8-15-2016

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Ariel Yin
Rowan University

Karina Goldberg
Rowan University

Archana Mupparapu
Rowan University School of Osteopathic Medicine

Edward Retzbach
Rowan University School of Osteopathic Medicine

Kingsley Yin
Rowan University School of Osteopathic Medicine

See next page for additional authors

Recommended Citation

Yin AC, Goldberg KH, Mupparapu A, Retzbach EP, Yin K, Yang CF, Goldberg GS. Low Molecular Weight Components in Aqueous Echinacea Purpurea Leaf Extract Inhibit Melanoma Cell Growth. *Journal of Cancer Biology and Therapeutics*. 2016 Aug 15;2(1):109 – 117. doi: 10.18314/gjct.v2i1.93.

ISSN: 2379-5972

Gratis

Journal of Cancer Biology and Therapeutics

Research Article

Low Molecular Weight Components in Aqueous *Echinacea Purpurea* Leaf Extract Inhibit Melanoma Cell Growth

Ariel C Yin[1], Karina H Goldberg[3], Archana Mupparapu[1], Edward P Retzbach[1], Kingsley Yin[2], Catherine F Yang[3,4] and Gary S Goldberg[1*]

[1]Department of Molecular Biology and Graduate School of Biomedical Sciences, Rowan University School of Osteopathic Medicine, Science Center, 2 Medical Center Drive, Stratford, NJ 08084, USA

[2]Department of Cell Biology, Rowan University School of Osteopathic Medicine, Science Center, 2 Medical Center Drive, Stratford, NJ 08084, USA

[3]Department of Chemistry and Biochemistry, Rowan University, 201 Mullica Hill Road, Glassboro, NJ 08028, USA

[4]Medical School, California Northstate University, 9700 West Taron Dr., Elk, Grove, CA 97575, USA

Received: June 25, 2016; Accepted: August 11, 2016; Published: August 15, 2016

Abstract

Melanoma is a skin cancer associated with high mortality. The three year survival rate from advanced melanoma is between 10-15%. One reason for this high mortality rate is that melanoma cells are resistant to traditional chemotherapeutics. *Echinacea* is a plant genus native to North America with putative anticancer properties. Here, we examined effects of aqueous *Echinacea purpurea* leaf extract on the growth of melanoma cells and nontransformed fibroblasts. This aqueous extract reduced B16 mouse melanoma cell growth at concentrations that did not inhibit the growth of nontransformed NIH3T3 fibroblasts, suggesting that the extract had biological specificity against transformed cells. We also examined the effect of different fractions of the extract on melanoma cell growth. These data indicate that components less than 3 kD in size exhibited the greatest inhibitory action on melanoma cell growth. In addition, these data indicated that larger components in the extract ameliorate the ability of these low molecular weight compounds to inhibit melanoma cell growth. Furthermore, *Echinacea* extract inhibited the growth of v-Src transformed LA25 cells without reducing Src kinase activity. Taken together, these results suggest that aqueous *Echinacea purpurea* extract contains low molecular weight compounds that preferentially inhibit tumor cell growth in the face of oncogenic tyrosine kinase activity. These data suggest future studies to better define bioactive compounds in *Echinacea purpurea* and evaluate their therapeutic efficacy *in vivo*.

Keywords: Cancer; Phytochemicals; Src; Fractionation

***Correspondence:** Gary S Goldberg, Department of Molecular Biology and Graduate School of Biomedical Sciences, Rowan University School of Osteopathic Medicine, Science Center, 2 Medical Center Drive, Stratford, NJ 08084, USA, Tel: +1-856-566-6718; E-mail: gary.goldberg@rowan.edu

Introduction

Melanoma is a cancer associated with a high mortality rate. The three year survival rate for advanced melanoma is only 10-15% [1,2]. A major reason for the high mortality is that melanoma cells are resistant to standard chemotherapeutic treatments [3,4]. The World Health Organization estimates that approximately 65,000 people die from melanoma worldwide each year [5]. New compounds with antineoplastic activity against melanoma are urgently needed to treat this patient population.

