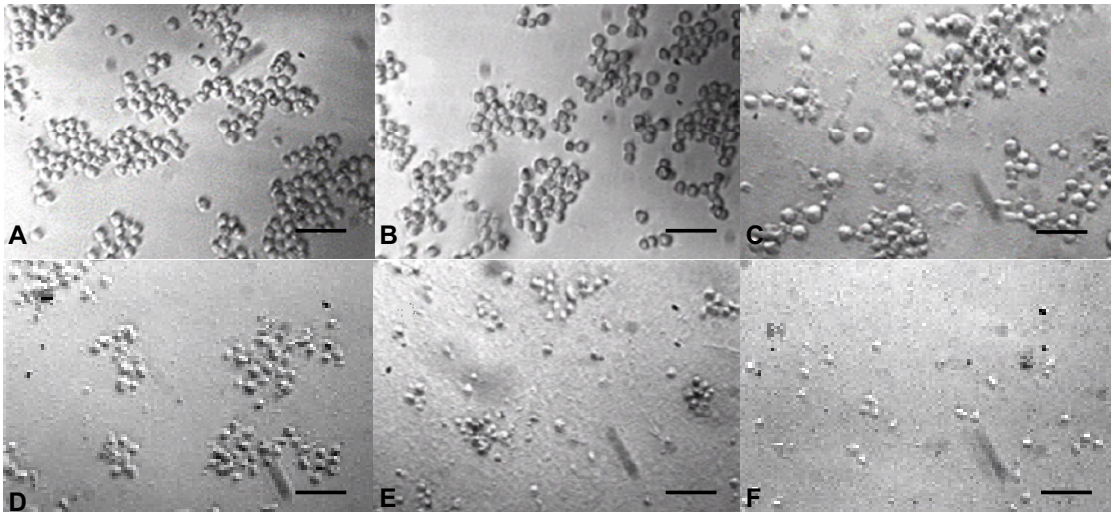

Fig. 2 Microscopy images of murine myeloma P3X cells proliferation. (**A**) Control (untreated) cells; (**B**) vehicle treated cells; (**C**) cells treated with 3 µL/mL; (**D**) cells treated with 5 µL/mL; (**E**) cells treated with 10 µL/mL; (**F**) cells treated with 30 µL/mL of *R. ulmifolius* leaf extract. Cell proliferation was increasingly reduced at higher concentrations of extract. Scale bar = 30 µm.

