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Template version : 6.7
Revised: 12/21/2011

Article : MPA21552
Creator : matombocon
Date : Tuesday February 14th 2012
Time : 14:35:19
Number of Pages (including this page) : 11

Copyeditor: Aleli L. Anacay

Selective Induction of Apoptosis and Autophagy Through Treatment With Dandelion Root Extract in Human Pancreatic Cancer Cells

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Objectives: Pancreatic cancer has a 100% mortality rate; the aim of this study is to evaluate the efficacy of dandelion root extract (DRE) in inducing apoptosis and autophagy in aggressive and resistant pancreatic cancer cells.

Methods: The effect of DRE was evaluated using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay. Apoptotic cell death was confirmed by nuclear condensation by Hoechst staining and externalization of phosphatidylserine to the outer leaflet of the plasma membrane by Annexin-V binding assay. Loss of mitochondrial membrane potential was observed using the JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide) dye. The induction of autophagy was detected using a monodansylcadaverine assay and this was confirmed by immunofluorescence for light chain 3-II.

Results: BxPC-3 and PANC-1 pancreatic cells were sensitive to aqueous DRE. This extract induces selective apoptosis in a dose- and time-dependent manner. Dandelion root extract caused the collapse of the mitochondrial membrane potential, leading to prodeath autophagy. Normal human fetal fibroblasts were resistant at similar doses.

Conclusions: We demonstrate that DRE has the potential to induce apoptosis and autophagy in human pancreatic cancer cells with no significant effect on noncancerous cells. This will provide a basis on which further research in cancer treatment through DRE can be executed.

Key Words: pancreatic cancer, dandelion root extract, apoptosis, autophagy

Abbreviations: DRE - dandelion root extract, LC3-II - light chain 3-II, MDC - monodansylcadaverine

(*Pancreas* 2012;00: 00–00)

In Canada, it is estimated that approximately 173,800 new cases of cancer will be diagnosed and 76,200 Canadians will die of cancer in 2010.[1] The risk of developing this disease increases with age; nonetheless, it can affect all ages. Pancreatic cancer, a very aggressive and highly resistant form of cancer, is a cancer that originates in the pancreas.[1] It is the fourth leading cause of cancer-related deaths in the world today.[2] In 2010, it was estimated that 4000 new cases would be diagnosed and 3900 deaths will be caused by pancreatic cancer in Canada. Treatment of pancreatic cancers includes the already-known forms of chemotherapeutics such as 5-fluorouracil and gemcitabine,[3] surgery, and radiation therapy. Although they show initial efficacy, these treatments do not remain effective for extended periods and are usually accompanied by severe adverse events.[4,5] Furthermore, in the early stages of pancreatic cancer, there are no clear signs or symptoms.[6] Depending on the location of the tumor in the pancreas, signs and symptoms begin to appear once surrounding tissues become affected, and surgical resection of the tumor is only successful if the disease is diagnosed in its early stages when the cancer has not metastasized.[2] Early prognosis is therefore one of the leading areas of research in this field because of its difficult identification.[1] Most commonly, pancreatic cancer is diagnosed in very late stages, and survival is dismal at best. With one of the highest-mortality-to-incidence-rate ratios,[7] there is an important need to introduce a safe and effective mode of targeting both early and late stage pancreatic cancers.

One of the most common hallmarks of cancer involves the ability to evade the physiological process of programmed cell death, also known as apoptosis.[8,9] Cancer cells have also been found to use autophagy, a catabolic process used to degrade cytoplasmic materials, as a prosurvival technique to overcome stressors such as starvation and chemotherapy.[10] There has been a lot of controversy surrounding autophagy, where it has been shown to play dual roles, as both a prosurvival and prodeath phenomenon.[10] Recent studies have shown that prodeath autophagy and apoptosis have interconnected pathways and can be regulated by the same proteins, including the proapoptotic Bcl-2 family of proteins.[11] It is therefore necessary to investigate these prodeath pathways so as to harness their properties for targeting various types of cancers.

In current cancer therapies, natural compounds such as paclitaxel (Taxol) and Navelbine have been widely used. However, many of these compounds are genotoxic or nonselective and therefore cause damage to normal cells as well.[12] Dandelions are very common weeds found in almost every part of the world,[13] the leaves and root of which have been studied for their effects on digestion and gastrointestinal diseases.[14] Recent investigations have shown that dandelion root extract (DRE) has the ability to selectively induce apoptosis in human melanoma and leukemia cells, with no toxicity to noncancerous peripheral blood mononuclear cells.[15,16] Whether DRE can induce programmed cell death in highly aggressive and resistant human pancreatic carcinoma cell lines remains unknown. In this study, we show the efficacy of DRE in inducing apoptosis in a dose- and time-dependent manner in aggressive human pancreatic cell lines (BxPC-3 and PANC-1). In parallel, similar experiments in noncancerous normal human fetal fibroblasts indicate that DRE selectively targets human pancreatic cancer cells, confirming results from previous studies.[15,16] Early activation of caspase-8 and subsequent activation of caspase-3 indicate that apoptosis induction by DRE is caused by activation of the extrinsic pathway

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Received for publication June 10, 2011; accepted January 6, 2012.
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This study was supported by the Knights of Columbus Chapter 9671 (Windsor, Ontario) and Seeds4Hope grant from Windsor and Essex County Cancer Centre Foundation.
The authors declare no conflict of interest.

of apoptosis. Interestingly, we observed that DRE induced a prodeath form of autophagy in human pancreatic cancer cells. This induction of autophagy corresponds with the destabilization of the mitochondrial membrane potential, which was observed after treatment with DRE. Through revival experiments, it was shown that the signal to commit suicide was retained once the cells had been exposed to DRE. Although we are unsure of the active ingredients in DRE responsible for the induction of cell death, our work provides novel evidence of the selective anticancer effects of DRE in human pancreatic cancer cells.

MATERIALS AND METHODS

Dandelion Root Extraction and Cell Treatment

Dandelion root extract was prepared from the roots of collected dandelion weeds found in localized open grasslands. After repeated washing with water, the roots were air-dried and ground to a fine powder. The powder was then homogenized in 200 mL of distilled water and filtered at room temperate using NITEX nylon mesh filters (LAB PAK; Sefar BDH Inc, Chicoutimi, Quebec, Canada). The filtrate was then spun down at 8000*g* at 25°C for 5 minutes, and the supernatant was filtered by 0.45 and 0.22 μm filters. The final filtrate was lyophilized, and 100-mg/mL stock aqueous extract was prepared.