There is a great interest in phytochemicals for melanoma management. Phytochemicals offer fewer adverse effects, less toxicity, and lower costs than many standard chemotherapy agents [6]. The use of these phytochemicals could therefore offer opportunities for efficacious therapy for melanoma, and possibly other cancers.

Echinacea is a genus of plant native to eastern North America. It was used by Native Americans and American settlers for medicinal purposes [7]. There are 9 species of *Echinacea*, and 3 are used medicinally. These three include *Echinacea pallida, Echinacea purpurea*, and *Echinacea angustifolia. Echinacea* has mainly been used for the homeopathic treatment of infections because of its antibacterial and immune boosting properties. *Echinacea* products are currently among the bestselling homeopathic remedies in the industrialized world [8].

Previous studies with organic solvent extracts from *Echinacea* roots or flowers found that *Echinacea* contains components that can reduce colon and pancreatic cancer cell growth [9-11]. These studies suggested that polyenes [9,10] or cichoric acid [11] are biologically active cytotoxic components responsible for these effects, and are abundant in these organic solvent *Echinacea* extracts. Studies also indicate that *Echinacea* root ethyl acetate extract is significantly more cytotoxic to HeLa cells than hexane extract, suggesting that water soluble compounds may play a part in *Echinacea* anticancer effects [12]. Interestingly, the use of aqueous extracts of *Echinacea* leaves, were found to be effective in increasing antibody response to snake venom [13]. Taken together, these studies suggest that aqueous extracts of *Echinacea* may have significant biological activity. It should be noted that many anticancer agents need to be given via the intravenous route in order to achieve adequate efficacy; thus, low water solubility is a barrier to obtain an effective chemotherapeutic agent [14].

Here, we report that aqueous extracts of *Echinacea purpurea* leaves had greater inhibitory action on melanoma cells than on nontransformed fibroblasts. Furthermore, we report that aqueous *Echinacea purpurea* extract contains low molecular weight compounds (<3 kD) that inhibited melanoma cell growth. The inhibitory activity of this low molecular weight fraction was greater than unfractionated extract. We also show that aqueous *Echinacea purpurea* extracts inhibited cell growth of Src transformed cells without affecting Src kinase activity. These data suggest that *Echinacea purpurea* leaves contain low molecular weight compounds that may be useful to combat melanoma, and possibly other cancers.

Materials and Methods

Echinacea purpurea aqueous extract preparation

Dried *Echinacea purpurea* leaves were incubated with 10 volumes (w/v) of 100°C water for 20 minutes and cooled to room temperature. This 10% solution was then clarified by centrifugation, sterilized by filtration through 0.2 micron filters (Millipore), frozen, and lyophilized to dryness. The dried extract was suspended in distilled water to a final concentration of 50 mg/ml.

Extract Fractionation

To examine bioactivity of different sized components of *Echinacea*, aqueous extract was fractionated as previously described [15]. Briefly, components greater than 50 kD were concentrated by filtering over centrifugal membranes with a 50 kD nominal molecular weight pore size (EMD Millipore Amicon UFC5050). Filtrates were subsequently concentrated over centrifugal

membranes with a 3 kD nominal molecular weight pore size (EMD Millipore Amicon UFC5003) to concentrate components between 3 and 50 kD, and obtain material below 3 kD as filtrates. Sizes of the different fractions were confirmed by electrophoresis on 4-18% SDS gels stained with SilverQuest dye (Invitrogen LC6070). Concentrated material, filtrate, and unfractionated extract were diluted to achieve final concentrations equivalent to 2 mg/ml of original unfractionated extract for cell culture studies.