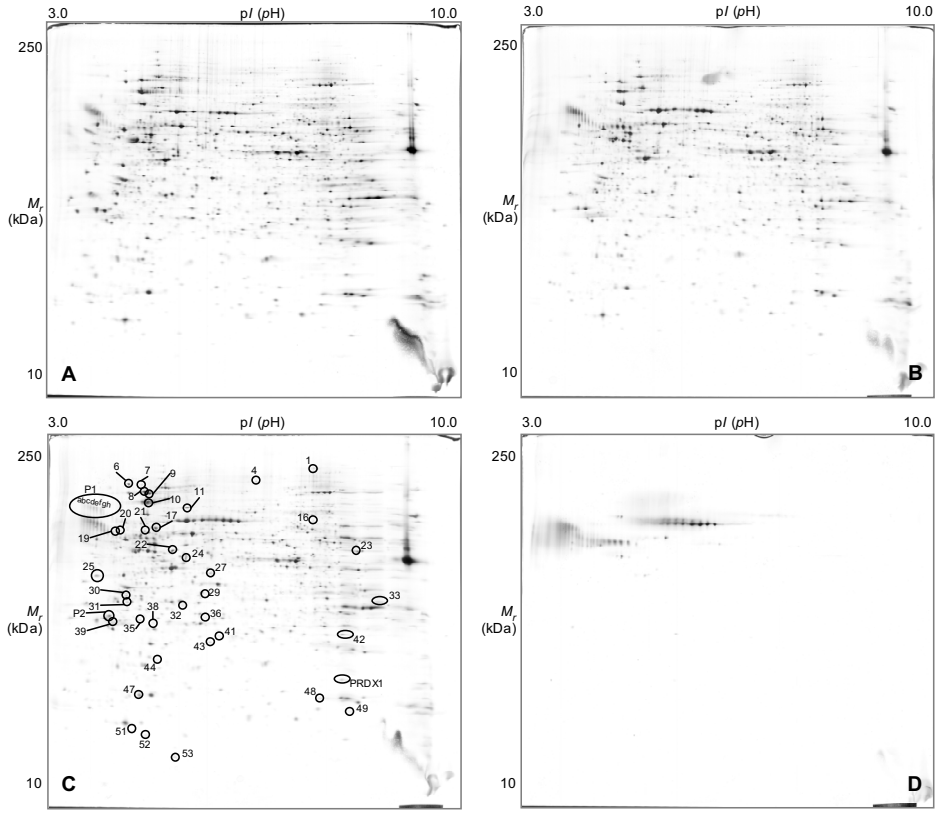

Fig. 3 Comparative proteomics of murine myeloma P3X cells. (**A**) Control (untreated) cells; (**B**) vehicle treated cells; (**C**) cells treated with 5 µL/mL and (**D**) with 30 µL/mL of *R. ulmifolius* leaf extract.

Cytotoxicity of purified *R. ulmifolius* leaf extract

The cytotoxicity of *R. ulmifolius* was shown *in vitro* against P3X murine myeloma cells using increasing concentrations of leaf extract, ranging from 3 to 30 µL/mL of culture. Higher concentrations were shown to increasingly inhibit cell proliferation (**Figs. 1, 2**).

Comparative proteomics

Silver staining allowed to reveal nearly 2500 spots in P3X untreated cells (control) (**Fig. 3A**), P3X cells treated with vehicle alone (vehicle control) (**Fig. 3B**) and P3X cells treated with *R. ulmifolius* leaf extract (5 µL extract/mL culture) (**Fig. 3C**). Conversely, the proteomic map of P3X cells treated with a higher extract concentration (30 µL/mL culture) revealed only a very limited amount of protein spots (220) (**Fig. 3D**), likely indicating a massive proteolysis under such extreme culture conditions. This phenomenon may in turn suggest the induction of apoptosis, though further analyses are needed to confirm such a hypothesis.

Gel matching with reference maps (Smales *et al.* 2004; Dinnis *et al.* 2006) allowed the identification of 29 gene products. Proteomic maps were also calibrated for p*I* and M_r values. Once set statistically significant thresholds ≥ 2.0 and ≤ 0.5 for fold-change values in protein relative abundance ratio (to highlight over-expressed and under-expressed proteins, respectively), the quantitative analysis indicated that control (untreated P3X cells) and vehicle control (ethanol-treated P3X cells) proteomic maps were almost

Table 1 Proteins differently expressed in P3X murine myeloma cells treated with *R. ulmifolius* leaf extract (5 µL/mL of culture).

Spot	p*I*	M_r	Fold-change (treated/vehicle)*
PRDX1 (Peroxiredoxin 1)	7.19	21820	2.28
#1	6.29	153893	2.01
#4	5.95	132077	7.92
#6	4.80	112603	2.79
#7	4.98	111857	2.02
#8	5.10	94714	2.57
#9	5.03	96640	4.18
#10	5.07	84647	2.11
#11	5.29	78773	3.64
#17	5.24	67175	6.59
#19	4.63	65255	3.12
#20	4.68	65773	3.82
#21	5.03	65773	0.48
#22	5.44	56296	3.50
#23	7.49	56148	2.01
#24	5.29	49498	3.10
#25	4.35	43056	2.29
#27	5.47	43694	2.32
#29	5.44	37805	3.18
#30	4.77	37253	2.57
#31	4.78	35644	2.34
#32	5.33	34694	2.00
#33	8.05	35731	2.06
#35	4.87	32074	2.00
#36	5.43	31917	3.42
#38	5.17	30614	2.24
#39	4.58	30840	2.28
#41	5.53	29005	4.00
#42	6.99	27481	2.18
#43	5.47	26749	2.79
#44	5.29	23315	2.83
#47	4.95	18287	2.12
#48	6.40	17844	2.83
#49	7.24	16255	2.20
#51	4.87	14203	3.61
#52	5.04	13690	2.22
#53	5.41	11700	4.30
P1a	3.92	80926	present
P1b	4.04	80202	present
P1c	4.13	79484	present
P1d	4.24	78419	present
P1e	4.35	77718	present
P1f	4.41	76333	present
P1g	4.45	72650	present
P1h	4.50	71777	present
P2	4.53	32311	present

***** Fold-change values indicate the ratio in protein spot % relative volumes calculated as treated cells [map (C) in **Fig. 3**]/vehicle control [map (B) in **Fig. 3**]. Values higher than 2.0 indicate over-expressed proteins, values lower than 0.5 indicate under-expressed proteins in treated cells.

superimposable.

