Eugenol Causes Melanoma Growth Suppression through Inhibition of E2F1 Transcriptional Activity

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Rita Ghosh§§, Nagalakshmi Nadiminty¶¶, James E. Fitzpatrick**, William L. Alworth**, Thomas J. Slaga‡, and Addanki P. Kumar‡

From the ¶Department of Cancer Causation and Prevention, AMC Cancer Research Center, Denver, Colorado 80214 and the University of Colorado Comprehensive Cancer Center, Denver, Colorado 80045, the ‡Department of Dermatology, University of Colorado Health Sciences Center, Denver, Colorado 80045, and the **Department of Chemistry and Tulane Cancer Center, Tulane University, New Orleans, Louisiana 70118

Metastatic malignant melanoma is an extremely aggressive cancer, with no currently viable therapy. 4-allyl-2-methoxyphenol (eugenol) was tested for its ability to inhibit proliferation of melanoma cells. Eugenol but not its isomer, isoeugenol (2-methoxy-4-propenylphenol), was found to be a potent inhibitor of melanoma cell proliferation. In a B16 xenograft study, eugenol treatment produced a significant tumor growth delay (p 0.0057), an almost 40% decrease in tumor size, and a 19% increase in the median time to end point. More significantly, 50% of the animals in the control group died from metastatic growth, whereas none in the treatment group showed any signs of invasion or metastasis. Eugenol was well tolerated as determined by measurement of bodyweights. Examination of the mechanism of the antiproliferative action of eugenol in the human malignant melanoma cell line, WM1205Lu, showed that it arrests cells in the S phase of the cell cycle. Flow cytometry coupled with biochemical analyses demonstrated that eugenol induced apoptosis. cDNA array analysis showed that eugenol caused deregulation of the E2F family of transcription factors. Transient transfection assays and electrophoretic mobility shift assays showed that eugenol inhibited the transcriptional activity of E2F1. Overexpression of E2F1 restored about 75% of proliferation ability in cultures. These results indicate that deregulation of E2F1 may be a key factor in eugenol-mediated melanoma growth inhibition both in vitro and in vivo. Since the E2F transcription factors provide growth impetus for the continuous proliferation of melanoma cells, these results suggest that eugenol could be developed as an E2F-targeted agent for melanoma treatment.

Melanoma is one of the fastest growing cancers in the developing world with the incidence having tripled in the last three decades (1). Chemotherapy, immunotherapy, and vaccines have all produced limited benefits especially since the responses are typically short-lived, with no significant effect on overall survival. As a first step toward developing new compounds for effective melanoma management, we screened a panel of naturally occurring compounds for antiproliferative activity toward melanoma cells. From this screen, we have identified 4-allyl-2-methoxyphenol (eugenol)1 as a potent inhibitor of both anchorage-dependent and anchorage-independent growth of melanoma cells representing the different stages of melanoma progression. The structures of eugenol and the isomeric isoeugenol are shown in Fig. 1.

Eugenol is found in reasonable quantities in the essential oils of different spices such as Syzygium aromaticum (clove), Pimenta racemosa (bay leaves), and Cinnamomum verum (cinnamon leaf). Eugenol has been used as an antiseptic, antibacterial, analgesic agent in traditional medical practices in Asia as well as in dentistry in cavity-filling procedures. Eugenol has been demonstrated to inhibit prostaglandin biosynthesis (2) and to block COX-2 activity with an IC50 value of 129 μmol (3). In long term carcinogenicity experiments by various groups in CD-1 mice and F344 rats, eugenol was not associated with tumor formation (4). Based on numerous long term carcinogenicity studies, the National Toxicology Program concluded that eugenol was not carcinogenic to rats and that there was no evidence that unequivocally proved the carcinogenic nature of eugenol in mice (National Toxicology Program). More recently, in a skin carcinogenesis study using the initiating agent 7,12-dimethylbenz(a)anthracene followed by three times weekly cutaneous applications of eugenol for 63 weeks in a group of female ICR/HA Swiss mice, no carcinomas were found (5). In a skin painting study by Van Duuren and Goldschmidt (6), eugenol was reported as being partially effective in inhibiting benzo[a]pyrene-induced skin carcinomas. Eugenol was shown to inhibit DMBA-croton oil-induced papillomas by about 84% (7). Eugenol is not mutagenic, although the incidence of sister chromatid exchange was found to increase in Chinese hamster ovary cells (8). Eugenol has neither been previously reported to be effective against melanoma nor been systematically tested in other common cancers.

