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PRINCIPAL INVESTIGATOR: Joseph M. Wu, Ph.D.

CONTRACTING ORGANIZATION: New York Medical College
Valhalla, NY 10595

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| 14. ABSTRACT Purpose: To test the hypothesis that resveratrol, a grape derived polyphenol, exerts its chemopreventive properties against prostate cancer by interacting with specific cellular targets, denoted resveratrol targeting proteins (RTPs). Scope: To explore the existence of RTPs in androgen-dependent LNCaP and androgen-independent (PC-3) cells, by chemically immobilizing resveratrol on epoxy-activated agarose to generate a biospecific affinity matrix for isolating and purifying RTPs from cell extracts. Major findings: By combining resveratrol affinity chromatography with MALDI-TOF mass spectrometry, we have identified dihydronicotinamide riboside:quinone reductase (NQO2) as a distinct RTP from LNCaP and PC-3 cells. Up-to-date progress: As mentioned in the annual report, we are in the process of possibly identifying another RTP. This goal has been achieved in PC-3 cell extracts by combining resveratrol affinity column chromatography with mass spectrophometry. The second RTP is glutathione sulfotransferase GST-Pi. Significance: we have identified two resveratrol RTPs, respectively, NQO2 and GST-Pi, both with function in detoxification reactions. Our findings suggest that resveratrol might function as a chemopreventive agent by interacting and modulating activity and/or stability of detoxification enzymes. | | | | | | |
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Table of Contents

| | |
|--|-----------|
| Introduction..... | 4 |
| Body..... | 5 |
| Key Research Accomplishments..... | 11 |
| Reportable Outcomes..... | 12 |
| Conclusions..... | 13 |
| References..... | 14 |
| Appendices..... | 16 |

INTRODUCTION

This project focuses on the identification of targeting proteins of chemopreventive agent resveratrol, denoted RTPs [1, 2], in androgen-dependent LNCaP and androgen-independent PC-3 prostate cancer cells, whose growth was previously reported by us to be strongly inhibited by resveratrol [3, 4]. Our working hypothesis is that resveratrol “docks” with RTPs at distinct “motifs” created through their mutual interactions. We tested this hypothesis by immobilizing resveratrol on epoxy-activated agarose to form a biospecific affinity platform for capturing and purifying RTPs from cell extracts. A salient feature of this approach, which we name ligand-select proteomics (LSP), is that it provides a panoramic display of proteins having varying binding affinities and therefore possibly differential relevance to the biological attributes of resveratrol [2]. A combination of LSP with MALDI-TOF mass spectrometry has demonstrated dihydronicotinamide riboside:quinone reductase (NQO2) as a distinct RTP from LNCaP and PC-3 cells [1, 2]. The same strategy has also recently identified a second RTP in PC-3 cell extract, as glutathione sulfotransferase (GST)-Pi. Both NQO2 and GST-Pi are detoxification enzymes; therefore our findings, to be detailed below, suggest that resveratrol may exert its chemopreventive property by binding and interacting with detoxification enzymes as to modulate their activity and/or stability. These new findings deserve further investigations.

BODY

Task 1 - To demonstrate proteins binding resveratrol in prostate cancer cells.

Overall research activities and accomplishments. As indicated in SOW, the primary focus of Task 1 was to test the hypothesis that proteins capable of interacting and binding resveratrol, denoted RTPs, exist in both androgen-dependent and –independent prostate cancer cells. Our experimental strategy for detecting RTPs, as proposed in Task 1, sought to determine whether [³H]resveratrol forms a stable complex with RTPs, as monitored by (i) retention of complex and not the free ligand on nitrocellulose filters, and (ii) shift in molecular weight of complex on gel filtration columns. Pilot experiments to test the feasibility of these approaches used extracts prepared from LNCaP and PC-3 cells. Low though reproducible uptake of [³H]resveratrol into LNCaP and PC-3 cell extracts were observed. In neither cell types, however, was uptake sufficiently robust to allow the demonstration of complexes based on retention on nitrocellulose filters. This negative research outcome may be attributable to scarcity of RTPs and/or low stability of [resveratrol.RTP] complexes.

Task 1a aimed to develop a nitrocellulose filter-binding assay for detecting RTPs in androgen-dependent LNCaP cells.

Rationale. Previous studies of interferon-inducible enzymes - 2',5'-oligoadenylate dependent ribonuclease L (RNase L) and the double-stranded RNA-activated 2',5'-oligoadenylate synthetase (2-5AOS) - by the PI utilized assays based on binding of radioactive ligands to low abundance RNase L and 2-5AOS; formation of ligand:enzyme complex can be detected by retention on nitrocellulose membranes [5-10]. We reasoned that the existence of RTPs might be similarly ascertained and established by testing whether a complex between [³H]resveratrol and RTPs forms with sufficient stability as to show quantitative retention on nitrocellulose filters. Repeated attempts using LNCaP cell extracts yielded negative results. Uptake studies to quantify the cellular uptake of [³H] resveratrol provided an explanation for the difficulties we encountered. As illustrated in Table 1, little [³H] resveratrol – typically only 300-750 cpm in 0.1 ml cell extracts containing 200-300 µg total protein - was taken up by LNCaP cells, suggesting that this experimental objective was unlikely to be successfully completed using this approach. Despite the noted technical challenges, it is worth mentioning that a time dependent increase in [³H] resveratrol assumed to be in complex with proteins in LNCaP cell lysates and in amounts significantly above background radioactivity, was repeatedly observed, thus providing support for the notion that RTPs are present in CaP cells.

Table 1. Amount of [³H] resveratrol bound to LNCaP cell extracts

| <i>LNCaP</i> | <i>Labeling condition</i> | <i>Label used</i> | <i>Cytosol (cpm/100 µl)</i> | <i>pellet</i> |
|---------------|---------------------------|-------------------|-----------------------------|---------------|
| Control-2 day | 1 h (pulse) | 60 µl (1.8 µCi) | 350 | 150 |
| Control-3 day | 3 h (pulse) | 60 µl | 750 | 0 |
| Control-4 day | 3 h (pulse) | 45 µl | 300 | 0 |
| Control-4 day | 3 days (continuous) | 60 µl | 450 | 0 |

[³H] resveratrol: 30µCi/ml H₂O; 7.5 µCi/250 µl (specific activity, 15 Ci/mmol)

Conclusions: The above results are consistent with the interpretation that to demonstrate the presence of RTPs using binding of [³H] resveratrol to cell lysates is unlikely to be a meaningful and productive experimental approach.

Task 1b aimed to confirm binding of resveratrol to RTPs by gel-filtration

Because of inadequate uptake of [³H] resveratrol into cell extracts as detailed above, we reasoned that to use gel filtration column to further ascertain [³H] resveratrol.RTP complex formation, as we had originally proposed, would be futile. Accordingly, task 1b was not performed as planned. Instead, we focused on the development of a ligand (resveratrol)-linked affinity column strategy, as was proposed in Task 2a. Development of such an affinity matrix will afford a one-step enrichment of low abundance RTPs. Details of our progress in this objective are described as part of Task 2 below.

Task 1c focused on the demonstration of the existence of resveratrol target proteins in androgen-independent PC-3 cells, based on the use of binding assays proposed in task 1a

We also performed uptake studies of [³H] resveratrol using PC-3 cell extracts. Compared to LNCaP cells, more [³H] resveratrol was taken up and bound to proteins in the cytosol, as illustrated by results in Table 2.