Conversely, 45 protein spots showed significantly altered levels in P3X cells treated with *R. ulmifolius* leaf extract (5 µL/mL culture) when compared to vehicle-treated control (**Table 1**). Among these, we were able to discriminate: 1) Only one protein spot under-expressed in treated cells (spot #21); 2) 36 protein spots over-expressed in treated cells; 3) Nine protein spots present exclusively in treated cells and absent in vehicle control map (spots #P1a-h and P2).

The identification of differently expressed proteins is still in its very infancy. Nevertheless, we were able to identify, among the over-expressed proteins, Peroxiredoxin 1 (Prdx1). This is a protein critically involved in redox regulation of the cell, since Prdx1, which is expressed in the cytosol of many types of cells and tissues, is thought to play important roles in the defence from oxidative stress by eliminating peroxides and maintaining a proper redox balance (Ishii *et al.* 1993; Rhee *et al.* 2005). A growing body of evidence indicates that an imbalance in redox homeostasis with elevated reactive oxygen species (ROS)/oxidative stress plays pivotal roles in a number of cellular metabolic and signalling processes. Though acting as regulatory mediators in signalling processes at low to moderate concentrations, at high concentrations ROS can be extremely hazardous for living organisms, as they can oxidatively damage cellular components (lipids, DNA and, especially, proteins). A well recognized role is also shown in cancer, since elevated ROS promote can tumorigenesis (Cao *et al.* 2009); however, the exact mechanisms of such a phenomenon are still unclear.

Prdx1 might also participate in the signalling cascades of growth factors and tumor necrosis factor-alpha by regulating the intracellular concentrations of H_2O_2. The ablation of Prdx1 is related to an increased susceptibility to Ras-induced breast cancer and recently it has been designated as a safeguard for the lipid phosphatase activity of PTEN, which is essential for its tumour suppressive function (Cao *et al.* 2009). Additionally, Prdx1-deficient fibroblasts in culture show decreased proliferation and increased sensitivity to oxidative DNA damage (Neumann *et al.* 2003). Prdx1 has been also shown to play tumor-suppressor activities, since it has been demonstrated that $Prdx1^{-/-}$ mice develop an increased frequency of multiple malignant cancers as they age, presumably as a consequence of an aberrant accumulation of oxidative damage (Neumann *et al.* 2003). Additionally, an important role of Prdx1 as a downregulator of inflammation has recently been shown (Kisucka *et al.* 2008).

CONCLUSION

Many supplements and foods enriched in polyphenols appear to have health benefits, although the molecular basis of these actions in target tissues has only begun to be addressed. Yet it is likely that the actions of dietary supplements could be correlated with changes in the target tissues in specific proteins, either in their expression or in their post-translational modifications (Deshane *et al.* 2004). The concept that food and phytochemicals can prevent both deficiency and chronic diseases dates back in the human history. Current nutrition research may rely on sophisticated technologies to reveal the mechanisms underlying these assumptions and provide their basis at molecular level. For instance, the use of proteomics (2D-PAGE for the separation of proteins and MS techniques for their identification) provides researchers with invaluable tools to investigate how specific cell types/tissues/organs may be affected by such compounds and for the identification of proteins mediating biological effects (Kim 2005). Such an approach is currently quite unexploited in the analysis of the effects of plant extracts, being one of the most remarkable exception the proteomic analysis of rat brain modification by grape seed extracts recently proposed by Deshane *et al.* (2004).

Similarly, the proteomic analysis of myeloma cells is still in its infancy, as only incomplete and fragmentary information is provided mainly deriving from cell line models or depicting individual signal pathways involved in disease development, the influence of microenvironmental conditions or the response to pharmacological treatments (Cumova *et al.* 2011). Advances in the field seem to be a great challenge, which however deserves further investigation. In the case of *R. ulmifolius* leaf extracts, for instance, the identification of differently expressed proteins would be fundamental to assess the molecular basis of the cytotoxicity against P3X murine myeloma cells. In the light of the results shown here about the modulation of Prdx1 expression, a redox proteomic analysis providing the identification of oxidized proteins could be undertaken as well.

Both basis cancer research and targeted chemotherapeutic discovery can ultimately involve the search for specific proteins. In this light, proteomics represent the elective tool to identify changes in protein expression or their modifications in response to specific stimuli, the high-throughput nature of the technology and the lack of a priori predictions constituting remarkable advantages (Kim 2005).

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