The E2F proteins are a family of transcription factors with an important role in regulating cell cycle progression (9). It has been shown that deregulated transcriptional activity of the E2F family in autonomously growing melanoma cells provides the impetus for continuous proliferation of melanoma cells. Specifically, E2F2 and E2F4 are predominant in malignantly growing melanoma cells and identified 4-allyl-2-methoxyphenol (eugenol) as a potent inhibitor of both anchorage-dependent and anchorage-independent growth of melanoma cells representing the different stages of melanoma progression. The structures of eugenol and the isomeric isoeugenol are shown in Fig. 1.

1 The abbreviations and trivial names used are: eugenol, 4-allyl-2-methoxyphenol; isoeugenol, 2-methoxy-4-propenylphenol; CDK, cyclin-dependent kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; RT, reverse transcription; pRb, retinoblastoma tumor suppressor protein.

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§ To whom correspondence should be addressed: Dept. of Cancer Causation and Prevention, AMC Cancer Research Center, 1600 Pierce St., Denver, CO 80214. Tel.: 303-239-3526; Fax: 303-239-3534; E-mail ghosh@amc.org.

¶ Present address: Dept. of Microbiology, State University of New York at Buffalo, Buffalo, NY.
Inhibition of E2F1 by Eugenol

E2F1 in eugenol-mediated melanoma management.

The growth inhibitory effect of eugenol is partially abrogated by overexpression of E2F1. These results suggest a potential role for eugenol in melanoma management. A clinical agent that can target the continuous cycling of melanoma cells would be an attractive tool for effective inhibition of melanoma cell growth. Our results show that eugenol is a potent inhibitor of both anchorage-dependent and anchorage-independent growth of melanoma cells in culture, causing significant tumor growth delay (p = 0.0057), decreases size of tumors, and inhibits melanoma invasion and metastasis in B16 xenograft animals. Eugenol also arrests cells in the S phase of cell cycle, induces apoptosis, and inhibits E2F1 transcriptional activity. The growth inhibitory effect of eugenol is partially abrogated by overexpression of E2F1. These results suggest a potential role for E2F1 in eugenol-mediated melanoma management.

EXPERIMENTAL PROCEDURES

Materials—Eugenol was purchased from Sigma; all the polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CellTiter96 96-well plates were provided by Promega Corp. (Madison, WI). All other reagents were molecular biology grade from Sigma. The E2F1 plasmids were a gift from Dr. David G. Johnson at University of Texas M. D. Anderson Cancer Center, Smithville, TX.

Cell Lines—The human melanoma cells that represent disease progression (Sbcl2-primary melanoma; WM3211-radial growth phase; primary RGP, WM98-1-radial and vertical growth phase; primary RGP, WM1205Lu-metastatic melanoma) were a gift from Dr. Meenhard Herlyn at the Wistar Institute in Philadelphia. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin/streptomycin. All melanoma cell lines were maintained in a humidified incubator with 5% CO2 at 37 °C.

Animal Study—Female B6D2F1 mice bearing established B16 melanomas (∼50 mm3) were randomized into a control and a treatment group of eight animals each at Piedmont Research Center in Seneca, SC. The treatment group received 125 mg of eugenol/kg of body weight twice a day. Animals were euthanized when their tumors reached the end point volume of 2,000 mm3, and the time to end point was used as treatment outcome (defined as the percentage of increase in median time to end point of treated versus control mice). Significance of efficacy was calculated using log rank analysis. All animal procedures were conducted in strict adherence to recommendations of the Guide for Care and Use of Laboratory Animals.

Proliferation Assay—Actively growing human melanoma cells were plated in 96-well plates at a density of 4 × 103 cells/well in triplicates. After 24 h at 37 °C with 5% CO2, cells were treated with different concentrations of eugenol (0.5–2.5 μM), isoeugenol (0.5–5.0 μM), or the solvent (ethanol). Cell proliferation following treatment was carried out using the CellTiter96 Aqueous One solution assay (Promega Corp.) as described elsewhere (11). Briefly, plated cells were incubated with the dye solution containing tetrazolium at 37 °C for 4 h. The reaction was terminated with a stop solution that solubilizes the formazan product formed. Absorbance at 570 nm was recorded using a SpectraMaxPlus plate reader (Molecular Devices). Proliferation assays were performed five times in triplicate wells. The trypan blue exclusion assay was initially used to measure cell viability.