Table 2. Amount of [³H] resveratrol bound to PC-3 cell extracts

| <i>PC-3</i> | <i>Labeling condition</i> | <i>Label used</i> | <i>Total (cpm/100 μl)</i> | <i>Net uptake (cpm/100 μl)</i> |
|---------------|---------------------------|-------------------|---------------------------|--------------------------------|
| Control-2 day | label for ~ 0 min | 60 μl | 1300±38 | |
| Control-2 day | label for 15 min | 60 μl | 1355±23 | 55 |
| Control-2 day | label for 30 min | 60 μl | 1910±45 | 610 |

Table 2 shows considerable background of [³H]-resveratrol bound to cell extracts, evident by 1300 cpm/100 μl [³H]-resveratrol measured when labeling was immediately followed by cell harvesting and processing. By subtracting this amount of radioactivity from [³H]-resveratrol taken up into cell extracts after 15 or 30 min labeling, it can be seen that uptake of [³H]-resveratrol was initially slow and then increased progressively. The net increase of [³H]-resveratrol in PC-3 cells was significantly more than LNCaP cells, suggesting that RTPs might be present more abundantly in PC-3 cells.

To test whether RTPs in PC-3 cells may be subject to modulation by resveratrol, PC-3 cells were first treated with varying doses of unlabeled resveratrol for 2 days, after which cells were washed to remove unlabeled resveratrol. Resveratrol-treated cells processed in this manner were then labeled with [³H]-resveratrol and uptake of [³H]-resveratrol into resveratrol-treated PC-3 cells was compared to untreated cells using the same approach shown in Table 2. The results are illustrated in Table 3.

Table 3. Uptake of [³H] resveratrol using resveratrol-treated PC-3 cell extracts

| <i>PC-3</i> | <i>Labeling Condition</i> | <i>Amount label</i> | <i>Total (cpm/100 μl)</i> | <i>Net uptake (cpm/100 μl)</i> |
|-----------------------|---------------------------|---------------------|---------------------------|--------------------------------|
| 2μM resveratrol-2 day | label for ~ 0 min | 60 μl | 1760±60 | |
| 2μM resveratrol-2 day | label for 15 min | 60 μl | 1830±58 | 70 |
| 2μM resveratrol-2 day | label for 30 min | 60 μl | 3410±80 | 1650 |

Although uptake of [³H]-resveratrol showed a lag of 15 min, similar to what was observed for untreated PC-3 cells (Table 2), treatment of PC-3 cells for 2 days with 2 μM resveratrol stimulated the uptake of [³H]-resveratrol by 276% (1650 cpm for resveratrol-treated cells as opposed to 610 cpm for control cells, both labeled for 30 min), suggesting that treatment of PC-3 cells by resveratrol increases the level of expression of RTPs.

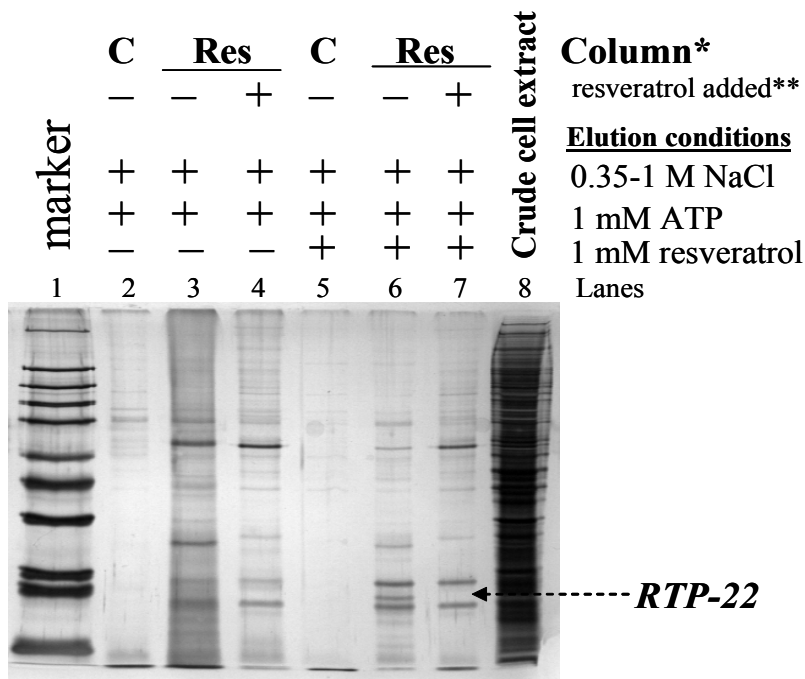
Conclusions: Uptake of [³H]-resveratrol was more robust in PC-3 cells, compared to LNCaP cells. Treatment of PC-3 cells by resveratrol increases the uptake of [³H]-resveratrol, suggesting the induction of RTPs by resveratrol.

Task 2. To identify and characterize RTPs in prostate cancer cells.

Overall research activities and accomplishments. Task 2 of SOW was designed to covalently link resveratrol to an immobile matrix. This was accomplished by immobilizing resveratrol on epoxy-activated agarose, resulting in a ligand (resveratrol)-linked affinity matrix with capability to interact with and specifically retain RTPs from cultured extracts. A purification scheme, involving sequential elution with high salt (0.35 M followed by 1.0 M NaCl), 1 mM ATP, and finally 1-2 mM resveratrol, for RTPs having different binding affinities for resveratrol was developed empirically [2]. We have named this approach ligand-select proteomics (LSP). By combining LSP with MALDI-TOF mass spectrometry, we have identified dihydronicotinamide riboside:quinone reductase (NQO2, also referred to as RTP-22 in Fig 1) as a distinct RTP from LNCaP and PC-3 cells [2]. The usefulness of this method was further validated by purifying NQO2 from cultured human melanoma cells [11]. The same strategy has also allowed the recent identification of a second RTP in PC-3 cell extract, as glutathione sulfotransferase (GST)-Pi.

Tasks 2a and 2b. To develop resveratrol-affinity columns for the analysis of RTPs. To demonstrate the existence and presence of resveratrol target proteins, RTPs, which we hypothesize will allow us to gain additional information on its chemopreventive properties, we prepared an affinity column using resveratrol as the ligand, as follows: One gram of epoxy-activated agarose was suspended in ice-cold water for 5 minute and washed extensively to remove preservatives. Resveratrol (23 mg) dissolved in 2.5 ml of 0.1 M NaOH was added to 1 ml of resuspended epoxy-activated agarose, followed by an overnight incubation at room temperature to allow chemical coupling of resveratrol to the resin. To stop the reaction, 6 ml of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT) was added to the mixture to neutralize unreacted epoxy groups and prevent further oxidation of resveratrol. Immobilized resveratrol resin was washed successively with 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT and 70%, 30%, 10% and 0% ethanol, respectively. Controls consisted of mock-treated beads (identical procedure except that no resveratrol was added) or beads immobilized with tyrosine as the ligand. Details and application of this approach for the isolation of RTPs from PC-3 cell extracts are illustrated in Fig 1. A sequential elution protocol was developed by first using increase concentration of NaCl from 0.35 M up to 1.0 M to displace proteins with lower affinity for resveratrol. This elution step was followed by a more stringent condition of elution with ATP. The final elution step used high concentration of resveratrol (1 mM) and was designed to select for proteins with significantly higher affinity for resveratrol. In this purification scheme, proteins eluted with 1.0 M NaCl were regarded as being less tightly binding, compared to proteins that eluted with 1 mM resveratrol [2].

