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4-methylcatechol-Induced Oxidative Stress Induces Intrinsic Apoptotic Pathway in Metastatic Melanoma Cells

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Abstract

There has been a steady rise in fatalities associated with thick melanomas (>4mm). Although understanding of the biology of the disease has improved, effective treatment strategies for patients with advanced metastatic melanoma remain elusive. Therefore, more intensive testing of agents with therapeutic potential are needed to improve survival of patients with metastatic malignant melanoma. We have tested the ability of 4-methylcatechol, a metabolite of quercetin; a naturally occurring compound that is commonly found in a variety of fruits for its potential as an anti-melanoma agent. Our results show that 4-methylcatechol inhibits proliferation of melanoma cells in culture while not affecting the growth of normal human epidermal melanocytes. Further, the ability of metastatic melanoma cells to form colonies on soft agar was also inhibited. 4-methylcatechol caused the accumulation of cells in G2/M phase of the cell cycle and induced apoptosis. There was an increase in reactive oxygen species following treatment with 4-methylcatechol that led to apoptosis through the intrinsic mitochondrial pathway. Treatment also inhibited cell survival mediated by Akt, a key player in melanoma cell survival. Taken together our results suggest that 4-methylcatechol exhibits cytotoxicity towards metastatic malignant melanoma cells while sparing normal melanocytes and should be tested further as a potential drug candidate for malignant melanoma.

Keywords

Melanoma; 4-methylcatechol; reactive oxygen species; cell cycle arrest; apoptosis; survival

Introduction

Malignant melanoma has more than tripled in the last two decades among people with white skin. Further, fatality related to thick melanomas continues to rise [1]. In the United States an estimated 69,000 people developed melanoma and more than 53,000 cases of melanoma *in situ* were expected in the last year. These numbers are not considered to be accurate because primary melanomas are diagnosed and treated in dermatological setting and are not

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reported to cancer registries. Recent evidence also points to an increase in thick melanoma in the Hispanic population especially among Hispanic women [2]. Currently, dacarbazine is the standard of care for metastatic melanoma with about 10% response rate. Clinical trials with high dose interferon alpha (IFN α) show increased relapse-free survival but no increase in overall survival. However the high toxicity associated with the use of IFN α , has limited its widespread use. Melanoma vaccines are a strategy for treatment of melanoma patients. Recently the antibody, Ipilimumab, directed to CTLA4 has improved survival of patients with advanced melanomas by 6 to 10 months [3]. Better understanding of melanoma biology has led to the development of a small molecule (PLX 4032) which inhibits downstream signaling through mutant BRAF^{V600E}, an activation mutation commonly found in melanoma. PLX4032 reduced the size of tumors in a majority of patients [4]. However, issues with resistance have already surfaced. Therefore every effort needs to be made to develop additional viable therapeutic strategies for malignant melanoma. Our approach towards this end has focused on natural compounds that have the ability to inhibit proliferation and induce apoptosis in metastatic melanoma cells. 4-methylcatechol (also known as 3, 4-dihydroxytoluene) is a polyphenolic substrate that is produced during the metabolism of quercetin, a natural compound found in various fruits including apple, apricot, avocado, mango, and peach [5, 6]. 4-methylcatechol has been found to produce reactive oxygen species such as hydrogen peroxide and this has been suggested to be a reason for the cytotoxicity of this compound [7]. Here we have examined the mechanism through which cytotoxicity from reactive oxygen species induces apoptosis in rapidly proliferating metastatic melanoma cells. Our results suggest that 4-methylcatechol may be a useful anti-melanoma agent for its ability to inhibit cell survival and induce apoptosis in melanoma cells.

Materials and methods

Materials

4-methyl catechol was purchased from Sigma-Aldrich (St. Louis, MO); CellTiter96 Aqueous One Solution was from Promega Corp., (Madison, WI); mitochondrial extraction kit was purchased from BioSource (Mountain View CA); Image-iT LIVE Green ROS detection kit was from Invitrogen (Carlsbad, CA) and the FITC-Annexin kit was purchased from EMD Chemicals (Gibbstown, NJ). The following antibodies were used: Caspase 3 (AM20) and Caspase 9 (AM47) from Oncogene Research Products), cytochrome C (ab53056) and Porin (ab15895) from Abcam Inc.), actin (sc-7210), Akt (sc-1618), Bax (sc-6236) and Bcl-2 (sc-783) from Santa Cruz Biotechnology, p-Akt (Ser473) (9271) from Cell Signaling Technology. All other reagents except where stated were molecular biology grade from Sigma-Aldrich (St. Louis, MO).