 Colony Formation Assay—A colony-forming assay as described by Kumar et al. (12) was used. Logarithmically growing melanoma cells were trypsinized and plated at a density of 8,000 cells/ml in 0.5% agarose plates in duplicate. 4 h after incubating for 14 days, colonies were stained with 0.02% p-diaminodihydroxyazoanilide. After 6 h, colonies containing more than 50 cells and stained dark pink were counted in eight different fields. The expected number was calculated using the Poisson distribution.

Flow Cytometric Analysis—Actively growing cells were plated at a density of 105 cells in 100-mm dishes. Cells at ~70% confluency were treated with either 0.5 μM eugenol in ethanol or solvent (ethanol) for 20 h. Cells were harvested and resuspended in 1 ml of Tri-HCl (pH 7.4) containing 0.5% Nonidet P-40, 50 mM NaF, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin, and 1 mM dithiothreitol. Lysed cells were passed through a 25-gauge needle, and the released material was centrifuged at 12,000 rpm for 30 min. Protein content in the supernatant was determined by a protein assay kit (Pierce). The lysate was then fractionated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Antibodies used were as follows: polyclonal anti-E2F1, anti-actin to normalize for loading differences. Western blotting was repeated twice with different batches of extracts. The membranes were blocked with 5% nonfat milk and probed with the respective antibodies at room temperature. Bands were visualized using the ECL detection system (Amersham, Arlington Heights, IL). Quantification and background subtraction were performed using a densitometric analysis software (Quantity One; Bio-Rad, Hercules, CA). The intensity of the bands was expressed relative to β-actin, which was used as a loading control.

DNA fragmentation analysis was performed using the apoptosis detection kit (Promega Corp.) as described previously (11). This assay utilizes a modification of the TUNEL assay to detect apoptotic cells in the tissue section. After the reaction was terminated, apoptosis was detected as the dark brown color of the horseradish peroxidase-labeled streptavidin biotinylated alkaline phosphatase complex. Two-color immunofluorescence analysis was performed in duplicate samples using equal numbers of viable B16 cells, and the amount of eugenol was based on the maximum tolerated dose of eugenol in different animal species. Animals were euthanized when their tumors reached the end point volume of 2,000 mm3, and the time to end point was used as treatment outcome (defined as the percentage of increase in median time to end point of treated versus control mice). Significance of efficacy was calculated using log rank analysis. All animal procedures were conducted in strict adherence to recommendations of the Guide for Care and Use of Laboratory Animals.
Eugenol. Fig. 2, A–D, eugenol addition inhibited the growth of all the human melanoma cell lines tested. The Sbcl2 and WM3211 cells showed 50% growth inhibition in 0.5 μM eugenol after 24 h, whereas the WM98-1 and WM1205Lu cells needed twice as much time for 50% growth inhibition at this concentration of eugenol. At 72 h, however, there was no significant difference in the G2/M phase cells following eugenol treatment (data not shown). Further we found the consistent appearance of a sub-G1 peak indicating that eugenol was well tolerated.

Tumor tissue (Fig. 3) from control group animals stained with hematoxylin and eosin (Fig. 3A) demonstrates numerous confluent collections of atypical cells with large pleomorphic nuclei, clamped chromatin, and atypical mitotic figures. Intersected between the melanoma cells are numerous melanin-containing melanophages. Following treatment with eugenol (panel ii), diffuse areas of tumor necrosis intermixed with occasional small aggregates of damaged melanocytes that demonstrate nuclear pyknosis and condensation of the cytoplasm are seen. Diffuse areas of tumor necrosis with no viable-appearing melanoma cells are also seen. We used the modified TUNEL assay to determine whether the tumors in the treatment group were undergoing apoptosis. In the modified TUNEL assay, streptavidin-labeled dUTP is incorporated into the 3′-OH ends of apoptotic DNA by the enzyme terminal deoxynucleotidyltransferase to produce brown staining in cells undergoing apoptosis. As shown in Fig. 3B (panel i), tumor sections of the control animals showed negligible brown staining. On the other hand, in Fig. 3B, panel ii, intense brown staining is visible, indicating that eugenol treatment induces apoptosis in melanoma tumors.