Evaluation of cell growth

Cell growth was analyzed as previously described [15]. Briefly, B16F10 mouse melanoma cells and nontransformed NIH3T3 cells were maintained in DMEM (Hyclone SH30021) supplemented with 25 mM HEPES (Hyclone SH3027) and FBS (Seradigm 1400-500) at 37°C in 5% CO_2 and 100% humidity [16-20]. Effects of aqueous *Echinacea* extract on cell viability were measured by plating 5,000 cells in each well, growing them overnight on standard 24 well tissue culture plates, and then treating them for 72 hours with the aqueous extract of *Echinacea*. Cells were analyzed on an inverted Zeiss Axiovert microscope and counted from images with the aid of Zeiss Axiovision software as previously described [15,18,19]. In fractionation studies, wells were trypsinized and cells removed and counted with the Coulter Counter (Beckman Coulter Z1 particle counter). LA25 cells were grown overnight at non-permissive temperature (40°C) before being incubated for 24 hours at permissive (33°C) and non-permissive (40°C) temperatures [16,17] with or without *Echinacea* extract, and then stained with Trypan blue to distinguish living and dead cells.

Western blotting

Protein was extracted from LA25 cells grown at permissive (33°C) and non-permissive (40°C) temperatures. The extracted protein was analyzed by Western blotting to detect total v-Src (Millipore 05-185), active Src (p-Src; phosphorylated at tyrosine 416) (Millipore 04-857), and ß-actin (Sigma A1978) as previously described [15-18].

Statistical analyses

Data was analyzed by one way ANOVA using GraphPad Prism V. Differences between groups were then tested using Bonferroni's post hoc test of significance and $p<0.05$ was considered significant.

Results

Aqueous *Echinacea purpurea* extract inhibits melanoma cell growth

Aqueous extracts of *Echinacea* substantially reduced B16 melanoma cell growth after 72 h. This effect was more pronounced in melanoma cells than in non-transformed NIH3T3 fibroblasts. As shown in Figure 1, all concentrations of *Echinacea* extract (1, 2, 8 mg/ml) inhibited melanoma cell growth. For example, 1 mg/ml extract inhibited growth by 86% and 8 mg/ml inhibited growth by 96%. In contrast, 1 mg/ml extract did not significantly reduce the growth of NIH3T3 cells. Only the highest concentration 8 mg/ml reduced nontransformed fibroblast growth by 69%.

Different molecular weight fractions have different biological activities on melanoma cell growth

Echinacea extract was fractionated by molecular weight, and different molecular weight fractions were added to melanoma cell cultures. All fractions significantly reduced cell growth. The largest molecular weight fraction (> 50 kD) inhibited growth by approximately 54% as shown in Figure 2. However, this level of inhibition was less than that of the unfractionated extract which reduced cell growth by 87%. Interestingly, the <3 kD fraction reduced cell growth by 97%, which was significantly more than the intact, unfractionated extract.

Echinacea extract inhibits Src transformed cell growth, but without inhibiting oncogenic Src kinase activity

We utilized LA25 cells transformed by a temperature sensitive oncogenic Src kinase construct to investigate