Cell Lines and Cell Culture Maintenance

The cancer cell line used in this study was human pancreatic cancer BxPC-3 and PANC-1, which were purchased from the American Type Culture Collection, Manassas, Va. The normal cell lines used were normal human fibroblast (NHF) cells, which were obtained from Coriell Institute for Medical Research, Camden, NJ. BxPC-3 cells were grown in RPMI-1640 media supplemented with 15% fetal bovine serum (FBS) and 10-mg/mL gentamicin (Gibco BRL, VWR, Mississauga, Ontario, Canada). The NHFs were cultured in Dulbecco modified eagle medium obtained from Sigma Chemical Company, Mississauga, Ontario, Canada, supplemented with 10% FBS, 2-mM L-glutamine, and 10-mg/mL gentamicin (Gibco BRL, VWR). PANC-1 cells were grown in Dulbecco modified eagle medium supplemented with 15% FBS and 10-mg/mL gentamicin (Gibco BRL, VWR). All cell lines were grown and maintained at 37°C, 95% humidity, and 5% CO_2 in a Forma Scientific CO_2 incubator equipped with a HEPA filter (Forma Scientific Inc, Marietta, Ohio). All cell lines were grown to approximately 50% confluence and were treated with either fresh DRE or lyophilized extract (0.5–7.5 mg/mL). Treated cells were allowed to grow over time up to 96 hours after treatment and were examined using several staining assays as described below.

Cell Staining

Hoechst Staining

To visualize apoptosis in the cells, a photosensitive DNA-binding stain called Hoechst 33342 (Sigma Chemical Company) was incubated with a final concentration of 10 μM with cells at room temperature for approximately 10 minutes. After incubation, the cells were viewed with a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany) and Northern Eclipse version 8.0 Imaging software at 100× and 400× objective. Apoptotic cells are characterized by brightly stained, condensed nuclei as compared to larger, round, and less-brightly stained nonapoptotic cells. The average percent apoptosis was calculated by counting and averaging the number of brightly condensed nuclei over the total number of cells observed over 5 fields at 400× objective.

Annexin-V Binding Assay

An early marker of apoptosis is the flipping of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane, which can be visualized through the use of an Annexin-V binding assay. Cells treated with various doses of DRE were scraped using a rubber policeman to removed adherent cells from the plate bottom and washed twice in phosphate-buffered saline (PBS). After washing with PBS, the cellular pellet was resuspended in Annexin-V binding buffer (10-mM HEPES, 10-mM NaOH, pH 7.5, 140-mM NaCl, 2.5-mM $CaCl_2$, and 50-nM sucrose), along with the Annexin-V Alexa Fluor 488 conjugate (Sigma Chemical Company), which binds to phosphatidylserine, at 1:50 with respect to the buffer. This was allowed to incubate in low-light conditions at room temperature for approximately 15 minutes. Hoechst 33342 dye was also incubated with the cells for the final 10 minutes to visualize nuclear morphology of positively stained Annexin-V cells. The cells were then visualized and images were taken using a fluorescence microscope (Leica DM IRB) at 400× objective.

Trypan Blue Exclusion Assay

To quantify the number of viable pancreatic cancer cells, trypan blue staining was used to visualize the cells. Trypan blue is a dye, which stains only dead cells whose plasma membranes are permeable to the dye. A 1:1 mixture of cell suspension and 0.4% trypan blue dye (Sigma Chemical Company) was loaded onto a hemocytometer (Hausser Scientific, Horsham, Pa), where nonviable blue-stained cells and viable unstained cells were counted. The average number of viable cells was expressed as number of cells per milliliter.

Cell Viability Assay

To determine cell viability, a colorimetric dye called WST-1 ([2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, Roche Diagnostics, Mannheim, Germany) was used. In metabolically active, viable cells, WST-1 produces formazan, which can be measured for absorbance. First, a trypan blue count was done to seed equal number of cells into 96-well plates. Approximately 5000 cells were seeded with a total volume of 200 μL in each well and treated following attachment of the cells overnight. After treatment, WST-1 dye was added to each well (20 μL per 200 μL) and incubated at 37°C for 4 hours. Absorbance was read at 590 nm with a Victor Plate Reader (Wallac, Turku, Finland). Absorbance values of the treated cells were calculated as a percentage of absorbance values of control.

Mitochondrial Membrane Potential

To visualize mitochondrial membrane permeability in BxPC cells, JC-1 dye (5,5′,6, 6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide, Sigma Chemical Company) was incubated with treated cells. JC-1 forms aggregates in mitochondria with intact membrane potential. The dye was diluted 2:50 in PBS to a concentration of 0.5 μM and was incubated with treated cells for 45 minutes at 37°C. During the final 10 minutes of incubation, Hoechst dye was added to the cells. After incubation, the cells were then visualized and images were taken using a fluorescence microscope at 400× objective. Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR) was used for PANC-1 cells to measure mitochondrial membrane potential. PANC-1 cells were grown on coverslips. After treatment period, the cells were incubated for 45 minutes with 200-nM TMRM at 37°C. The coverslips were then placed on microscope slide and pictures were taken at 40× objective using inverted fluorescence microscope (Leica DM IRB).

MDC Assay

To detect autophagy, a monodansylcadaverine (MDC, Sigma Chemical Company) assay was preformed. Monodansylcadaverine is a fluorescent compound that is incorporated into autophagic vacuoles and produces a bright punctate stain. Propidium iodide (PI) is used as a co-stain with MDC to visualize dead cells. First, 100-μM MDC is diluted 1:25 in PBS and added to the cells along with PI, then incubated at 37°C for 15 minutes. After incubation, the cells were visualized and images were taken using a fluorescence microscope at 400× objective.