Eugenol Blocks Cell Cycle Progression and Induces Apoptosis—Logarithmically growing WM1205Lu cells were treated with eugenol and subjected to flow cytometry analysis as described under “Experimental Procedures.” Results in Fig. 4A show that there was a 40% increase in cells in the S phase accompanied by a decrease in the G1 phase cells with no significant change in the G2/M phase cells following eugenol treatment. Further these data also show that the cells remain blocked in the S phase up to 36 h. Other human melanoma cells (Sbcl2, WM3211, and WM98-1) also showed a similar pattern of S phase block upon eugenol treatment (data not shown). Further we found the consistent appearance of a sub-G1 peak during flow cytometry analysis that is indicative of apoptotic cells (data not shown).

We treated melanoma cells with 0.5, 1, and 2.5 μM eugenol for 18 h and then observed the cells under a phase contrast microscope. Fig. 4B shows the morphological changes that occur in a representative cell line WM1205Lu following eugenol treatment. As shown in Fig. 4B, panels ii–iv, all the cells treated with eugenol showed blebbing of membranes, shrinkage of cytoplasm, and condensation of nuclear material, as well as gradually lifting off the dishes. These characteristic features of apoptosis occurred in a dose-dependent manner and were absent in untreated cells (Fig. 4B, panel i). To confirm the other melanoma cells at 0.5 μM eugenol. Taken together, these results show that eugenol inhibits both anchorage-dependent and anchorage-independent growth of melanoma cells.

Eugenol Causes Significant Tumor Growth Delay, Decreases Tumor Size, and Prevents Tumor Metastasis in B16F10 Xenograft Mice—We tested the effect of eugenol in vivo in the B16 melanoma xenograft model system as shown in Table I. As shown in Table II tumors in the vehicle group grew rapidly to the end point volume, and four mice died due to metastasis between days 8 and 14. The median time for the tumor to grow to end point for the control group was 12.6 days. There was a highly significant 19% tumor growth delay (p = 0.0057) in the group treated with eugenol. The size of tumors in the treatment group was about 62% of that of the tumors of control animals on day 15. Very significantly, 50% of the animals in the control group developed non-treatment-related metastases, whereas none of the animals in the treatment group showed any signs of invasion or metastasis. Daily examination and body weight measurements of the animals showed no difference in body weight between control and treatment group animals, indicating that eugenol was well tolerated.

RESULTS

Eugenol Inhibits Anchorage-dependent and -independent Growth of Melanoma Cells in Culture—The CellTiter96 proliferation assay was used to measure proliferation of the cells for up to 72 h following the addition of increasing concentrations of eugenol. As shown in Fig. 2, A–D, eugenol addition inhibited the growth of all the human melanoma cell lines tested. The Sbcl2 and WM3211 cells showed 50% growth inhibition in 0.5 μM eugenol after 24 h, whereas the WM98-1 and WM1205Lu cells needed twice as much time for 50% growth inhibition at this concentration of eugenol. At 72 h, however, there was no difference in response between the different cell lines using 0.5 μM eugenol. Fig. 2E shows that isoeugenol, an isomer of eugenol, did not inhibit the growth of any of the human melanoma cell lines at concentrations up to 5 μM.