Cells

Normal human epidermal melanocytes were purchased from the American Type Culture Collection and grown in dermal cell basal media supplemented with melanocyte growth kit (ATCC, Manassas, VA). Human melanoma cells, Sbcl2 and 1205Lu were a gift from Dr. Meenhard Herlyn at the Wistar Institute in Philadelphia. These cells were cultured in DMEM medium (Mediatech Inc., Manassas, VA) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA), 0.5% insulin (Sigma-Aldrich (St. Louis, MO) and maintained in a humidified incubator with 5% CO₂ at 37°C as described previously [8].

Proliferation Assay

A modified MTT assay was used as previously described [9]. Actively growing cells were plated in 96 well plates at a density of 4×10^4 cells/ well in triplicates. After 24h, cells were either treated with increasing concentrations of 4-methylcatechol (0.5-20 μ g/ml) or the

solvent (DMSO). To measure cell proliferation following treatment with 4-methylcatechol, we used the CellTiter96 Aqueous One Solution assay according to the manufacturer's instructions (Promega Corp., Madison, WI). Briefly, plated cells were incubated with the dye solution containing tetrazolium and incubated at 37°C for 4h. The reaction was terminated with a stop solution that solubilized the formazan product formed. Absorbance was recorded at 570 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). The experiment was repeated thrice with cells from different passages.

Survival Assay

Actively growing cells were plated in 60 mm dishes at a density of 1×10^5 cells in triplicate dishes. After 24h, cells were either treated with increasing concentrations of 4-methylcatechol (2.5-25 $\mu\text{g/ml}$), or the solvent control (DMSO) or left untreated. 24h later the 4-methylcatechol containing media was replaced with 4-methylcatechol-free media and cells were allowed to grow in the tissue culture incubator. 14 days later cells were fixed with ethanol, and stained with 0.005% crystal violet. The plate was divided into quadrants and surviving cells were counted. Cell survival relative to untreated cells was calculated. The experiment was repeated twice.

Colony formation assay

Colony forming assay was carried out as previously described [9]. Logarithmically growing melanoma cells were plated at a density of 8,000 cells/ ml in 0.5% agarose in duplicate. After incubating for 14 days, colonies were stained with 0.02% p-iodonitrotetrazolium. 6 h later colonies containing more than 50 cells and stained dark pink were counted in eight different fields. The experiment was repeated twice in duplicate.

Detection of Reactive Oxygen Species

Approximately 1,000 actively growing cells were plated in chamber slides and allowed to attach overnight in the tissue culture incubator. Cells were treated with either increasing concentration of 4-methylcatechol, solvent or *tert*-butylhydro peroxide (TBHP; positive control) for 8h and the Image-iT™ LIVE Green ROS detection kit (Invitrogen, Carlsbad CA) was used to determine ROS generated by the different treatment. Cells were washed and labeled with 25 μM carboxy-2', 7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{-DCFDA}$) for 30 minutes at 37°C in the dark. Cells were washed and imaged immediately using neutral density filters. The experiment was repeated thrice with cells from different passages.

Flow Cytometry Analysis

Actively growing cells were plated at a density of 10^6 cells in 100 mm dishes. Cells at approximately 70% confluency were either treated with 10 $\mu\text{g/ml}$ 4-methylcatechol or left untreated for different time periods (4h, 16h and 24h). Cells were harvested and resuspended in 1 ml of Krishan stain containing 1.1 mg/ml sodium citrate, 46 $\mu\text{g/ml}$ propidium iodide, 0.01% NP40 and 10 $\mu\text{g/ml}$ RNase [10]. Data was acquired on the FACS Calibur (using Cellquest software) and analyzed using ModFit software at the University of Texas Health Science Center at San Antonio flow cytometry shared resources core facility. The experiment was repeated thrice.