Immunofluorescence

BxPC-3 cells were plated on poly-L-lysine-coated coverslips and treated with DRE for 48 hours to stimulate the induction of autophagy. Treated cells were fixed in cold methanol, then cold acetone for 5 seconds and allowed to air-dry. After fixation, the cells on the coverslips were incubated with PBS containing 0.05% Tween 20 and 5% normal goat serum for 10 minutes. Cells were then incubated with anti–light chain 3 (LC3) antibody (1:500 dilution) (Novus Biologicals, Cat. No. NB100–2220, Littleton, Colo), overnight at 4°C. On the following day, the cells were subjected to two 5-minute washes with PBS containing 0.05% Tween 20 and then incubated with anti-rabbit antibody, Alexa Fluor 488 conjugate (1:1000) (Cell Signaling Technologies, Cat. No. 4412, Pickering, Ontario, Canada), for an hour at room temperature. After 2 washes with PBS-Tween 20 for 5 minutes, cells were counterstained with Hoechst for 10 minutes and visualized and images were taken using a fluorescence microscope at 400× objective.

Caspase Activity

The caspase assays were performed using a previously published method.[17] To determine caspase activity, the total protein from BxPC-3 and PANC-1 cell lysates were incubated with the fluorogenic substrates corresponding to the substrate cleavage site, specific for each caspase, DEVD-AFC for caspase-3

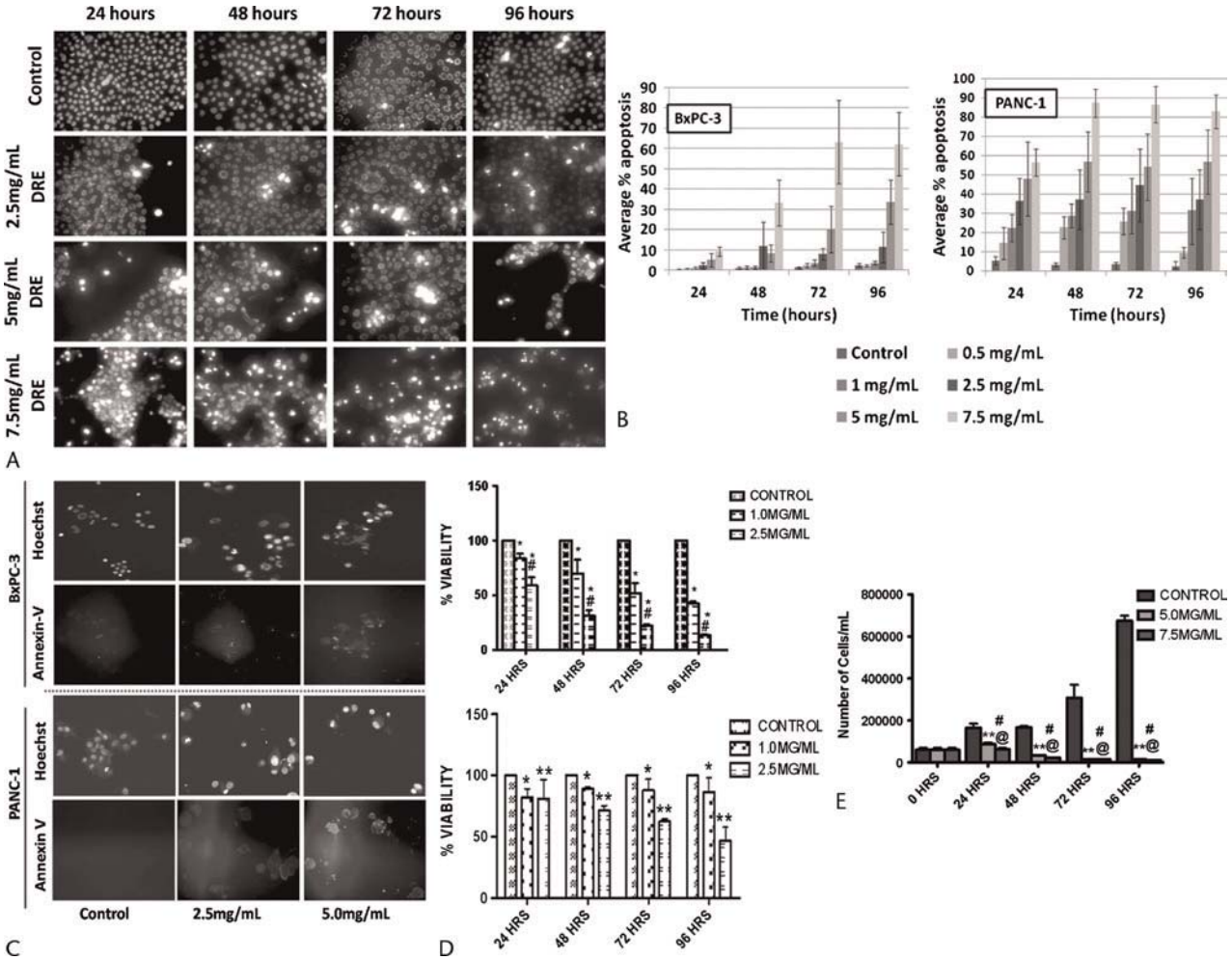

FIGURE 1. Efficacy of DRE in inducing apoptosis in human pancreatic cancer cells. A, An increase in brightly stained, condensed nuclei indicative of apoptosis is seen with increasing doses and periods following treatment with DRE. B, Manual quantification of Hoechst pictures. In comparison to the results seen in (A), there is an increase in the average % apoptosis in a dose- and time-dependent manner. C, Brightly stained apoptotic cells seen in Hoechst images are visualized by Annexin-V binding to exposed phosphatidylserine in the lower panel of images in both BxPC-3 and PANC-1 cells. D, Treatment with DRE led to a drastic decrease in the percent of viable cells after treatment at the indicated time points; (i) BxPC-3 ($^{*}P < 0.05$; $^{*\#}P < 0.0001$) and (ii) PANC-1 ($^{*}P < 0.05$; $^{**}P < 0.0001$) hours with DRE. E, Exposure to of BxPC-3 cells to DRE for 48 hours halted the cell growth following removal of treatment. Cells were unable to revive growth after exposure to DRE and retained the signal to commit suicide after removal of DRE (**, $^{\#@}P < 0.0001$).

and IETD-AFC for caspase-8 and -9. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm using a Spectra Max Gemini XS (Molecular Devices, Sunnyvale, Calif). Caspase activity was calculated as activity per microgram protein, and protein concentration was determined with BioRad protein assay reagent (BioRad, Mississauga, Ontario, Canada) using bovine serum albumin as a standard. Readings were analyzed using GraphPad Prism 5.0 288 software.