Since anchorage-independent growth is a hallmark of cancer cells, we tested to see whether eugenol could inhibit colony formation on soft agar. Data presented in Fig. 2F show that eugenol inhibited colony formation in all the human melanoma cells. The metastatic melanoma cell line WM1205Lu showed the lowest inhibition in colony formation as compared with the
induction of apoptosis, we performed the modified TUNEL assay to detect apoptotic cells in situ using the metastatic melanoma cell line, WM1205Lu. As shown in Fig. 4C, panels ii–iv, increasing the concentration of eugenol from 0.5 to 2.5 μM produced an increase in the number of brown stained cells, indicating that these cells were undergoing apoptosis. In Fig. 2, Eugenol inhibits growth of melanoma cells. In A–D, the anchorage-dependent proliferation of cells representing the different phases of progression Sbcl2 (primary melanoma), WM3211 (primary radial growth phase), WM98-1 (primary vertical growth phase), and WM1205Lu (metastatic melanoma) are shown. Cells were treated with either ethanol or 0.5, 1, or 2.5 μM eugenol as described under “Experimental Procedures.” CellTiter96 Aqueous One solution assay was used to determine the conversion of tetrazolium salt into a formazan product in proliferating cells. Absorbance of the formazan product was measured at 570 nm every 24 h. E shows the effect of isoeugenol on human melanoma cell proliferation. Cells were treated with increasing concentrations of isoeugenol (from 0.5 to 5 μM) for 72 h, and proliferation was measured with the CellTiter96 Aqueous One solution. The data shown here are an average ± S.D. of five independent experiments performed in triplicates. F shows the anchorage-independent growth of human melanoma cells, Sbcl2, WM3211, WM98-1, and WM1205Lu. Cells were treated with increasing concentrations of eugenol (0, 0.5, 1.0, 2.5, 5, and 10 μM) and plated as described under “Experimental Procedures.” After 14 days, stained colonies containing 50 or more cells were counted in five different fields. The data shown are an average ± S.D. of two independent experiments performed in duplicate.
Table I

| Group | Treatment Regimen | Schedule |
|-------|-------------------|----------|
|       | Number | Agent | Weight | Route |                  |
| n     |         | mg/kg |        |        |                  |
| 1     | 8       | Corn oil | 0   | Intrapertoneal | 2×/week |
| 2     | 8       | Eugenol | 125 | Intrapertoneal | 2×/week |

Table II

Summary of eugenol treatment response

TTE is the time to endpoint (days) 2000 mm^3; T − C is the difference in TTE of treated and control group; %TGD = [(T − C)/C] × 100%; the log rank test is equivalent to the Mantel-Haenszel test, ** = p < 0.01; NTRm is non-treatment related death due to tumor metastasis.

| Group | Median TTE | T − C | %TGD | Log rank | Mean tumor size | Number of NTRm |
|-------|------------|-------|------|----------|----------------|----------------|
| 1     | 12.6       | 0     | 0    | None     | 3417.3 ± 107.1 mg | 4              |
| 2     | 15         | 2.4   | 19%  | ***      | 2135.8 ± 455.9 mg | 0              |

a **, p value = 0.0057.

Fig. 3. A, B16 melanoma tumor sections stained with hematoxylin and eosin. Sections of melanoma tumors from three different animals in the control (i) and eugenol treatment (ii) group were stained with hematoxylin and eosin. Panel i is a representative section from a control animal and demonstrates an admixture of necrotic and viable tumor cells. Arrows indicate the area where there are numerous viable cells. Stars indicate the area where necrotic cells are present. Panel ii is a representative section from a eugenol-treated animal that demonstrates extensive necrosis with no viable melanoma cells. Original magnification, ×400 using an Olympus microscope and digital camera. B, photomicrographs of control cells (panel i) treated with 1 μM eugenol (panel ii) show some nuclear condensation, and cells treated with 1 μM eugenol (panel iii) and 2.5 μM eugenol (panel iv) are also shown in this panel. Apoptotic cells show blebbing of membrane, shrinking of cytoplasm, and nuclear condensation. Most of the cells have lifted off the culture dish in panel iv. The data shown are a representative of three independent experiments.

Fig. 4. A, flow cytometry analysis in response to eugenol treatment. WM1205Lu cells were treated with 1 μM eugenol and subject to flow cytometry analysis. The percentage of cells in the different phases of the cell cycle is shown at 0, 20, and 36 h. The data shown are a representative of three independent experiments. B, eugenol induces apoptosis in WM1205Lu cells. Photomicrographs of control cells (panel i) treated with 0.5 μM eugenol (panel ii) show no nuclear condensation, and cells treated with 1 μM eugenol (panel iii) and 2.5 μM eugenol (panel iv) are also shown in this panel. Apoptotic cells show nuclear condensation, and the number of dark brown stained cells increases. Photographs are at ×20 magnification taken through a Zeiss microscope using a CCD camera. Photographs shown are a representative of three independent experiments.