It should be noted that a significant number of proteins became bound to the affinity column, most of which, however, were displaced from the column using increasing concentrations of NaCl concentrations, as were clearly evident in the silver stained patterns of salt eluted fractions (Fig 1). Apparently, some additional proteins were eluted using ATP. By contrast, a more limited, though distinct proteins were eluted with resveratrol. As an illustration of the use of this strategy, we have found using this fractionation scheme that estrogen and aryl hydrocarbon receptors were both bound to the resveratrol affinity column, and were quantitatively displaced using 1.0 M NaCl (data not shown). By focusing on proteins having the highest and selective affinity for resveratrol (low Kd), i.e., ones which can be displaced from the affinity column only with the use of high concentration of resveratrol (1 mM), we demonstrated that dihydronicotinamide riboside:quinone reductase (NQO2, identified as RTP-22 in Fig 1) is a distinct protein eluted from the resveratrol affinity column using resveratrol [2]. Utility of resveratrol affinity column chromatography for capturing and purifying NQO2 was further demonstrated using cultured human melanoma cells [11].



*C: control resin; Res: resveratrol affinity resin

** : 1 mM resveratrol added during binding to the column

Fig 1. Demonstration of binding of specific proteins from PC-3 cell extracts to immobilized resveratrol affinity columns and isolation and identification of RTPs. Cell lysates were fractionated on mock control column and resveratrol affinity column. Proteins were eluted from the column using sodium chloride (0.35M or 1M NaCl) followed by 1 mM ATP and 1 mM resveratrol. Lanes 2-6 show silver stained elution profiles between control (lanes 2 and 5) and resveratrol affinity column (lanes 3,4,6,7) with (lanes 4 and 7) or without (lanes 3 and 6) competition by resveratrol. Note that RTP-22 was competed by adding resveratrol during binding to the column. RTP-22 was identified as dihydronicotinamide riboside quinone reductase (NQO2).

Task 2c. Purification of resveratrol binding proteins RTPs from control and resveratrol treated LNCaP cells.

Further evidence of the utility of resveratrol affinity columns for identifying and purifying RTPs was obtained using LNCaP cell extracts (Fig 2). Fig 2 also shows data from experiments designed to test whether this affinity matrix approach might serve to identify new proteins subject to control by resveratrol. Accordingly, LNCaP cells, with and without 2-day treatment with 25 μM resveratrol, were lysed. Lysates from control and treated LNCaP cells were separately fractionated on resveratrol affinity column. Representative RTP profiles depicted as silver stained patterns Fig 2 show a significant difference in the

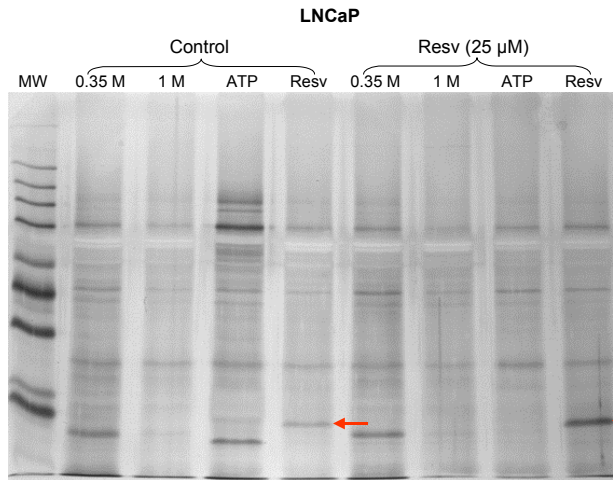
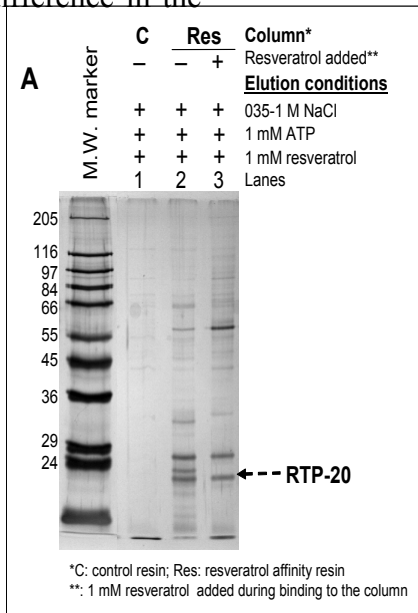


Fig 2. Identification of RTPs in control and resveratrol treated LNCaP cells. Cell lysates were fractionated resveratrol affinity columns according to the scheme in Fig. 1. Protein profiles in control and 25μM resveratrol treated LNCaP cell extracts, eluted with NaCl, ATP and 1 mM resveratrol are shown and putative RTPs are indicated by arrows.

silver stained patterns of fractions eluted with 1 mM ATP and resveratrol. Identity of some of these proteins has yet to be determined.

Task 2d. To identify resveratrol-binding proteins using mass spectrometry.

As indicated above, we have identified dihydronicotinamide riboside quinone reductase 2 (NQO2) as the first RTP [1, 2, 11]. It is of interest to determine whether other RTPs can be purified using the same approach. Comparative analysis of silver stained pattern of resveratrol-eluted



B

Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi)

| Mass | Almond | Human | Aluminate sequence |
|---------|----------------------|-------|--------------------|
| 1335.86 | AKKGGED ^b | D26a | ASGHYGGQPK |
| 1337.86 | | 0.26 | RSCTVGGPKR |
| 1537.96 | | 0.23 | PSHIVVNPAGK |
| 1884.04 | | 0.23 | FQDGDINVEQSKILR |
| 1889.07 | | 0.39 | PDGSHENWQSNNGR |
| 2064.37 | | 0.39 | KSLASHPNVKCHDNGK |
| 2154.17 | 2154.05 | 0.12 | YISLIYTNVEAGKDDYVK |

fractions between PC-3 and LNCaP cells show that whereas a triplet shows in the molecular weight range of 20-25-kDa in PC-3 cell extracts, doublet usually shows in LNCaP cells. Missing from the LNCaP cell is a 20-kDa protein, denoted RTP-20, which is found in abundance in PC-3 cells. A curious property of RTP-20 relative to NQO2 is that whereas NQO2 binding to the resveratrol affinity columns can be completely competed by the addition of resveratrol to the crude extract prior to fractionation, RTP-20 is only weakly competed using the same approach (see Fig 3). Despite this anomaly, we were interested in identifying RTP-20. Accordingly, silver-stained RTP-20 was excised from the gel and subjected to in-gel trypsin digestion to generate peptide fragments that were further determined by MALDI-TOF mass spectrometry. This procedure generated signature patterns that were used in database searches to match predicted tryptic peptide masses of proteins with known identity, resulting in the identification of RTP-20 as GST-Pi (Fig 3).

To further confirm the identity of RTP-20 as GST-Pi, we took advantage of its reported absence in LNCaP cells [12-17]. Immunoblot analysis using GST-Pi antibody to check the expression level of GST-Pi from four different prostate cancer cell lines confirmed that there was negligible expression of GST-Pi in LNCaP cells (Fig 4).