Apoptosis detection

Logarithmically growing cells were treated with increasing doses of 4-methylcatechol (5, 10, 25 $\mu\text{g/ml}$) and observed for morphological changes at 4h, 8h, 16h and 24h. Photographs were taken with a digital camera mounted on an inverted microscope at the 24h time point. Since the cells showed morphological changes associated with apoptosis, kinetics of

apoptosis induction was determined with Annexin V-FITC as previously described [11]. This assay was also used to determine apoptosis induction as a function of escalating doses and both sets of experiments were repeated thrice.

Preparation of whole cell and mitochondrial extracts

Logarithmically growing cells at approximately 80% confluency was treated with solvent or 5, 10 and 25 $\mu\text{g/ml}$ 4-methylcatechol for 18h. For whole cell extracts cells were harvested and lysed in a buffer containing 50 mM Tris.Cl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1mM NaVO₄, 1mM phenyl methyl sulfonyl fluoride, 25 $\mu\text{g/ml}$ leupeptin, 25 $\mu\text{g/ml}$ aprotinin, 25 $\mu\text{g/ml}$ pepstatin and 1 mM DTT. Lysed cells were passed through a 25G needle and centrifugation was carried out at 12,000 rpm for 30 min at 4°C. Protein content in the supernatant was determined according to Bradford [12]. Mitochondrial extracts were prepared with a kit from Biovision (San Francisco, CA) according to the manufacturer's instructions.

Western Blot analysis

Equal amounts of whole cell or mitochondrial extracts were fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was essentially carried out as described [9]. The method of detection was enhanced chemiluminescence using the Western Lightning-Plus ECL according to the manufacturer's protocol (Perkin Elmer, Waltham, MA). All whole cell extract blots were probed with anti- β -actin antibody and mitochondrial extract blots were probed with anti-porin antibody to normalize for loading.

Results

4-methylcatechol inhibits growth of melanoma cells

To assess the effect of 4-methylcatechol (structure shown in figure 1) on growth of melanoma cells, we first carried out experiments to test its effect on cell proliferation using the modified MTT assay. In this assay reducing equivalents such as NADH produced by actively growing cells reduces the tetrazolium compound to formazan product which is detected by measuring absorbance at 570 nm. The dose for this experiment was based on cell viability results obtained using trypan blue (data not shown). As shown in figure 2 (panel A), treatment with 4-methylcatechol for 72h inhibited growth of melanoma cells in a dose-dependent manner. 10 $\mu\text{g/ml}$ 4-methylcatechol was sufficient to produce 50% growth inhibition in metastatic cells. There was no significant effect of low doses of 4-methylcatechol on the proliferation of non-metastatic (Sbcl2) cells; at least 10 $\mu\text{g/ml}$ was needed to produce 50% growth inhibition. 4-methylcatechol did not inhibit the growth of normal human epidermal melanocytes under the same conditions (up to 20 $\mu\text{g/ml}$ 4-methylcatechol). Since we found that 4-methylcatechol treatment inhibited proliferation of metastatic melanoma cells (1205lu) most effectively we used these cells in subsequent experiments. We then tested the ability of these metastatic melanoma cells to survive following 4-methylcatechol treatment. Actively growing cells were treated as described in methods section for 24h and allowed to recover and grow in 4-methylcatechol-free media for 14 days. Surviving cells were stained with crystal violet and counted. As shown in figure 2 (panel B), treatment of cells with 4-methylcatechol for 24h, inhibited their recovery and survival in a dose dependent manner. While approximately 70% solvent treated cells survived at the end of 14 days, only 25% and 15% cells treated with 10 and 25 $\mu\text{g/ml}$ 4-methylcatechol respectively survived during this time period. Since a hallmark of cancer cells is their ability to grow in an anchorage-independent manner, we tested the ability of 4-methylcatechol to inhibit colony formation on soft agar. As described in methods, cells were plated on media containing solidified agarose with increasing concentration of 4-

methylcatechol. 14 days later colonies were stained and counted. Dark pink stained colonies with more than 50 cells were scored during data collection. Analyses of the collected data showed that 5 µg/ml 4-methylcatechol inhibited anchorage-independent growth by about 75% (figure 2, panel C). At 10 µg/ml there were few colonies that met our selection criteria of 50 or more cells; however there were many small colonies of less than 10 cells. At 25 µg/ml 4-methylcatechol there were no colonies that met the cut off and very few small colonies. The results presented above shows that 4-methylcatechol inhibits anchorage-dependent and -independent growth of metastatic melanoma cells in culture.