Eugenol Modulates Expression of E2F Family Members—

Since we found that eugenol blocks cells in the replication phase of the cell cycle, we examined the cell cycle regulatory genes involved in the eugenol response using the pathway-specific gene expression system as described under “Experimental Procedures.” The raw data shown in Fig. 5A were corrected for background by subtracting the signal intensity of pUC18 (negative control) and normalizing to peptidylprolyl isomerase A (PPIA; housekeeping gene), and the relative abundance of transcripts between control and eugenol-treated samples was determined (Fig. 5B). To determine genes that are experimentally and biologically relevant, we filtered the data for those genes whose expression level increased or decreased at least 2-fold. Based on this analysis, we determined that members of the E2F family of transcription factors are modulated by eugenol treatment. Quantitative-RT-PCR with glyceraldehyde-3-phosphate dehydrogenase as an internal control was used to validate the array results. Results presented in Fig. 5C show the amplification products of the E2F family (E2F1–E2F6) along with glyceraldehyde-3-phosphate dehydrogenase (bottom band). Transcript abundance was calculated as
the ratio of the target transcript to that of its internal control. A graphical representation of the relative abundance of transcripts in eugenol-treated and untreated cells is shown in Fig. 5D. We found that E2F1, E2F2, and E2F3 were all down-regulated 2-fold or more following treatment with 1 μM eugenol. E2F6 was the only E2F family member that was up-regulated in response to eugenol treatment. These results were also validated by Western blotting using whole cell extracts from eugenol-treated and control cells. As shown in Fig. 5E, protein levels of E2F1, E2F2, and E2F3 were down-regulated by eugenol treatment, confirming the array data. The down-regulation of E2F4 and E2F5 as well as the up-regulation of E2F6 were not discernable at the protein level.

We used immunocytochemical analyses to determine the level of E2F1 protein expression following eugenol treatment. Results shown in Fig. 6A show that more than 90% of the cells express E2F1 in untreated WM1205Lu cells (panel i), whereas the number of cells expressing E2F1 following 1 μM eugenol treatment decreased to less than 1% (panel ii). Nuclear extracts made from control and eugenol-treated cells showed a 90% decrease in the E2F1 protein following eugenol treatment (Fig. 6B). This was confirmed in melanoma tumor samples by immunohistochemistry. E2F1 expression was found to be greater in the control animals (Fig. 6C, panel i) as compared with the treatment group (Fig. 6C, panel ii). A comparison of the data presented in Fig. 6, A and C, shows that E2F1 immunoreactive staining was more diffuse in tumor tissues as compared with tumor cells in culture, indicating the heterogeneous nature of tumor growth.

Eugenol Inhibits E2F1 Transcriptional Activity—Since E2F1 is a key regulator of genes involved in cell cycle progression, we determined whether eugenol affected the transcriptional regulation of E2F1. We used transient transfection assays to determine the effect of eugenol on E2F1 transcription activity as described under “Experimental Procedures.” 24 h after transfection with wild type or mutant E2F1 reporter plasmid, WM1205Lu cells were treated with 1 μM eugenol for 6 h. Normalized Renilla Luciferase activity presented in Fig. 7A shows that eugenol treatment decreased the E2F1 transcriptional activity by more than 50% as compared with that of untreated cells. Cells transfected either with the pGL3 control vector or with the E2F1 mutant reporter showed no transcriptional activity and no response to eugenol treatment. This result clearly demonstrates that eugenol inhibits transcriptional activity of E2F1.
Procedures. Brown staining in themunocytochemistry was carried out as described under “Experimental
nol-treated WM1205Lu cells were fixed in paraformaldehyde, and im-
expression. In the eugenol-treated cells in the right panel, there is very little
staining, indicating that eugenol treatment decreases E2F1 protein
expression. The experiment was repeated twice. Photographs shown
are at ×40 magnification through a Nikon T1-SM microscope fitted
with a Coolpix Nikon digital camera. B, detection of E2F1 protein in
nuclear extracts. WM1205Lu cells were treated or not with eugenol as
described for immunocytochemistry. Nuclear extracts were made and
resolved by gel electrophoresis as described under “Experimental
rocessures.” Blots were probed with the E2F1 antibody, and the experi-
ment was repeated twice with extracts from two different passage cells.
C is control, and T is eugenol-treated. C, immunohistochemical detect-
tion of E2F1. Melanoma tumor sections from two different animals in
the control and treatment groups were subject to immunohistochemis-
ty as described. E2F1 staining in the tissue of the representative
control animal is shown in the left panel. Tissue from a representative
eugenol-treated animal is shown in the right panel. Photographs were
taken with a Coolpix Nikon digital camera attached to a Zeiss micro-
scope at ×20 magnification.