Statistical Analysis

All experiments were performed in triplicates and the results expressed as mean (SD). Statistical analysis was performed with GraphPad Prism 5.0 288 software.

RESULTS

Effect of DRE on Highly Aggressive Pancreatic Cancer Cells

To determine the effect of DRE on pancreatic cancer cells, 2 different pancreatic cancer cell lines (BxPC-3 and PANC-1) were treated with DRE at doses of 0.5, 1, 2.5, 5, and 7.5 mg/mL and examined at different time points. To visualize morphological features of apoptosis induction in BxPC-3 and PANC-1 following DRE treatment, Hoechst, a dye that binds to the minor groove of DNA, was used to observe nuclear condensation. An increase in nuclear condensation was observed with an increase in concentration, as well as an increase in the time of exposure to DRE (Fig. 1A). Apoptotic cells and nonapoptotic cells were manually counted and quantified as average percentage apoptosis in both cell lines. The average percent apoptosis was found to increase with increasing concentration of DRE over time (Fig. 1B).

An early marker of apoptosis is the reorganization of the cell's membrane, which leads to externalization of the phosphatidylserine from the inner leaflet to the outer leaflet of the cell membrane. This can be observed with Annexin-V binding assay. After 72-hour treatment with DRE, there is an increase in bright green fluorescence indicative of apoptosis in a dose- and time-dependent manner in DRE-treated BxPC-3 cells. Annexin-V positive staining was observed as early as 24 hours in DRE-treated PANC-1 cells (Fig. 1C).

Quantitative cell viability as a function of cell metabolism was assessed through WST-1 assay. Dandelion root extract led to a 60% decrease in cell viability 48 hours following treatment with only 20% of BxPC-3 cells remaining viable after 96 hours of exposure to DRE. Similar results were observed in PANC-1 cells; with a gradual decrease in cell viability with an increase in dose of DRE compared to untreated control cells (Fig. 1D).

According to results of both qualitative and quantitative analyses, the EC_{50} for both pancreatic cancer cell lines is around 5.0 mg/mL.

To investigate the fate of cells exposed to DRE but do not show apoptotic morphology following treatment for 48 hours (40%), BxPC-3 cells were plated and treated for 48 hours at 5.0- and 7.5-mg/mL DRE. After 48 hours, equal numbers of BxPC-3 cells were seeded and allowed to grow in drug-free media, with growth observed every 24 hours by trypan blue exclusion assay. The untreated control cells were able to maintain constant growth over time. However, the DRE-treated cells were unable to revive growth in fresh media as time progressed. Analysis reveals a significant decrease in the number of cells per milliliter at 96 hours after treatment from 700,000 to 10,000 cells between the untreated control cells and the cells treated with DRE, respectively (Fig. 1E). Similar results were observed for the PANC-1 cell line following revival after treatment with DRE for 48 hours (data not shown). These results indicate that DRE led to the loss of cell viability and effectively induced apoptosis in human pancreatic cancer cells in a dose- and time-dependent manner. More importantly, cells that had been exposed to DRE retained the signal to commit suicide even after the removal of DRE.

Repeated Doses of DRE at Lower Concentrations Is More Effective Than a Single Low/High Dose in Inducing Apoptosis in Human Pancreatic Cancer Cells

Any treatment at high doses can be toxic and as can be observed from our previous results in Figure 1, higher doses of DRE were required to induce apoptosis in both pancreatic cancer cell lines. Treatment of these cells with repeated doses of low concentrations of DRE showed a higher efficacy than 1 single high dose. Exposure of BxPC-3 (Fig. 2A) and PANC-1 (Fig. 2B)

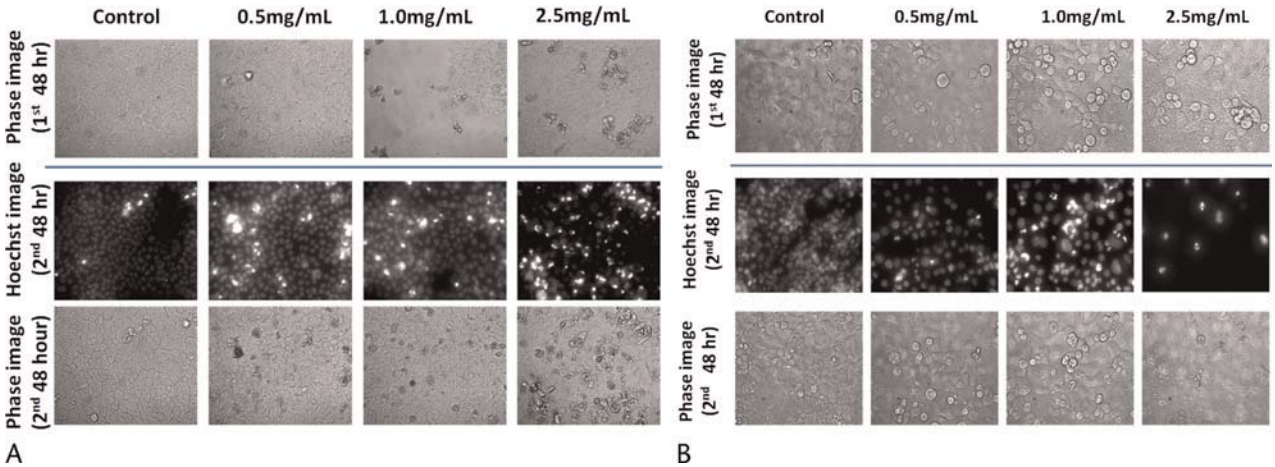

FIGURE 2. Repeated doses of DRE at lower concentrations in human pancreatic cancer cells. Treating BxPC-3 (A) and PANC-1 (B) twice for 48 hours at doses of 0.5, 1, and 2.5 mg/mL has a significantly higher effect than a single treatment for 48 hours. The proportion of apoptotic cells at 2.5 mg/mL following a second 48-hour treatment as compared to control reveals that multiple low doses of DRE are more effective at killing the pancreatic cancer cells than a single high dose.