As mentioned above, compared to binding of NQO2 to resveratrol affinity columns, the affinity of GST-Pi for resveratrol affinity columns was relatively weak and appeared to bind less quantitatively. This conclusion was based on immunoblot analysis of various fractions of PC-3 cell extracts, with and without prior competition by resveratrol, eluted with salt, ATP and finally resveratrol, and probed for the presence of GST-Pi and NQO2. Whereas GST-Pi is present in the unbound fraction as well as in much smaller quantities in the various fractions eluted with NaCl and ATP, with significantly more abundant GST-Pi being eluted in the resveratrol fraction, a different picture was found in the binding and elution pattern of NQO2. Specifically, NQO2, present copiously in unfractionated cell extracts, was quantitatively retained on the resveratrol affinity column and was only displaced when the column was eluted with 1 mM resveratrol (Fig 5). The difference between GST-Pi and NQO2 in regard to their binding

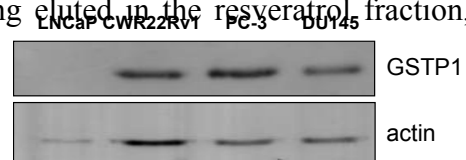


Fig 4. GST-Pi expression profile in prostate cancer cell lines.

to resveratrol may suggest need for a different conformational architecture in the binding of GST-Pi to resveratrol, relative to NQO2. Both NQO2 and GST-Pi are detoxification enzymes, our findings suggest that resveratrol may exert its chemopreventive property by binding and interacting with detoxification enzymes as to modulate their activity and/or stability.

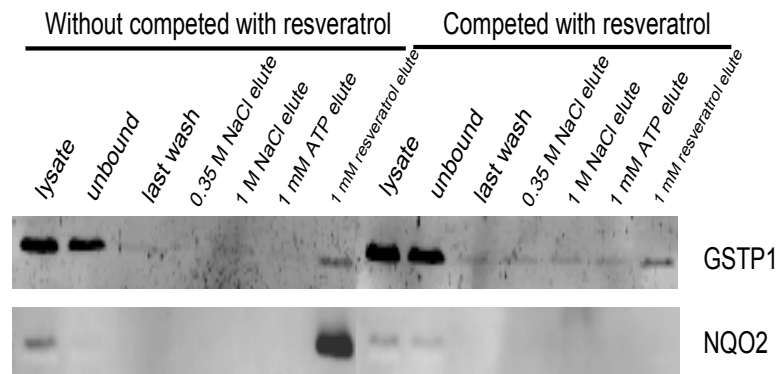


Fig 5. Binding of GST-Pi on resveratrol affinity columns

KEY RESEARCH ACCOMPLISHMENTS

- [³H]resveratrol can be taken into normal and prostate tumor cells, albeit with a definite time lag and rather inefficiently.
- Uptake of [³H]resveratrol is more robust in hormone-refractory human prostate PC-3 cells, compared to androgen-dependent LNCaP cells.
- Pretreatment of PC-3 cells for 2 days with 2 μM resveratrol results in a 2.7-fold increase in uptake of [³H]resveratrol, compared to untreated PC-3 cells.
- Using fractionation procedures that separate cell extracts into cytosol, organelle, nucleus and cytoskeleton, we observed that the majority of radioactive resveratrol in both untreated and 2-day resveratrol-treated cells, can be traced to the organelle fraction.
- By chemically coupling resveratrol to epoxy-activated agarose to form a resveratrol affinity column, we have been able to isolate and identify dihydronicotinamide riboside quinone reductase 2 (NQO2) as novel resveratrol targeting protein in prostate cancer cells. The same strategy may lead to the isolation of additional resveratrol targeting proteins the identify of which remains to be fully elucidated. Salient features of this innovative and facile development, which we have named *ligand (resveratrol)-capture proteomics*, include significant attributes listed below.
 - Enable detection and analysis of low abundance proteins that often are challenging to visualize and characterize by conventional biochemical methods.
 - Generate RTP profiles characteristic of androgen-dependent LNCaP and androgen-independent PC-3 cells
- A combination of LSP with MALDI-TOF mass spectrometry has also recently identified a second RTP in PC-3 cell extract, as glutathione sulfotransferase GST-Pi.
- Both NQO2 and GST-Pi are detoxification enzymes, therefore our findings, to be detailed below, suggest that resveratrol may exert its chemopreventive property by binding and interacting with detoxification enzymes as to modulate their activity and/or stability.

REPORTABLE OUTCOMES

A manuscript summarizing the findings in this report is in preparation. In addition, we are also considering submitting an IDEA grant to the U.S. Army Medical Research and Material Command that builds on the observations reported herein.

CONCLUSION

We have made significant progress towards defining the identity and nature of cellular proteins, denoted RTPs that specifically interact with resveratrol using cancerous prostate cells. The identity of a specific RTP has been elucidated as quinone reductase 2 (NQO2). The same strategy has also recently identified a second RTP in PC-3 cell extract, as glutathione sulfotransferase GST-Pi. Both NQO2 and GST-Pi are detoxification enzymes, therefore our findings, to be detailed below, suggest that resveratrol may exert its chemopreventive property by binding and interacting with detoxification enzymes as to modulate their activity and/or stability. These new findings deserve further investigations.

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List of personnel receiving pay from the research effort.

Joseph M. Wu, Ph.D.

Tze-chen Hsieh, Ph.D.

Yan Chen

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APPENDICES

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Inhibition of melanoma cell proliferation by resveratrol is correlated with upregulation of quinone reductase 2 and p53

Tze-chen Hsieh^a, Zhirong Wang^a, Carl V. Hamby^b, Joseph M. Wu^{a,*}

^a Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

^b Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595, USA

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Abstract

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a grape-derived polyphenol under intensive study for its potential in cancer prevention. In the case of cultured human melanoma cells, no one to our knowledge has investigated whether resveratrol exerts similar anti-proliferative activities in cells with different metastatic potential. Therefore, we examined the effects of this polyphenol on the growth of weakly metastatic Line IV clone 3 and on autologous, highly metastatic Line IV clone 1 cultured melanoma cells. Comparable inhibition of growth and colony formation resulted from treatment by resveratrol in both cell lines. Flow cytometric analysis revealed that resveratrol-treated clone 1 cells had a dose-dependent increase in S phase and a concomitant reduction in the G₁ phase. No detectable change in cell cycle phase distribution was found in similarly treated clone 3 cells. Western blots demonstrated a significant increase in the expression of the tumor suppressor gene p53, without a commensurate change in p21 and several other cell cycle regulatory proteins in both cell types. Chromatography of Line IV clone 3 and clone 1 cell extracts on resveratrol affinity columns revealed that the basal expression of dihydronicotinamide riboside quinone reductase 2 (NQO2) was higher in Line IV clone 1 than clone 3 cells. Levels of NQO2 but not its structural analog NQO1 were dose-dependently increased by resveratrol in both cell lines. We propose that induction of NQO2 may relate to the observed increased expression of p53 that, in turn, contributes to the observed suppression of cell growth in both melanoma cell lines.

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Keywords: Resveratrol; Resveratrol targeting protein NQO2; Immobilized resveratrol affinity columns; Melanoma carcinogenesis

Cancer presents clinically as a constellation of diseases and at all stages. From primary prevention to therapeutic management and treatment, nutrition is a key factor. Decades of epidemiological studies have established a direct link between consumption of plant-based diets and a reduction in risk of cancer [1–4]. In recent years, efforts directed at the identification of bioactive ingredients in those diets and delineation of their mechanism(s) have demonstrated that even ubiquitous, non-nutritional secondary plant metabolites, such as flavonoids and polyphenolics widely present in

foods consumed in the US, have significant health consequences [5–10].