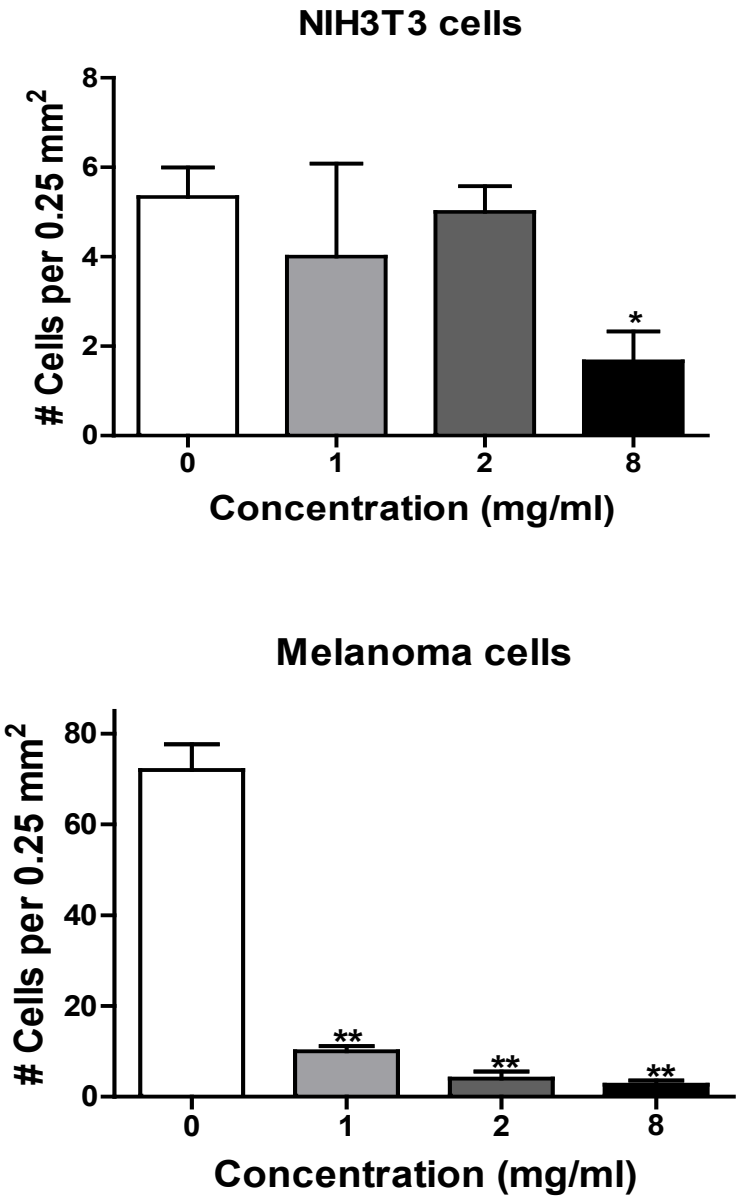

Figure 1: Aqueous Echinacea purpurea extract inhibits melanoma cell growth.

Nontransformed NIH3T3 fibroblasts and B16F10 melanoma cells (5,000 cells per well) were grown overnight before being incubated for 72 hours with indicated concentrations of *Echinacea* extract and counted in a 500×500 micron area in the center of each well. Only the highest concentration of aqueous Echinacea extract inhibited growth of NIH 3T3 fibroblasts. In contrast, all concentrations of *Echinacea* extract reduced melanoma cell (B16F10) growth. Data are presented as mean+SEM (n=3). Single and double asterisks denote $p<0.05$ and $p<0.01$ compared to untreated control cells, respectively. These data are representative of experiments that have been repeated with similar results.

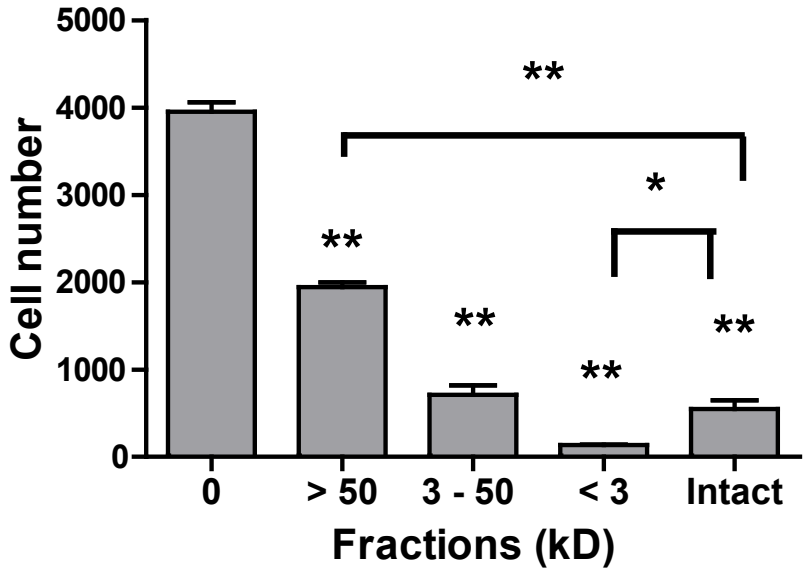

Figure 2: Different fractions of Echinacea extract inhibit melanoma cell growth.