4-methylcatechol induces ROS in metastatic melanoma cells

We determined ROS production in live cells following treatment with 4-methylcatechol as described in methods. In this assay formation of carboxy-DCF is used as a measure of ROS. In live cells, carboxy-H₂DCFDA undergoes cleavage by cellular esterases and the reduced fluorescein compound is oxidized by cellular ROS to emit bright green fluorescence. As shown in figure 3, there was a dose-dependent increase in ROS production following treatment with 4-methylcatechol. At the lowest treatment dose (5 µg/ml for 8h) ROS production was slightly higher than the untreated cells showing that there is a background level of ROS produced in these melanoma cells. Solvent treated cells did not cause a significant increase in ROS production. We used TBHP as a positive control for ROS induction and as shown in figure 3 ROS production following 100 µM TBHP treatment was comparable to that of cells treated with 10 µg/ml 4-methylcatechol for 8h. At the higher treatment doses (10, and 25 µg/ml) ROS production increased significantly ($p=0.006$ and 0.003 respectively) compared with the solvent treated samples.

Effect of 4-methylcatechol on cell cycle progression

Exponentially growing metastatic melanoma cells (1205Lu) were treated with 10 µg/ml 4-methylcatechol and cells were harvested immediately for the 0h time point. Subsequently samples were collected at 4h, 16h and 24h. Propidium iodide stained cells were assayed for DNA content using FACS analysis. As shown in figure 4, at time 0h approximately 70% of the cells were in G1 phase and this did not change substantially after 4h of 4-methylcatechol treatment. However, at 16h only about 50% of the cells were in G1 phase; which did not change at 24h. There was no substantial change in the percentage of replicating cells following 4-methylcatechol treatment. About 10% of the cells were in the G2/M phase at time 0h and this did not change after 4h of treatment. However, after 16h of treatment the number of cells in G2/M phase doubled; and at 24h about 35% of the cells were in the G2/M phase compared with approximately 10% at time 0h (at the time of treatment). These results suggest that following 4-methylcatechol treatment although a significant number of cells pass from the G1 through S phase they are blocked in the G2/M phase of the cell cycle.

4-methylcatechol induces apoptosis in metastatic melanoma cells

We followed the cells treated with 4-methylcatechol by observing morphological changes. We found that increasing doses of 4-methylcatechol showed characteristic features of apoptosis including blebbing of cell membrane, condensed nuclear material followed by gradual rounding of cells and lifting off the culture plate. Photographs of cells treated with 10 µg/ml 4-methylcatechol at 0h, 4h, 16h and 24h shown in figure 5 (panels A-D) demonstrate that the number of dead cells increase as a function of time. Subsequently we used Annexin V-FITC assay to determine whether cell death mediated by 4-methylcatechol was through apoptosis in these cells. In this assay necrotic cells also bind Annexin V-FITC. Propidium iodide was used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. Necrotic cells bound to Annexin V-FITC and stained with propidium iodide while propidium iodide was excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Data was plotted for early apoptotic cells and are shown

here. Results of dose-response experiments (figure 5; panel E), show dose-dependent increase in apoptosis when cells were treated with increasing doses of 4-methylcatechol for 12h. We found that following treatment with 10 µg/ml 4-methylcatechol about 10% of cells underwent apoptosis and at 20 µg/ml 4-methylcatechol there was a 2.5 fold increase in apoptotic cells compared with 10 µg/ml treatment. We also carried out time course experiments with 10 µg/ml 4-methylcatechol which is shown in figure 5 (panel F). The number of apoptotic cells at 2h was minimal; which increased to about 10% at 6h and by 24h approximately 25% of the cells were FITC-Annexin-positive for early apoptosis. Approximately 5-7% of the cells treated with 4-methylcatechol underwent necrosis. Taken together these results demonstrate that treatment with 4-methylcatechol induces apoptotic cell death in metastatic melanoma cells in culture.