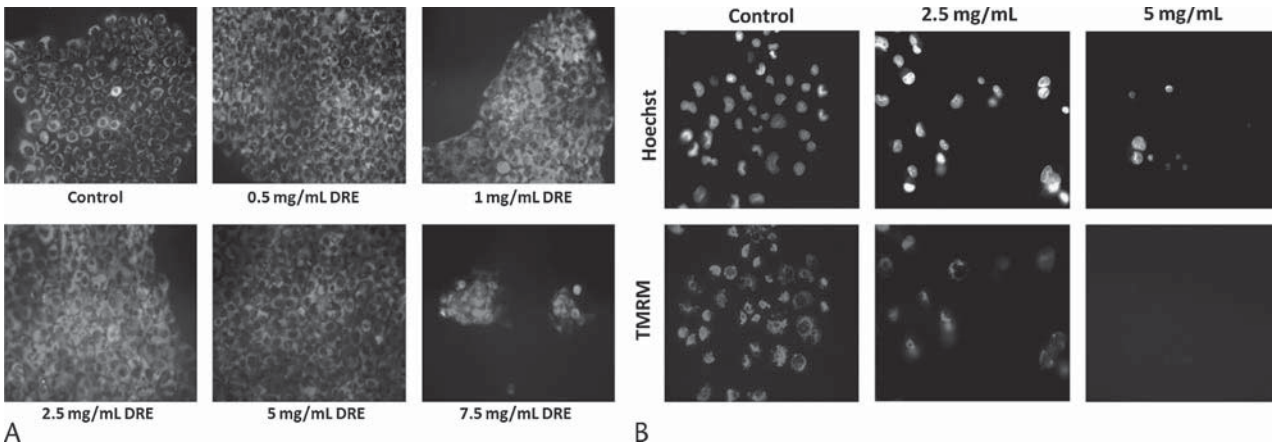

FIGURE 3. Dandelion root extract destabilized the mitochondrial membrane potential of human pancreatic cancer cells. Pancreatic cancer cells were grown on coverslips and treated with DRE for 48 hours and stained with either JC-1 (A) or TMRM (B), which are mitochondrial permeable dyes that aggregate in healthy mitochondria and fluoresce red. The loss of dye aggregation is observed in DRE-treated BxPC-3 (A) and PANC-1 (B) cells.

to low concentration (0.5, 1.0, and 2.5 mg/mL) of DRE for 48 hours and then a second treatment for another 48 hours reveals that 2 treatments are more effective than treating once with a high dose. Comparing the control and 2.5 mg/mL treated phase images shows a drastic difference in the number of dead cells following the second 48-hour treatment (Fig. 2). These results indicate multiple doses of low concentrations of DRE are more effective in inducing apoptosis than 1 single high dose in human pancreatic cancer cells.

DRE Destabilizes the Mitochondrial Membrane Potential in Human Pancreatic Cancer Cell Lines

To determine the mechanism of action of DRE, we wanted to observe the effect of DRE on the mitochondria of human pancreatic cancer cells. Both the extrinsic and intrinsic pathways of apoptosis converge on and permeabilize the mitochondria leading to the release of proapoptotic factors.[18] Evaluation of mitochondrial membrane potential is done through visualization of red fluorescence of JC-1 or TMRM mitochondria permeable dyes in healthy, intact mitochondria. Dandelion root extract led to the destabilization of the mitochondrial membrane potential in BxPC-3 cells as observed by the loss of red aggregates of JC-1 dye in DRE-treated cells after 48 hours of treatment (Fig. 3A). F3 Similar results were observed in DRE-treated PANC-1 cells, stained with TMRM (Fig. 3B). These results indicate that the mitochondria of highly aggressive pancreatic cells are vulnerable to DRE, leading to permeabilization of the mitochondrial membrane potential, characteristic of apoptosis.

DRE Activates the Death Receptor-Mediated Extrinsic Pathway of Apoptosis

The loss of mitochondrial membrane potential in itself does not provide us with information regarding the pathway of apoptosis induction. To get more information on the pathway of

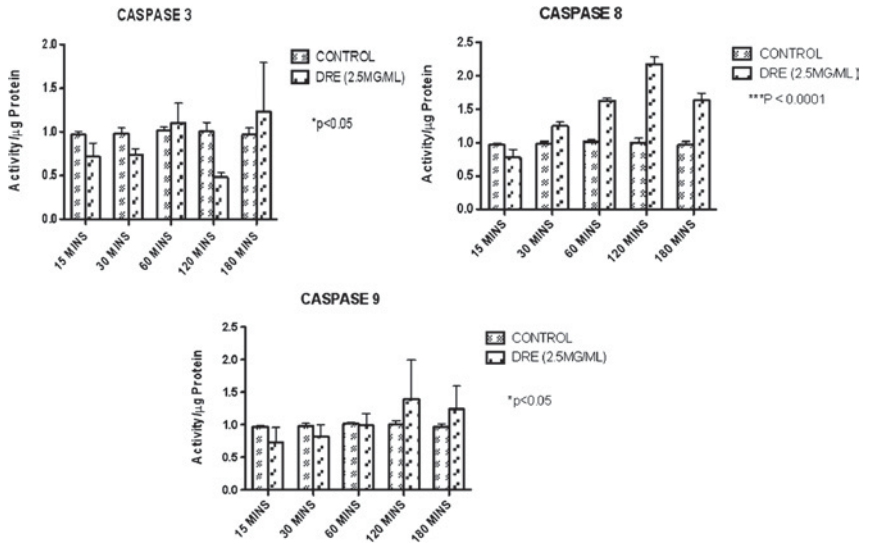

FIGURE 4. Dandelion root extract induces extrinsic apoptosis in human pancreatic cancer cells. BxPC-3 cells were treated with DRE at the indicated time points. Subsequently, cells were collected, washed, and incubated with lysis buffer to obtain cell lysate. The cell lysate was incubated with caspase substrates, specific to each caspase (3, 8, and 9) and incubated for an hour. Fluorescence readings were obtained using a spectrofluorometer. An average of 6 readings per well and a minimum of 3 wells were run per experiment. The results here are reported as activity per microgram of protein (in fold), and the average of 3 experiments are shown.