Resveratrol is a phytoalexin found in grapes, nuts, and red wine. Various studies have reported that resveratrol has antioxidant, antithrombotic, anti-inflammatory, anti-aging, cardioprotective, and anti-tumorigenic properties [11–18]. We had previously proposed that part of its diverse biological attributes might relate to the ability of resveratrol to interact with specific cellular proteins, denoted resveratrol targeting proteins (RTPs) [19]. To test this hypothesis, we designed an affinity matrix with resveratrol immobilized on epoxy-activated agarose beads that served as a selection platform for the isolation and identification of RTPs. The efficacy of this approach was recently demonstrated

* Corresponding author. Fax: +1 914 594 4058.
E-mail address: Joseph_Wu@nymc.edu (J.M. Wu).

by isolating a 22-kDa polypeptide, designated RTP-22, from prostate and erythroleukemia K562 cancer cells that was identified as dihydronicotinamide riboside quinone reductase 2 (NQO2) by MALDI-TOF mass spectrometry and cloning [19,20].

Melanoma is the most common fatal cutaneous malignancy in Caucasian populations. The frequency of melanoma has increased by a factor of 15 over the past 60 years and it is continuing to rise at an annual incidence rate of 5–7% in fair-skinned individuals despite advances made in skin cancer detection and diagnosis [21–23]. The prognosis for individuals with advanced cutaneous malignant melanoma is dismal due to the lack of effective treatment options [24–26].

Chemoprevention has recently been proposed as a new approach in the control of melanoma [27,28]. In the case of cultured human melanoma cells, no one to our knowledge has investigated whether resveratrol exerts similar anti-proliferative activities in cells with different metastatic potential. Therefore, we tested the effects of resveratrol on the growth of highly metastatic Line IV clone 1 cultured melanoma cells and on autologous, weakly metastatic Line IV clone 3 cells derived from the same donor. These two cell lines have previously been used in a model system to identify differentially expressed cellular antigens associated with the metastatic phenotype [29,30]. We found that treatment by resveratrol significantly inhibited the proliferation of both cell types. The suppression of proliferation in Line IV clone 1 cells was correlated with a threefold increase in the S and a corresponding 75% reduction in G₁ phases of the cell cycle. However, reduction in growth of Line IV clone 3 was not associated with changes in cell cycle events. Immunoblot analysis showed that NQO2 expression was significantly higher in Line IV clone 1 than clone 3 cells, and that its levels were dose-dependently increased by resveratrol to a greater degree in clone 1 than clone 3. The increase in NQO2 was correlated with upregulation of expression of tumor suppressor gene protein p53. We propose that the induction of NQO2 by resveratrol may be coupled to an increase in stability of p53 that, in turn, contributes to the observed suppression of cell growth in both human melanoma cell lines.

Materials and methods

Materials. Resveratrol was purchased from LKT Laboratories (St. Paul, MN) or Sigma Chemical (St. Louis, MO). Epoxy-activated agarose resin (12 atom linker, 33 μmol of epoxy group per ml of packed gel) was also obtained from Sigma Chemical. Other biochemical and molecular biology grade reagents were purchased from various commercial vendors. Stock solutions of resveratrol (12.5 mM) were prepared in dimethyl sulfoxide (DMSO) and kept at –20 °C.

Cell cultures. Autologous human melanoma Line IV clone 1 and clone 3 cells were sub-clones of parental Line IV cells, originally

established from a primary malignant melanoma lesion [29]. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. Cells were split once a week and media were changed every 3–4 days.

Cell proliferation and colony formation assays. Line IV clone 1 and clone 3 cells were seeded at 5×10^4 cells/ml in T-75 flasks, allowed to attach overnight, and treated for 72 h with various doses of resveratrol. Control and resveratrol-treated cells were harvested by trypsinization and cell numbers were determined using a hemocytometer. Cell viability was assayed by trypan blue dye exclusion [31–34].

Colony formation or clonogenicity was performed as described [33,34]. Two milliliter aliquots of Line IV clone 1 and clone 3 cells adjusted to a density of 200 cells/ml using RPMI 1640 containing 10% FBS were dispensed into individual wells of six-well tissue culture dishes, followed by addition of different concentrations of resveratrol or 0.2% DMSO. Following another 14 days in culture, the cells were fixed and stained with 0.1% crystal violet to visualize colonies.

Flow cytometry. Line IV clone 1 and clone 3 cultures were treated with increasing concentrations of resveratrol for 3 days and harvested. Cells were washed once with PBS and stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM MgCl₂, and 0.1% Triton X-100 (Sigma) at pH 6.8 as previously described [31,32,34,35]. The DNA-specific DAPI fluorescence was excited with UV light and collected with appropriate filters in an ICP-22 (Ortho Diagnostic, Westwood, MA) flow cytometer. The cell cycle distribution was obtained by deconvoluting the DNA content frequency histograms with the use of Multicycle software (Phoenix Flow, San Diego, CA).

Preparation of immobilized resveratrol affinity column. A chromatographic matrix with resveratrol as the immobilized affinity ligand was prepared as described [19]. In brief, resuspended epoxy-activated agarose was reacted with resveratrol dissolved in 0.1 M NaOH. Chemical coupling of ligand to the solid support was terminated by addition of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT). The affinity resin containing immobilized resveratrol was stored in 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT. Control resins consisted of mock-treated beads (identical procedure except that no resveratrol was added) or beads immobilized with tyrosine as the ligand.

Fractionation of cytoplasmic extracts on immobilized resveratrol affinity columns. Cytoplasmic extracts from cultured melanoma cells were prepared as described previously [19,31,35]. In brief, cells were suspended in buffer containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM Mg(OAc)₂, 1 mM DTT, 0.5% NP-40, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μl/ml of the protease inhibitor cocktail from Sigma Chemical. Cells were lysed by vortexing in three freeze–thaw cycles and cell-free extracts were obtained by a 10-min centrifugation in a refrigerated microcentrifuge. The protein concentration of cytoplasmic extracts was determined using a protein assay kit, purchased from Pierce Chemical. All samples were stored in aliquots at –80 °C.

To characterize proteins bound to the resveratrol affinity column, extracts containing 0.6–1.0 mg of protein in 200 μl of lysis buffer were individually mixed with 50 μl control (mock-treated or tyrosine-linked) or resveratrol immobilized agarose beads in a 1.5-ml Eppendorf tube. The tube was incubated overnight at 4 °C with mild tumbling. The gel slurry containing protein extract was loaded onto a minicolumn (from Pierce Chemical) and washed with 10–20 ml of lysis buffer to remove unbound proteins. The column was next eluted five times, each time with 0.5 ml lysis buffer containing 0.35 M NaCl, and was followed by the same number of washings using 1 M NaCl supplemented buffer. Next, the column was re-equilibrated with the lysis buffer, and eluted with 1 mM ATP. The last step involved elution with 1–2 mM resveratrol dissolved in 2% DMSO. Specificity of binding was demonstrated by mixing extracts with 1 mM resveratrol prior to binding to the affinity resin, for ascertaining competition of binding of distinct RTPs. Proteins eluted by salt, 1 mM ATP, and finally resveratrol in the last

step were analyzed by 10% SDS-PAGE and visualized by silver staining.

Western blot analysis. To analyze the expression of specific proteins in control and treated cell extracts, 10 μg proteins was boiled for 5 min in Laemmli buffer and separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then transferred to nitrocellulose membranes by a semi-dry transfer method. After blocking with TBST containing 5% low-fat milk, the membranes were probed for the level of expression of cyclin D1, Rb, PCNA, p53, p21, quinone reductase types 1 and 2 (NQO1 and NQO2), and β -actin using monoclonal or polyclonal antibodies, as described [31–34]. Specific immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) or color reaction using procedures detailed in the manufacturer's protocol (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Activity assay of dihydronicotinamide riboside (NRH):quinone reductase 2, NQO2. NQO2 was assayed as NRH-dependent reduction of menadione, assayed spectrophotometrically as the coupled reduction of MTT at 610 nm at 25 °C in the presence of dicoumarol (inhibitor of NQO1). The assay mixture contained 100 mM Tris-HCl, pH 8.5, 10 μM menadione, 160 μM NRH, 0.3 mg/ml MTT, and 10 μM dicoumarol.