4-methylcatechol downregulates cell survival and anti-apoptotic proteins

Western blotting was used to determine changes in key pro- and anti-apoptotic as well as survival proteins. Either whole cell or mitochondrial extracts were made from cells treated with increasing concentration of 4-methylcatechol. As shown in figure 6, level of anti-apoptotic protein Bcl2 decreased upon treatment with 4-methylcatechol; while the level of pro-apoptotic Bax increased. We also found that the level of cytochrome c decreased in the mitochondrial extracts following treatment with 25 µg/ml 4-methylcatechol suggesting that cytochrome C was released upon treatment. We also found dose-dependent increase in the cleaved form of caspase 3 and caspase 9 following treatment indicating the involvement of the activation of the caspase cascade. While the level of total Akt did not change with 4-methylcatechol, the level of the active form of Akt (pAkt Ser⁴⁷³) was found to decrease suggesting that the compound inhibited cell survival activity mediated by Akt. All of the western blotting results taken together suggest that 4-methylcatechol induces apoptosis by disrupting the survival and apoptosis balance in metastatic melanoma cells and that apoptosis is mediated through the mitochondrial pathway through the generation of reactive oxygen species.

Discussion

Although the recovery rate of localized cutaneous melanoma is >90%, survival decreases as the melanoma spreads locally and is poor in patients with disease that has dispersed to distant organs. The use of currently available therapeutic agents for dispersed disease is limited in part due to high toxicity resulting in low response rates and survival benefit. With the lack of standard of care that benefits all melanoma patients it becomes imperative for patients with advanced melanoma to enroll in clinical trials. A handful of therapeutic agents including drugs and vaccines are in various phases of clinical testing. It is expected that only a fraction if any of these test agents can be successfully developed for melanoma therapy. In addition to this the fact remains that not all advanced melanoma patients will benefit from the small number of agents that may be developed for clinical use. Further, melanoma is notorious for developing therapeutic resistance. Given that it takes between 7-15 years for development of therapeutic agents, development of a pipeline of laboratory based testing of potential drug candidates should be actively pursued. In this effort we report the biological effect of 4-methylcatechol on malignant melanoma cells.

4-methylcatechol (also known as 3, 4-dihydroxytoluene) is a well-known metabolite of the flavonoid quercetin that is found to occur naturally in fruits and vegetables. It is produced in the intestinal tract following the consumption of quercetin and has been shown to inhibit lipid peroxidation and cholesterol biosynthesis in rat hepatocytes [13]. There are numerous published reports on the ability of the parent compound, quercetin to benefit human health including prevention of cancer and cardiovascular disease [14]. It has been suggested that the parent polyphenolic compounds have protective antioxidant effects and therefore inhibit

tumorigenesis. Further, these parent compounds inhibit growth of a variety of cancer cells including leukemia and non-small cell lung cancer [15-18]. Quercetin protected colorectal adenoma cells from fatty acid hydroperoxide-induced oxidative stress but in colorectal tumor cells quercetin increased oxidative stress and induced apoptosis in about 40-60% of the cells [19]. In osteoblastic cells, quercetin was found to induce caspase-dependent apoptosis [16]. Quercetin has been found to inhibit tumorigenesis, invasion and induce apoptosis in a variety of cultured melanoma cells and xenograft models [20-22]. Quercetin metabolism was found to selectively sensitize melanoma cells that express tyrosinase to increase ROS and induce apoptosis [23]. These data taken together with the reported ability to inhibit lipid peroxidation suggest that the compound has antioxidant effects. On the other hand the metabolites of these parent compounds are known to produce cytotoxic effects. However whether these biological activities are due to quercetin or its metabolites is unclear. Data presented here show that 4-methylcatechol inhibits proliferation of primary and metastatic melanoma cells while it does not significantly affect growth of normal human epidermal melanocytes. These results suggest that 4-methylcatechol perhaps targets pathways in rapidly proliferating cells as is in the case during melanoma progression. Published reports demonstrate the cytotoxicity of 4-methylcatechol in murine tumor cells is associated with production of hydrogen peroxide [7]. 4-methylcatechol was also found to decrease LPS-induced NO, TNF production, expression of iNOS, and LPS-induced nuclear translocation of NF κ B in microglia cells but did not protect neuroblastoma cells in the co-culture from hydrogen peroxide-induced cytotoxicity [24]. These results suggest that 4-methylcatechol can be protective and cytotoxic depending on the cellular context. In normal melanocytes, melanosomes not only produce the pigment, melanin but also serve as efficient quenchers of reactive oxygen species produced during cellular metabolism as well as ultraviolet radiation. Therefore, despite the production of ROS, the ability of melanosomes to quench ROS in normal melanocytes may prevent them from succumbing to the cytotoxic effects of ROS. On the other hand, in melanoma cells melanosomes are known to undergo structural changes that reverse ROS quenching capabilities resulting in generation of more ROS. Therefore it has been thought that the use of compounds that enhance ROS generation can facilitate apoptosis in melanoma cells by increasing free radical-induced DNA damage. In this regard, a recent phase II trial in which elesclomol (an agent that generates ROS), was used in combination with paclitaxel, led to improved patient survival suggesting that this may be a viable approach to treat patients with malignant melanoma [25-26].