Fractionated and unfractionated *Echinacea* extract were diluted to concentrations equivalent to 2 mg/ml of starting material and incubated with B16F10 melanoma cells for 72h. Cells were then removed from wells by trypsin and counted by Coulter counter. All fractions and unfractionated extract inhibited cell growth. The >50 kD fraction inhibited cell growth by 54%. This level of inhibition was less than that of the unfractionated extract which reduced cell growth by 87%. In addition, the <3 kD fraction reduced cell growth by a significantly greater extent than unfractionated extract (97% versus 87% respectively). Data are presented as mean+SEM (n=3). Single and double asterisks denotes $p<0.05$ and $p<0.01$ compared to untreated control cells and between groups as indicated. These data are representative of experiments have been repeated with similar results.

the effects of *Echinacea* extract on Src transformed cells and kinase activity [15,16]. *Echinacea* aqueous extract selectively inhibited Src kinase transformed cells grown at permissive temperature as shown in Figure 3a. However, this treatment did not decrease Src kinase activity of these cells as seen in Figure 3b.

Discussion

Results from this study indicate that aqueous extract of *Echinacea purpurea* can reduce melanoma cell growth. At lower concentrations (1-2 mg/ml), the aqueous extract reduced melanoma cell growth without significantly inhibiting growth of nontransformed fibroblasts. Additionally, different molecular weight fractions of *Echinacea* extract inhibited melanoma growth to different extents. Components less than 3 kD show greater inhibitory activity than the intact unfractionated extract, while fractions above 50 kD in molecular weight showed less activity than the unfractionated extract.

This study presents the novel finding that concentrations of aqueous *Echinacea* extract can selectively inhibit melanoma cell growth. These findings are consistent with previous reports of *Echinacea* components with anticancer effects [9-11]. However, this current study examined the effects of aqueous extract while previous studies examined the effects of organic solvent extracts on cell growth. In addition, the present studies examined the effect of *Echinacea* on melanoma cells while previous studies examined the