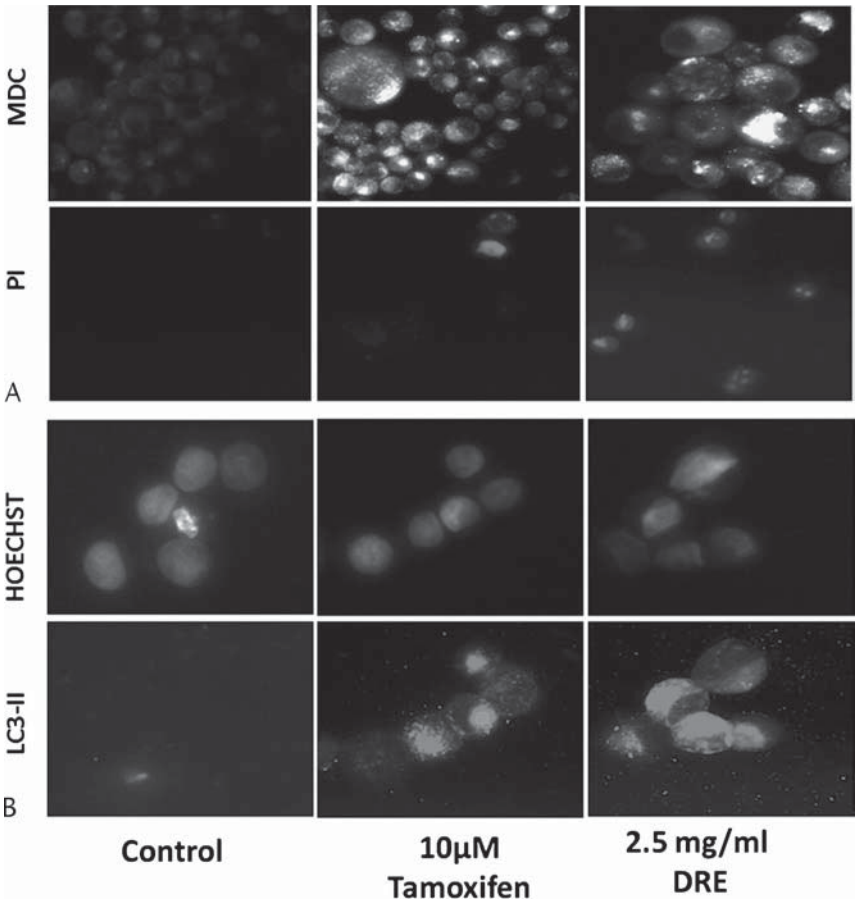

FIGURE 5. Dandelion root extract induces prodeath autophagy in human pancreatic cancer cells. A, BxPC-3 cells were seeded onto coverslips in 6-well plates and treated with DRE at 2.5 mg/mL for 48 hours. Tamoxifen is a known inducer of prosurvival autophagy and was used as a positive control for autophagic induction in pancreatic cancer cells. Following treatment, cells were stained with MDC and counterstained with PI to indicate the dead cells. B, BxPC-3 cells were plated on coverslips and treated with DRE or tamoxifen for 48 hours. After treatment, coverslips were incubated with primary antibody specific to LC3-II overnight at 4°C, then incubated with appropriate secondary antibody for an hour at room temperature. The cells were counterstained with Hoechst and imaged.

apoptosis induction, which would shed more light on the mechanism of induction of apoptosis by DRE, we analyzed the chronological activation of caspases. BxPC-3 cells were treated with 2.5-mg/mL DRE, at the indicated time points, and subsequently analyzed for the activation of caspases, using substrates specific to each type of caspase. Our results show that there is a very rapid activation of caspase-8 in BxPC-3 cells (Fig. 4), suggesting an extrinsic mode of cell death. Similar results were observed in PANC-1 cells.

DRE Induces Prodeath Autophagy in Human Pancreatic Cells

Along with apoptosis, autophagy is a physiological process of cell death involved in the maintenance of cellular homeostasis.[19] Cells undergoing autophagy form autophagic vacuoles, which can be visualized through the use of MDC stain.[20] The corresponding PI stains reveals which of the autophagic cells are dead. These dead cells signify that the autophagy occurring is a prodeath form of autophagy. Tamoxifen is known to induce prosurvival autophagy[21] and therefore is used as a positive control. We observed a clear induction of autophagy in the treated cells at increasing doses of DRE over time as compared to the positive tamoxifen control in BxPC-3 cells (Fig. 5A). When autophagy is induced, microtubule-associated protein 1 LC3-I, usually localized in the cytosol, is conjugated to phosphatidylethanolamine. This conjugation results in the lipidated protein LC3-II that is recruited to autophagosomal membranes and can be used as another marker for autophagy detection.[22] To confirm the results of autophagy seen by MDC staining, immunocytochemical analysis was used to detect the conversion of LC3-I to LC3-II. BxPC-3 treated with DRE at 2.5 mg/mL had a similar effect on the conversion of LC3-I to LC3-II, compared to the tamoxifen-treated cells. Both the tamoxifen- and DRE-treated cells incubated with antibody against LC3-II gave positive results, which confirmed the autophagy observed in Fig. 5A (Fig. 5B). Treated PANC-1 cells with DRE doses of 2.5 mg/mL showed similar results.

DRE Is Not Toxic to Noncancerous NHFs

Dandelions have been used as an herbal medicine for centuries, and there has been no reported evidence of toxicity.[23] To further investigate whether DRE selectively targets cancerous cells, NHFs were treated with DRE under the same conditions as the pancreatic cancer cells. Treatment of noncancerous NHFs with DRE at doses of 1.0, 2.5, and 5 mg/mL for 96 hours reveals no morphological signs or characteristic of apoptosis as seen by Hoechst staining (Fig. 6 top panel). In comparison to pancreatic cancer cells treated with DRE, NHFs retain their viability in the