Results

Suppression of growth and clonogenicity of Line IV clone 1 and IV clone 3 human melanoma cells by resveratrol

We first tested the anti-proliferative effects of resveratrol on in vitro growth of Line IV clones 1 and 3 cells by adding varying concentrations of resveratrol to tissue culture media and following the growth of these cells. At various times, control and treated cells were harvested by trypsinization. Cell viability was determined by incubation with trypan blue and the numbers of blue (dead) cells and transparent (live) cells were counted in a hemocytometer. Fig. 1 depicts a 72-h treatment experiment that demonstrated suppression of proliferation by resveratrol in both Line IV clone 1 and Line IV clone 3 cells. The Line IV clone 1 cells appeared to be slightly more sensitive to the grape-derived polyphenol than

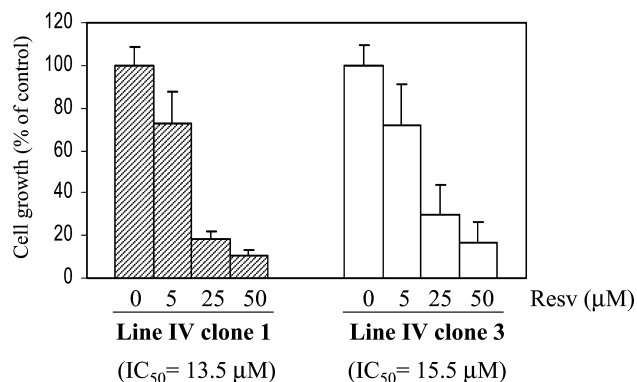


Fig. 1. Effects of resveratrol on growth of Line IV clone 1 and Line IV clone 3 human melanoma cells. Growth of Line IV clone 1 and clone 3 cells is inhibited by adding varying doses of resveratrol as described under Materials and methods. Results represent the average of 2–3 experiments.

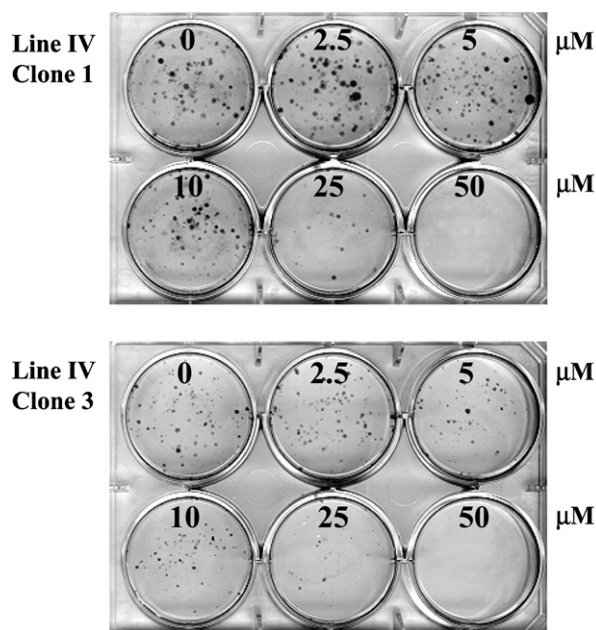


Fig. 2. Effects of resveratrol on clonogenicity of human melanoma Line IV clone 1 and clone 3 cells. The clonogenicity of Line IV clone 1 and clone 3 cells is reduced in a dose-dependent manner by resveratrol treatment. The clonal growth assay was performed as described under Materials and methods. Colony formation was visualized by fixing and staining the cells with 0.1% crystal violet. Experiments were performed in duplicate or triplicate.

Line IV clone 3 cells. Additional evidence for the growth inhibitory activity of resveratrol on human melanoma cells was based on assessment of the ability of tumor cells to grow and form foci, i.e., an indirect measurement of the propensity of tumor cells for neoplastic transformation. This assay is known as colony formation or clonogenicity and is typically performed by plating a fixed number of Line IV clone 1 and clone 3 cells onto multi-well tissue culture dishes, with and without addition of various doses of resveratrol. Colony formation after 14 days in culture can be visually inspected by fixing and staining the cells in 0.1% crystal violet. Fig. 2 shows that clonogenicity of both Line IV clone 1 and clone 3 melanoma cell lines was significantly inhibited by resveratrol, as evident by a marked reduction in both the number and the size of colonies at $\geq 10 \mu\text{M}$ resveratrol. Notably, Line IV clone 1 cells formed larger and greater number of colonies, possibly a reflection of their higher metastatic potential as compared to Line IV clone 3 cells.

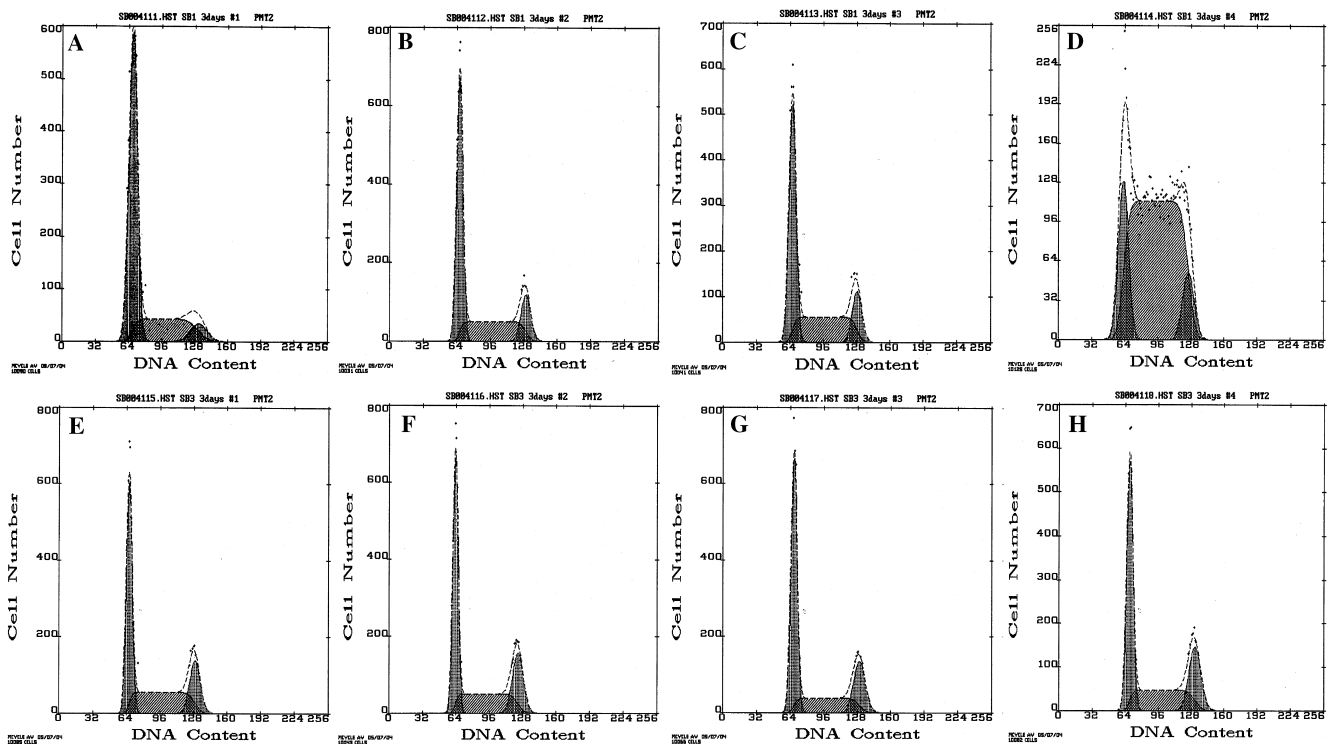
Effects of resveratrol on cell cycle progression and induction of apoptosis in melanoma cells