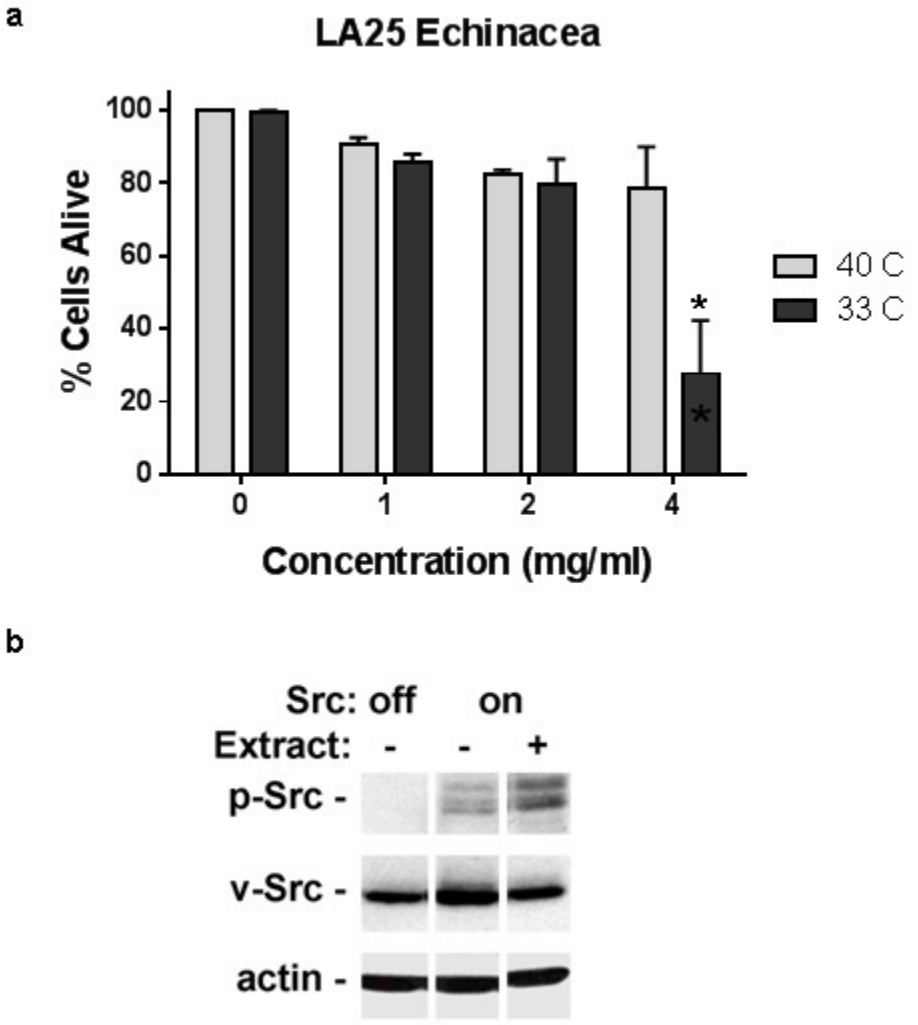

Figure 3: Effects of Echinacea extract on Src transformed cells and kinase activity.

LA25 cells were grown overnight at non-permissive temperature (40°C). Cells were then incubated at nonpermissive (40°C) or permissive (33°C) temperatures with different concentrations of aqueous *Echinacea* extract for 24 h as indicated. Cells were then stained with Trypan blue to measure cell death. **(a)** The highest concentration (4 mg/ml) of *Echinacea* extract significantly inhibited transformed cell growth at permissive temperature, but not non-permissive temperature. Data are shown as mean+SEM (n=3) with single asterisk indicating $p<0.05$. **(b)** LA25 cells grown at non-permissive and permissive temperatures were incubated with and without 4 mg/ml *Echinacea* aqueous extract. Protein was extracted and examined by western blotting to detect total v-Src, active Src (p-Src), and β-actin as indicated.

actions of *Echinacea* on other cancers including colon and pancreatic carcinoma cells [9-11].

Previous studies suggest that cichoric acid is a possible anticancer component found in *Echinacea* [11]. Although cichoric acid is soluble in water and is less than 3kD in molecular weight, it is rapidly oxidized when *Echinacea purpurea* is extracted in water [21]; therefore, it is unlikely that this compound is the active component in the aqueous extract of *Echinacea* used here. Other studies suggest that polyacteylenes and polyenes extracted by hexane are active anticancer components of *Echinacea* [10]. These agents are not very water soluble and, therefore, are also unlikely to be active agents in aqueous extracts examined here.

Our studies also found that low molecular weight (<3 kD) compounds inhibited melanoma cell growth more than the unfractionated extract. In addition, the >50 kD fraction showed a significantly lower effect than the unfractionated extract. These results suggest that fractions with larger compounds (>50 kD) may contain molecules which modulate the inhibitory properties of the lower molecular weight (<3 kD) fraction. These results are different from those of aqueous hibiscus flower extract in which unfractionated extract inhibits melanoma cell growth more than fractionated material. Thus, *Echinacea* and hibiscus appear to work by different mechanisms: *Echinacea*, contains small bioactive compounds less than 3 kD in size, while Hibiscus contains active components that work in concert for optimum effect [15].

The Src tyrosine kinase has been shown to be involved in transformation, growth, and metastasis of many types of cancer including melanoma [22-26]. Our studies indicate that *Echinacea* extract inhibited Src transformed cell growth without affecting Src kinase activity in these cells. These data suggest that *Echinacea* extract reduces growth by a mechanism that is downstream or independent of Src kinase activity.

These studies suggest that aqueous *Echinacea purpurea* extract contains compounds that may be used as agents to prevent or treat melanoma, and possibly other cancers. Interestingly, low molecular weight compounds (<3 kD) inhibited transformed cell growth to a greater extent than unfractionated extract. Future investigations may be performed to identify these specific bioactive compounds and elucidate their modes of action, as well as their efficacy *in vivo*.

Acknowledgements

This work was funded in part by support from the Osteopathic Heritage Foundation and the Graduate School of Biomedical Science at Rowan University-School of Osteopathic Medicine.

Conflict of interest

The authors declare no conflict of interest.

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