Data presented here shows that 4-methylcatechol arrests cells in G2/M phase and induces apoptosis that is mediated by the mitochondrial pathway. A central occurrence in the induction of apoptosis is the activation of caspases that lead to the systematic demise of the cell. In the intrinsic pathway of apoptosis mitochondria are primarily involved in that collapsed mitochondrial membrane potential leads to release of cytochrome c and activation of caspases cascade [27]. Release of cytochrome c leads to the formation of apoptosome and the recruitment of procaspase 9 and subsequent activation of caspase 9 which then activates the downstream caspases [27-28]. Deregulation of the p14^{ARF}/MDM2/p53 pathway in melanoma affects apoptosis through its effects on downstream effectors such as Bax/Bcl2; with higher expression of Bcl2 implicated in melanoma aggressiveness [29]. Therefore the ability of 4-methylcatechol to induce apoptosis in these cells through downregulation of Bcl2 protein level is of great significance.

Our results also suggest that 4-methylcatechol inhibits cell survival activity mediated by Akt. It is known that PI3K activation leads to activation of the serine/threonine kinase, Akt [30]. Akt is also known to activate NF- κ B, an important pleiotropic transcription factor involved in the control of cell proliferation and apoptosis in melanoma. It is believed that the loss of PTEN expression in melanoma cell lines with high Akt expression, could represent an important common pathway in the progression of melanoma possibly through enhancing

cell survival mediated by NF- κ B up-regulation and escape from apoptosis [31]. Activation of Akt is found in 40–60% of melanomas. Akt3 expression strongly correlates with melanoma progression, and depletion of Akt3 induces apoptosis in melanoma cells and reduces the growth of xenograft tumors [32]. Several mechanisms have been found to be associated with the ability of Akt to suppress apoptosis including the phosphorylation and inactivation of pro-apoptotic proteins, such as BAD (Bcl-2 antagonist of cell death, a Bcl2 family member, caspase-9, MDM2, forkhead family of transcription factors, as well as the activation of NF- κ B [33–39]. Activating mutations of Akt are nearly always absent in melanoma and the silencing of Akt function by targeting PI3K has been shown to inhibit cell proliferation and reduce sensitivity to UV radiation [40]. Therefore, the ability of 4-methylcatechol to inhibit Akt activity and simultaneously upregulating reactive oxygen species-induced apoptosis is of great significance for melanoma cells. Work is in progress to identify whether Akt is the direct target of 4-methylcatechol in melanoma cells. These results suggest a potential role for combining a ROS-inducing agent such as 4-methylcatechol with chemotherapeutic drugs in light of the positive observations obtained with combining elesclomol with paclitaxel.

In summary the key findings of this study are that a naturally occurring compound inhibits growth of melanoma cells while not affecting growth of normal human melanocytes. Inhibition of growth is associated with increased ROS generation which leads to apoptosis induction through downregulation of pro-apoptotic Bcl2 as well as pro-survival pAkt. All these results taken together suggest that 4-methylcatechol could be developed as a potential drug candidate for advanced melanomas and requires further testing in preclinical models of advanced melanoma.

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Figure 1.
Structure of 4-methylcatechol

Figure 2.

Growth inhibition in response to 4-methylcatechol treatment.