FIG. 6. A, immunocytochemical detection of E2F1. Control and eug-

enol-treated WM1205Lu cells were fixed in paraformaldehyde, and
munocytochemistry was carried out as described under “Experimental

Procedures.” Brown staining in the left panel represents E2F1 expres-
sion. In the eugenol-treated cells in the right panel, there is very little
staining, indicating that eugenol treatment decreases E2F1 protein
expression. The experiment was repeated twice. Photographs shown
are at ×40 magnification through a Nikon T1-SM microscope fitted
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control animal is shown in the left panel. Tissue from a representative
eugenol-treated animal is shown in the right panel. Photographs were
taken with a Coolpix Nikon digital camera attached to a Zeiss micro-
scope at ×20 magnification.

FIG. 7. Eugenol inhibits E2F1 transcriptional activity. WM1205Lu cells were transfected with E2F1 wild type (wt) reporter or
E2F1 mutant (mut) reporter plasmid and treated with eugenol for 4 h. Luciferase activity was measured and normalized to Renilla luciferase.
The graph shows normalized luciferase activity in pGL3 (control plasmid) treated with no eugenol and with 1 μM eugenol, E2F1 wild type
untreated and treated with 1 μM eugenol, E2F1 mutant untreated and treated with 1 μM eugenol. The data shown are an average of two
independent experiments ± S.D. with two different plasmid preparations
each time.

Overexpression of E2F1 Restores Melanoma Cell Proliferation—The observation that eugenol-induced inhibition of cell
proliferation decreased E2F1 expression and its transcriptional
activity suggested that E2F1 is involved in eugenol-mediated
inhibition of melanoma cell proliferation. Therefore we deter-
mined whether overexpression of E2F1 would restore cell pro-
liferation. Cells were transfected with increasing amounts of
the E2F1 expression plasmid (pCDNA3E2F1) or the vector
control. As shown in Fig. 8, vector control cells responded to
eugenol treatment with about 90% decrease in cell viability. In
cells that were transfected with increasing concentrations of
E2F1 cDNA, the percentage of viable cells increased to about
75% of vector transfected cells treated with eugenol (Fig. 8 and
data not shown). This indicates that overexpression of E2F1 is
able to reverse the growth inhibitory effect of eugenol in a
dose-response manner.

FIG. 8. Overexpression of E2F1 partially restores proliferation
capacity. WM1205Lu cells were transfected with increasing amounts
of E2F1 cDNA to determine whether overexpression of E2F1 would restore cell proliferation.

DISCUSSION
Melanoma incidence rate has been increasing at the rate of
2.8%/year at a time when most other cancer incidence rates are
either holding steady or declining. The target population for
melanoma is fairly young, with people in their 40s and 50s
being affected. Therefore it is estimated that the average pro-
ductive life lost from melanoma is about 18.8 years. Treatment
choices for malignant melanoma are very limited and hindered
by the short response rates that do not affect overall disease-
free survival. In an effort to develop molecular mechanism-
based treatment strategies, we have identified eugenol (a vol-
atile oil isolated from culinary spices) as a compound with the
ability to inhibit the growth of melanoma cells in culture. Our
results show that eugenol inhibits both anchorage-dependent
and anchorage-independent growth of human and mouse mel-
anoma cells. Surprisingly, however, isoeugenol, an isomer of
eugenol with similar structure and functionality, did not ex-
hibit this antiproliferative activity. This difference in activity of
the two related molecules may therefore be due to the differ-
ences in metabolism of the two isomers. In studies of skin
sensitization to eugenol and isoeugenol in mice, it was con-
cluded that eugenol was probably being demethylated and then
oxidized to a reactive o-quinone, whereas isoeugenol was prob-
bly being directly oxidized to a p-quinone methide (17). The
IC_{50} for eugenol at 72 h varied from 0.3 to 0.5 μM in human
melanoma cells to 75 μM in the B16 mouse melanoma cells.
Treatment of female BDF1 mice bearing B16 melanoma al-
lografts with 125 mg of eugenol/kg of body weight resulted in a
small but highly significant (p = 0.0057) 2.4-day tumor growth
delay. Furthermore the fact that the treated animals had no
deaths that were attributed to metastasis or tumor invasion is
indicative of the ability of eugenol to suppress melanoma metastasis. This has great significance for melanoma management since it is metastatic malignant melanoma that is fatal, accounting for about 78% of melanoma deaths. Eugenol was also well tolerated in this study based on the fact that there were no treatment-related deaths and changes in body weight were comparable with the control group animals. Tumor size in eugenol-treated animals was about 40% smaller than animals in the control group. Staining of the tumor tissue from the control animals demonstrated extensive areas of viable melanoma cells with only focal areas of necrosis, whereas the tumors from the eugenol-treated animals demonstrated extensive areas of tumor necrosis with only a few small areas of viable tumor cells. Resistance of melanoma cells to apoptosis induction is a common problem associated with successful melanoma therapy. Our results show that eugenol induces apoptosis in melanoma tumors as demonstrated by the TUNEL assay data presented in Fig. 3B. All of these results taken together provide evidence for the potential development of eugenol as an agent for the treatment of melanoma.