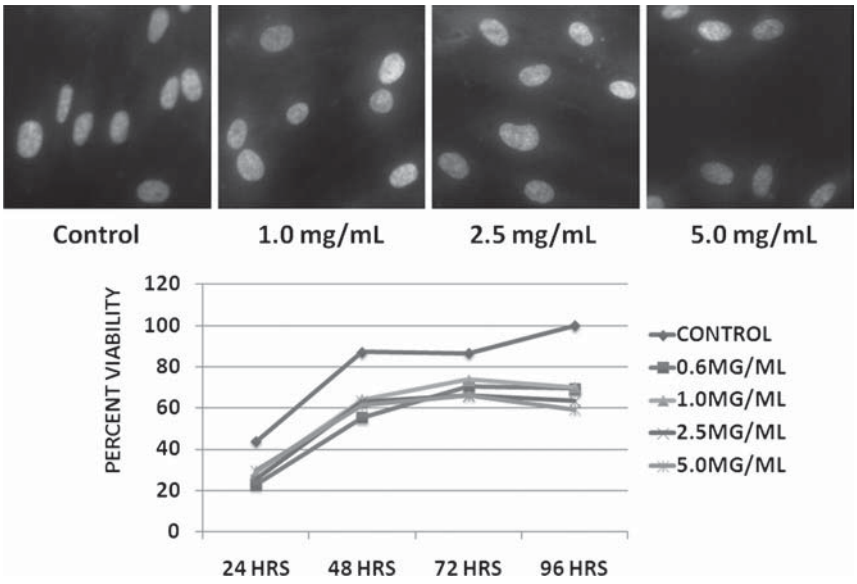

FIGURE 6. Dandelion root extract has selective toxicity to human pancreatic cancer cells. Normal human fibroblasts were treated with DRE at increasing concentrations for 96 hours. The cells were then stained with Hoechst dye for nuclear morphology. No nuclear condensation, characteristic of apoptosis, is observed in NHF-treated cells (top panel). WST-1 cell viability assay was also used to monitor the viability of NHFs after treatment with DRE (bottom panel).

presence of DRE as seen through WST-1 cell viability assay (Fig. 6 bottom panel). However, the treated cells are not as viable as the untreated control cells and plateaus at approximately 70% cell viability. The noncancerous NHF cells do not undergo apoptosis in the presence of DRE. These results reveal that DRE is nontoxic to noncancerous cells and selectively targets cancerous cells.

DISCUSSION

Natural compounds have shown significant anticancer activity, with reports showing their potential to inhibit the progression of several cancers by interfering with several key mechanisms employed by cancer cells.[24] In this study, we have shown that the root extract of the very common weed, dandelions, has the ability to selectively induce apoptosis and autophagy in very aggressive human pancreatic cancer cells.

The high mortality rate of pancreatic cancer patients is most likely due to the fact that the disease is usually asymptomatic until it metastasizes and becomes invasive, at which point it is incurable.[25,26] Therefore, a majority of pancreatic cancer patients presented in this state of advanced and/or metastatic condition are inoperable.[26] A lot of chemotherapeutic agents have been tested for their efficacy in treating this disease but only a few of them have shown moderate activity and produce very little survival benefit and severe adverse effects.[27]

We observed that the exposure of pancreatic cancer cells to DRE led to the loss of cell viability followed by the induction of apoptosis in a dose- and time-dependent manner. More importantly, we looked at the effect of initial exposure to DRE on the ability of these cells to revive growth. After treatment for 48 hours, cells were removed from the treated media and replated in fresh, drug-free media and allowed to grow. We observed that this initial exposure to DRE halted the ability of the cells to revive growth, indicating that these aggressive pancreatic cells retained the signal to commit suicide, induced by DRE (Fig. 1). Over the ages, people have relied on dandelions for therapeutic benefits in the treatment of various diseases ranging from diarrhea and other gastrointestinal diseases to more serious diseases, like hepatitis. To date, there has been no report of toxicity linked to usage of dandelion extracts.[14,28] Previous studies in our laboratory have shown that DRE is nontoxic to noncancerous peripheral blood mononuclear cells obtained from healthy volunteers.[15] To confirm these results, as well as corroborate what has been seen for centuries, we tested DRE on noncancerous normal fetal fibroblasts in parallel experiments. As expected, there was no significant effect on the viability of normal fetal fibroblasts. These cells did not show apoptotic morphology, characterized by nuclear condensation and fragmentation (Fig. 6). These results indicate that DRE is specific in its targeting of human cancer cells, without affecting the normal cells tested.

It is very well known that a lot of chemotherapies have very severe adverse effects, mostly because these drugs are not specific to cancer cells and are sometimes only effective at very high doses. Also, cancer cells, especially pancreatic cancer cells, develop resistance to conventional chemotherapies.[26] As mentioned earlier, the EC_{50} for DRE in highly aggressive pancreatic cancer cells is 5.0 mg/mL. Interestingly, we found that repeated treatment of pancreatic cancer cells with lower doses of DRE led to a greater induction of apoptosis than the single high dose (Fig. 2A, B). This might indicate that the active ingredient in the extract are not stable or effective for long periods in the cells, and a second dose may be required to effectively induce apoptosis in the cells that remain after the first treatment. In this way, repeated treatment with low dose may overcome the aggressiveness and resistance of these cells to DRE. Although DRE has shown no significant toxicity to noncancerous cells, these results indicate that giving a repeated dose of DRE at lower concentrations is more effective as well as reducing the chance of adverse effects (if any) seen in high-dose chemotherapies.

Not many studies have been done to elucidate the mechanism of action of natural extracts, specifically DRE, for their efficacy for disease treatment. We have previously shown that DRE targets the death receptor-mediated pathway of apoptosis

in human leukemia[16] and aggressive human melanoma cells.[15] Previously, pancreatic cancer cells have been shown to express Fas and TRAIL receptors.[29,30] We hypothesize that some components of DRE may imitate death ligands and might interact with the death receptors, activating the extrinsic pathway of apoptosis. In this study, we also confirmed this by observing the activation of caspases after DRE treatment (Fig. 4). These results corroborate the previous results obtained. Both the extrinsic and intrinsic pathway of apoptosis converges on the mitochondria.[31] The activation of both pathways leads to the permeabilization of the mitochondrial membrane for the release of proapoptotic factors such as apoptosis-inducing factor, cytochrome *c*, and endonuclease G, which are involved in the execution of apoptosis.[31] In this study, we observe the loss of mitochondrial membrane potential after caspase-8 activation, following treatment with DRE (Fig. 3A, B). This loss was seen as early as 48 hours, before we see the bulk of apoptotic cells, indicating that DRE may indirectly involve mitochondria destabilization in pancreatic cancer cells for the execution of apoptosis. Mitochondrial destabilization could lead to increased reactive oxygen species.[31]

Autophagy could be induced by the presence of such dysfunctional mitochondria, a process of "self-eating" activated under conditions of starvation and stress. Under these conditions, the cells attempt to deal with stress (such as damaged organelles and proteins) by engulfing and degrading these organelles and proteins and recycling the materials obtained from the degradation. Prolonged exposure to stressors lead to prolonged degradation of organelles and proteins eventually leading to cell death caused by autophagy.[10] There are also studies that have shown that molecular pathways involved in programmed cell death type I (apoptosis) and type II (autophagy) are interconnected, and proteins involved in 1 pathway could be involved in the other.[11] Indeed, we observed the induction of autophagy by DRE in both pancreatic cancer cells, concurrent with mitochondrial membrane destabilization (Fig. 5).