To explore the underlying mechanism for the observed anti-proliferative effects of resveratrol, cell cycle analyses were performed. Cell cycle phase distribution in Line IV clone 1 and clone 3 cells, with and without

treatment with 5, 25, and 50 μM resveratrol, is depicted in Fig. 3. Resveratrol had little effect on cell cycle transition in Line IV clone 3 cells, whereas it dose-dependently suppressed progression of clone 1 cells. This effect was most vividly illustrated in Line IV clone 1 cells treated with 50 μM resveratrol; the proportion of S phase cells increased almost threefold from 28.6% to 75.4%, and G₁ cells decreased by 75% from 64% to 16.6% (Fig. 3). Interestingly, although resveratrol elicited such a pronounced derangement in cell cycling, it had little to no effect on induction of apoptosis, recognized as the presence of cells with fractional DNA content, in either cell type.

To gain additional information on the cell cycle effects of resveratrol in Line IV clone 1 cells, changes in cell cycle regulatory protein levels were monitored by

immunoblot analysis. Representative results in Rb and p53 expression are shown in Fig. 4. The retinoblastoma tumor suppressor protein Rb, whose expression and state of phosphorylation play a pivotal role in the control of cell cycle checkpoints, was only slightly reduced by treatment with 50 μM resveratrol in both clone 1 and clone 3 cells. Although resveratrol did not affect cell cycling in clone 3 cells, it caused a more pronounced decrease in hyperphosphorylated Rb. The most dramatic effect of resveratrol was a dose-dependent increase in the expression of the tumor suppressor gene p53. Indeed, in the case of Line IV clone 1 cells, as little as 5 μM resveratrol sufficed to upregulate p53 (Fig. 4). Since p53 functions as a transcriptional activator of a number of genes, including the checkpoint regulator p21, we tested whether resveratrol correspondingly



| Treatment | Line IV clone 1 | | | | Line IV clone 3 | | | |
|-----------|-----------------|-----------------|------------------|------------------|-----------------|-----------------|------------------|------------------|
| | A | B | C | D | E | F | G | H |
| | Control | 5 μM | 25 μM | 50 μM | control | 5 μM | 25 μM | 50 μM |
| G1 | 64.1 | 52.9 | 48.4 | 16.6 | 45.0 | 46.3 | 53.8 | 46.6 |
| S | 28.6 | 33.0 | 37.6 | 75.4 | 36.6 | 32.0 | 25.9 | 31.1 |
| G2/M | 7.3 | 14.1 | 14.1 | 8.0 | 18.4 | 21.8 | 20.3 | 22.3 |

Fig. 3. Effects of resveratrol on changes in cell cycle phase distribution. (A–D) Cell cycle phase distribution of Line IV clone 1 cells and (E–H) results from Line IV clone 3 cells. Cells were treated for 72 h with 0, 5, 25, and 50 μM resveratrol. Results of the cell cytometric analysis appear in the table.

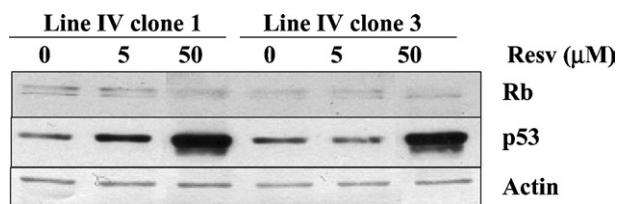


Fig. 4. Expression of Rb, p53, and actin in human melanoma Line IV clone 1 and Line IV clone 3 cells. Western blot analysis of extracts prepared from cells treated for 3 days with 0, 5, 25, and 50 μM resveratrol.

affected p21. Contrary to expectation, p21 levels were reduced by ≥ 5 μM resveratrol in Line IV clone 1, whereas in Line IV clone 3 cells only 50 μM resveratrol had an appreciable suppressive effect on p21 (data not shown).

Identification of cellular proteins distinguishing Line IV clone 1 and clone 3 cells and their differential responses to resveratrol by resveratrol affinity chromatography

To determine differences between Line IV clone 1 and clone 3 cells, and analyze and identify targets that might mediate the growth sensitivity and responsiveness of these two human melanoma cells to resveratrol, an affinity chromatography approach was used. Resveratrol was immobilized on epoxy-activated agarose beads, thereby providing an affinity platform to fractionate control and treated cell extracts. This resveratrol-directed fractionation strategy relies on the molecular prowess (specificity and affinity) of resveratrol and not a priori assumption of relevant cellular attributes of the melanoma cells in question; as such, it could generate protein profiles characteristic of the cell type being analyzed. Increasing salt concentrations and ATP were used to elute the column in order to reduce binding of nonspecific proteins to the affinity column. Cellular targets with distinct binding affinity to resveratrol can be displaced from the column using resveratrol. Results comparing fractionation of extracts prepared from untreated Line IV clone 1 and clone 3 cells are depicted in Fig. 5. Visual inspection of the silver-stained protein bands corresponding to the various elution conditions revealed the following: (1) major differences can be observed with respect to proteins eluted with 1 M NaCl, 1 mM ATP, and also resveratrol; (2) a protein, identified as RTP-22, was found in the resveratrol-eluted fraction and was present proportionately much more abundant in Line IV clone 1 than Line IV clone 3 extracts; (3) RTP-22 is identified as a resveratrol binding protein known as dihydronicotinamide riboside quinone reductase 2 (NQO2), on the basis of its migration in SDS-PAGE, its competition from binding to the resveratrol affinity column by adding excess resveratrol to the extract prior to fractionation (compare lanes 4 with 5 for Line IV clone 1 and lanes 8 with 9 for Line IV clone

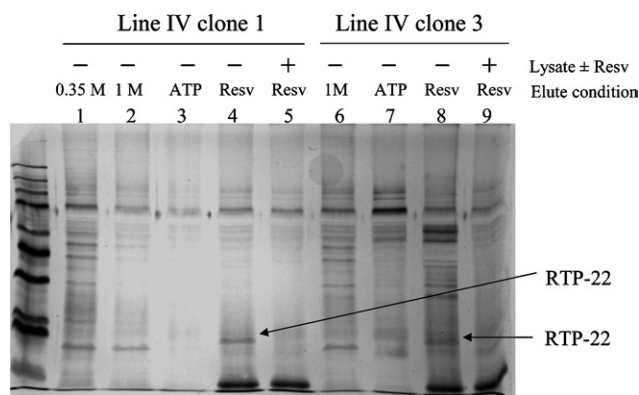


Fig. 5. Analysis of melanoma proteins on resveratrol immobilized affinity columns. Lysates from Line IV clone 1 and clone 3 cells were fractionated on resveratrol affinity columns, as detailed under Materials and methods. Fractions eluted with 0.35 M and 1 M NaCl, followed by elution using 1 mM ATP, and in the last step elution with 1–2 mM resveratrol were concentrated, separated by SDS-PAGE, and visualized by silver-staining. Samples of both Line IV clone 1 and clone 3 cell extracts, containing 1 mM resveratrol, were prepared prior to binding and fractionation on the resveratrol affinity column as specificity controls for protein binding to resveratrol.