Mechanistically, eugenol blocks cells in the replication phase, suggesting that cells stop to repair DNA damage and either re-enter the cell cycle or activate apoptosis in case of massive DNA damage. Results presented here show that melanoma cells treated with eugenol remain blocked in the S phase and undergo apoptosis. We also found that eugenol treatment upregulated numerous enzymes involved in the base excision repair pathway (data not shown). Using cDNA array analysis, we found that the E2F family of transcription factors is differentially regulated in response to eugenol treatment. Under normal growth conditions, these factors regulate cell cycle transition through the activity of the CDKs by phosphorylating and inactivating the pocket proteins, the retinoblastoma tumor suppressor protein (pRb), p107, and p130. The cyclin-dependent kinase inhibitors negatively regulate the CDK holoenzymes and their partner cyclins by site-specific phosphorylation and dephosphorylation (18, 19). Therefore the loss of these inhibitors facilitates the downstream hyperphosphorylation events that keep pRb phosphorylated and promotes uninterrupted cycling of cells through the cell cycle. Since the loss of functional p16 is common in melanoma (20–24), pRb is either constitutively hyperphosphorylated or expressed at extremely low levels in melanoma cells (25, 26). Hyperphosphorylation of the pocket proteins promotes the release of the E2F family of transcription factors. This leads to an increase in E2F transcriptional activity and up-regulation of genes involved in cell cycle progression, DNA synthesis, and transcription factors that participate in the induction of early and late responsive genes (27–31). E2F6, on the other hand, is not associated with the pocket proteins, and overexpression of E2F6 acts as a transcriptional repressor of E2F-responsive genes and arrests cell cycle transition (32–34).

It has been shown that deregulated E2F transcriptional activity in autonomously growing melanoma cells provides the impetus for the continuous proliferation of melanoma cells. Further it has been shown that the E2F2 and E2F4 proteins are predominant in actively proliferating melanocytes, melanoma cells, and freshly isolated melanoma tumors, that growth-arrested melanocytes manifested a E2F4-p130/pRb growth inhibitory complex, and that up-regulated E2F2 activity in melanoma cells was dependent on persistent CDK activity and inactivation of the pocket proteins. In addition, it has been found that members of the E2F family that are known to cause growth arrest and apoptosis, namely E2F1 and E2F6, are deregulated and therefore provide a growth advantage to melanoma cells (26). Therefore the ability of eugenol to disrupt the transcriptional activity of E2F1 demonstrated here underscores the importance of the potential use of eugenol in deregulating cell cycle progression in melanoma cells.

By overexpressing E2F1 in melanoma cells, we were able to restore the proliferation ability of the melanoma cells following eugenol treatment. We have, however, not identified other target(s) that work in concert with E2F1 to allow these melanoma cells to proliferate. Work is in progress to identify other members of the E2F family that may be involved and to identify the downstream targets of the E2F family that are involved in eugenol-induced cell cycle block and apoptosis.

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Eugenol Causes Melanoma Growth Suppression through Inhibition of E2F1 Transcriptional Activity

Rita Ghosh, Nagalakshmi Nadiminty, James E. Fitzpatrick, William L. Alworth, Thomas J. Slaga and Addanki P. Kumar

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