A. Proliferation inhibition in response to increasing dose of 4-methylcatechol. CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega Corp.) was performed as described in methods. Production of formazan product was measured as absorbance at 570 nm following 72h of treatment. Results are average of 3 separate experiments performed in triplicate. NHEM, normal human epidermal melanocytes; Sbc12, primary melanoma cells; 1205Lu, metastatic melanoma cells

B. Inhibition of cell survival following 4-methylcatechol treatment. Logarithmically growing 1205Lu cells were left untreated, or treated with either solvent or 4-methylcatechol as described in materials and methods. After 14 days cells were fixed with ethanol, stained with 0.005% crystal violet and counted. Percentage of cells that survived compared to the untreated cells is shown in the figure. The graph is an average $\pm$ SD of two separate experiments performed in triplicate.

C. Anchorage-independent growth of human melanoma cells 1205Lu. Cells were treated with increasing concentrations of 4-methylcatechol and plated on soft agar as described in methods. After 14 days colonies were stained with 0.02% p-iodonitrotetrazolium and colonies were counted in eight different fields. The data shown are an average $\pm$ SD of 2 independent experiments performed in duplicate.

Figure 3.

Induction of reactive oxygen species in response to 4-methylcatechol treatment. 1205Lu cells were treated with solvent control, *tert*-butylhydro peroxide (TBHP) and increasing concentration of 4-methylcatechol for 8h. 1µM Dichlorodihydrofluorescein Diacetate (DCFDA) was added to the cells as per vendor recommendations (Invitrogen). Fluorescence microscopy was used to record images of cells undergoing oxidative stress in response to treatment with 4-methylcatechol. Panel A, untreated cells; B, solvent treated cells; C, TBHP; D, 5µg/ml; E, 10µg/ml and F, 25µg/ml 4-methylcatechol. Images shown are a representative of three independent experiments. Average of three independent experiments $\pm$ SD is shown as a graph in panel 3G.

Figure 4.

Flow cytometry analysis in response to treatment with 4-methylcatechol. 1205Lu cells were treated with 10 $\mu\text{g/ml}$ 4-methylcatechol and harvested for flow cytometry analysis at 4h, 16h and 24h. 0h time point represents cells that were harvested immediately following treatment. The data shown are a representative of three independent experiments.

Figure 5.

Apoptosis induction following treatment with 4-methylcatechol.

A-D Photomicrographs of metastatic melanoma cells (1205Lu) following treatment with solvent control (DMSO; panel A) and treated with increasing doses (5, 10, and 25 µg/ml) of 4-methylcatechol (panels B-D respectively). Changes in cell morphology after 24h are shown. Apoptotic cells showed blebbing of membrane, shrinking of cytoplasm and nuclear condensation. Photographs were taken with a camera attached to a Nikon microscope. Photographs are at 10X magnification.

E. Logarithmically growing cells were treated with increasing doses of 4-methylcatechol for 18h (panel E). Cells were harvested and labeled with FITC-Annexin V as described in methods. Flow cytometry was used to detect Annexin V positive cells. The data shown are an average of three independent experiments $\pm$ SD.

F. Logarithmically growing cells were treated with 4-methylcatechol for increasing time periods (panel F). Cells were harvested and labeled with FITC-Annexin V as described in methods. Flow cytometry was used to detect cells apoptotic cells. The data shown are an average $\pm$ SD of three independent experiments.

Figure 6.

Western blot analysis of proteins involved in cell survival and apoptosis induction in response to 4-methylcatechol treatment. Whole cell or mitochondrial extracts were made from control and 4-methylcatechol treated (5, 10 and 25 $\mu\text{g/ml}$) cells that were treated for 18h. Equal amounts of extracts were fractionated by SDS-PAGE and probed with antibodies against Akt, pAkt (S473), caspase 3, caspase 9, Bax, Bcl2, and cytochrome C. Whole cell extract blots were normalized for loading with β -actin and mitochondrial blots were normalized with Porin. Western blotting and analysis was confirmed with three different sets of extracts. The Caspase 3 antibody reacts with 32, 28 and 17 kDa forms of Caspase 3 and additional non-specific bands are also detected with this antibody. The Caspase 9 reacts with the zymogen form of Caspase 9 (46-48 kDa) and the p32 subunit in apoptotic cells.