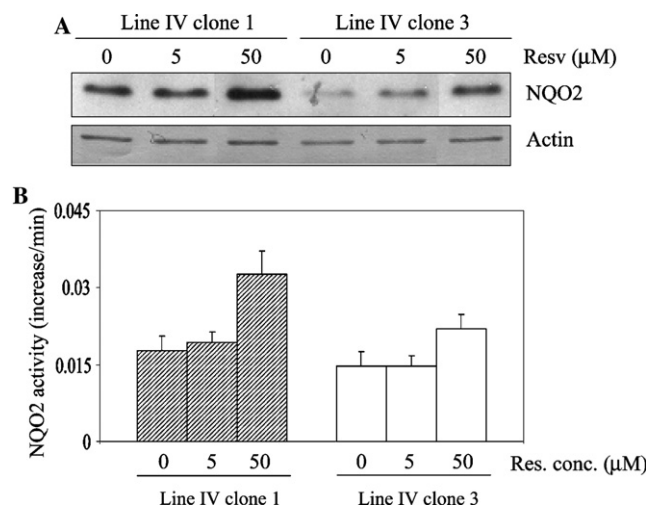


Fig. 6. Induction of NQO2 by resveratrol in human melanoma Line IV clone 1 and clone 3 cells. (A) Western blot analysis to determine the expression of NQO2 in Line IV clone 1 and clone 3 cells treated for 3 days with 0, 5, and 50 μM resveratrol. (B) Activity assay of NQO2 as detailed under Materials and methods.

3), NQO2 activity assays, and by Western blot analysis (Fig. 6).

Induction of NQO2 by resveratrol in Line IV clone 1 and clone 3 cells

Using activity assays and Western blot analysis, we also checked for effects of resveratrol on expression of NQO2 in both cell lines. These experiments showed that the levels of NQO2 were increased by resveratrol in a dose-dependent manner, and that the magnitude of

induction was slightly more pronounced in Line IV clone 1 than Line IV clone 3 cells (Fig. 6). Treatment with resveratrol had no appreciable effect on the expression of NQO1, a structural analog of NQO2, in these two human melanoma cell lines (data not shown). These results as a whole demonstrate qualitative and quantitative differences between Line IV clone 1 and clone 3 human melanoma cells. Since the magnitude of change in NQO2, in response to resveratrol, correlated well with the observed increase in p53, we propose that NQO2 levels may be coupled to elevated expression of p53 that, in turn, contributes to observed suppression of cell growth in both melanoma cell lines.

Discussion

We have demonstrated in the present studies that resveratrol is a potent inhibitor of proliferation and of colony formation in highly metastatic Line IV clone 1 and weakly metastatic Line IV clone 3 human melanoma cells. Notably, these two cell lines were sub-clones of the original Line IV cells established from a single primary melanoma lesion [29,30] whose qualitative differences in metastasis have been demonstrated by subdermal challenge in nude mice. Suppression of proliferation in these two melanoma cells by resveratrol was significantly more pronounced and occurred at a lower dose than what had been previously reported in prostate and breast cancer cells [32,36–38], and also in SK-mel28 melanoma cells [39] although comparable to that found in A375 melanoma cells [39,40]. Therefore, this grape-derived polyphenol should be further explored for its potential in the prevention of human melanoma.

Suppression of cell proliferation in resveratrol-treated cells was accompanied by an accumulation in S phase in Line IV clone 1 but not in clone 3 cells, which was most evident in cells treated with 50 μ M resveratrol (Fig. 3). This could be explained either by their arrest in S, increased transit time through this phase, or apoptosis in G₁ and G₂/M phases of the cell cycle. Since resveratrol had no demonstrated effect on the induction of apoptosis, based on flow cytometric analysis, in either Line IV clone 1 or clone 3 cells, it seems an unlikely explanation for the observed changes in cell cycle distribution. Western blot analysis of control and treated cell extracts showed a reduction in hyperphosphorylated and unphosphorylated forms of Rb, more substantially in 50 μ M resveratrol-treated Line IV clone 1 than clone 3 cells. Similarly, a striking increase was also seen in the expression of tumor suppressor gene p53, without a corresponding change in its downstream target p21 (Fig. 4). These results could contribute to the observed suppression in cell growth in both cell types, albeit by mechanisms unrelated to p53-dependent upregulation

of p21 expression. It is notable that resveratrol has been reported to inhibit ribonucleotide reductase and DNA polymerase δ [41,42], both enzymes that affect DNA replication, these effects could provide a mechanistic basis for the observed prolongation of resveratrol-treated cells in S phase and is an area of current research focus.

To obtain additional insights on cellular targets that might mediate the anti-proliferative mechanism of resveratrol, we tested the efficacy of resveratrol-tagged affinity columns to microcapture RTPs from weakly metastatic Line IV clone 3 cell extracts, for comparison with protein patterns generated from highly metastatic Line IV clone 1 cells. This is a strategy that permits probing of low abundance proteins and could generate protein profiles serving to distinguish normal from tumor cells and tumor cells with different metastatic potentials. It is noteworthy that this experimental approach has led to the capture and cloning of NQO2 in human PC-3 prostate cancer and K562 erythroleukemia cells [19,20]. Chromatography of extracts from Line IV clone 3 and clone 1 cells on resveratrol-immobilized affinity columns shows that NQO2 is present at a higher level in clone 1 than clone 3 cells (Figs. 5 and 6). Treatment of both cell types by resveratrol resulted in substantial induction of NQO2, as verified by activity assays and immunoblot analysis with a NQO2-specific polyclonal antibody (Fig. 6). Since resveratrol treatment did not induce changes in NQO1 (data not shown), a structural analog of NQO2, and because increased expression of NQO2 is highly correlated with induction of p53 by resveratrol in both melanoma cell lines, we hypothesize that NQO2 plays a critical role in the suppression of melanoma cell growth by resveratrol. The potential of NQO2 to bind to and stabilize p53 has been previously suggested by Jaiswal and coworkers [43].

In summary, resveratrol significantly reduces cell proliferation in highly metastatic Line IV clone 1 cells, probably by impairing the progression of cells through the S into G₂M phases of the cell cycle. Moreover, the growth suppressive effect of resveratrol was similarly found in the weakly metastatic Line IV clone 3 cells, by as yet undetermined mechanisms. Chromatography of both melanoma cell extracts on resveratrol-immobilized columns shows that there are quantitative and qualitative differences in the profile of proteins RTPs retained on this affinity platform. The identity of one RTP has been determined as NQO2, which shows a higher expression in Line IV clone 1 than Line IV clone 3 cells. Expression of NQO2 was copiously induced by resveratrol in both cell types. The identity and functions of other RTPs in both melanoma cell types remain to be elucidated. Conceivably, their identification and characterization may be of fundamental importance in the biology of melanoma, particularly in the context of metastasis and in regard to chemoprevention of melano-

ma by resveratrol. Since foods rich in resveratrol, such as grapes and peanuts, are popular items of the American diet, it is possible that resveratrol may be considered as a chemopreventive agent for patients at risk of recurrent melanoma.

Acknowledgments

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