We therefore hypothesize that components found in DRE activate the death receptor-mediated extrinsic pathway of apoptosis, which involves the early activation of caspase-8, followed by activation of the executioner caspase-3 as well as cleavage of BID and mitochondrial destabilization. Damaged mitochondria and the generation of reactive oxygen species could therefore lead to the induction of prodeath autophagy, coupled with apoptosis for the cell death we observe 96 hours after exposure of highly aggressive and resistant human pancreatic cancer cells to DRE.

At this point of the study, we are aware of some limitations to this work. The active ingredient responsible for the anticancer activity in DRE has not yet been identified; although natural products are usually considered to be complex botanicals and activity is not caused by a single active ingredient.[32] It is possible that the crude aqueous DRE contains multiple compounds working together in unison to promote its selective anticancer activity. We have standardized a protocol for extraction and after every extraction process, we assay for activity of the extract. The different batches of root extracted have shown similar activity throughout. More importantly, preliminary HPLC analysis of our extract has shown the presence of compounds (triterpenes and sesquiterpenes) previously reported to be present in dandelions (data not shown). Because natural aqueous DRE has been used as a traditional medicine for other ailments and is not associated with any toxicity, it represents a safer potential form of therapy for pancreatic cancer treatment. In an attempt to ensure that our experiments are well controlled, we have standardized an extraction procedure so as to ensure that we have approximately equal amounts of extracts in each treatment. Enrichment of the apoptosis-inducing fraction of DRE, characterization of compounds in this fraction, and evaluating its efficacy in an in vivo model of pancreatic cancer are the obvious next steps currently in progress in our laboratory.

ACKNOWLEDGMENT

The authors thank Ms Sabrina Ma for editing and proofreading this article.This article is dedicated to the memory of Kevin Couvillon, who lost his fight to cancer in 2010.

REFERENCES

1. Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics. Toronto: Canadian Cancer Society; 2010.
2. Castellanos EH, Cardin DB, Berlin JD. Treatment of early-stage pancreatic cancer. *Oncology.* 2011;25(2):182–189.
3. Skinner J. Adjuvant 5-FU/folinic acid and gemcitabine equally effective in pancreatic cancer. *Oncology.* 2010;19(10):1–3.
4. Kindler HL, Niedzwiecki D, Hollis D, et al. Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). *J Clin Oncol*. 2010;28(22):3617–3622.
5. Poplin E, Feng Y, Berlin J, et al. Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group. *J Clin Oncol*. 2009;27(23):3778–2785.
6. Tuveson D, Hanahan D. Cancer lessons from mice to humans. *Nature*. 2011;471:316–317.
7. Tempero MA, Berlin J, Ducreux M, et al. Pancreatic cancer treatment and research: an international expert panel discussion. *Ann Oncol*. 2011;22(7):1500–1506.
8. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*. 2002;108:153–164.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.
10. Rosenfeldt MT, Ryan KM. The multiple roles of autophagy in cancer. *Carcinogenesis*. 2011;32(7):955–963.
11. Thorburn A. Apoptosis and autophagy: regulatory connections between two supposedly different processes. *Apoptosis*. 2008;13:1–9.
12. Mukherjee AK, Basu S, Sarkar N, et al. Advances in cancer therapy with plant based natural products. *Curr Med Chem*. 2001;8(12): 1467–1486.
13. Koo HN, Hong SH, Song BK, et al. *Taraxacum officinale* induces cytotoxicity through TNF-α and IL-1α secretion in Hep G2 cells. *Life Sci*. 2004;74:1149–1157.
14. Yarnell E, Abascal K. Dandelion (*Taraxacum officinale* and *T. mongolicum*). *Integr Med*. 2009;8(2):35–38.
15. Chatterjee SJ, Ovadje P, Mousa M, et al. The efficacy of dandelion root extract in inducing apoptosis in drug-resistant human melanoma cells. *Evid Based Complement Alternat Med*. 2011:1–11.
16. Ovadje P, Chatterjee S, Griffin C, et al. Selective induction of apoptosis through activation of caspase-8 in human leukemia cells (Jurkat) by dandelion root extract. *J Ethnopharmacol*. 2011;133:86–91.
17. Naderi J, Hung M, Pandey S. Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: resistance of quiescent cells to oxidative stress. *Apoptosis*. 2003;8(1):91–100.
18. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med*. 2005;258(6):479–517.
19. Gottlieb RA, Carreira RS. Autophagy in health and disease: 5. Mitophagy as a way of life. *Am J Physiol Cell Physiol*. 2010;299:C203–C210.
20. Niemann A, Takatsuki A, Elsässer HP. The lysosomotropic agent monodansylcadaverine also acts as a solvent polarity probe. *J Histochem Cytochem*. 2000;48(2):251–258.

21. Qadir MA, Kwok B, Dragowska WH, et al. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Res Treat*. 2008; 112(3):389–403.
22. Ma D, Tremblay P, Mahngar K, et al. A novel synthetic C-1 analogue of 7-deoxypancratistatin induces apoptosis in p53 positive and negative human colorectal cancer cells by targeting the mitochondria: enhancement of activity by tamoxifen. *Invest New Drugs*. 2011; 29(2):(Epub ahead of print).
23. Kemper KJ. Dandelion (*Taraxacum officinalis*). The Longwood Herbal Task Force (http://www.mcp.edu/herbal/default.htm); The Center for Holistic Pediatric Education and Research. 1999;1–11.
24. Boik J. *Natural Compounds in Cancer Therapy.* 1st ed. Oregon Medical Press; 2000.
25. Koorstra JM, Hustinx SR, Offerhaus G, et al. Pancreatic carcinogenesis. *Pancreatology*. 2008;8:110–125.
26. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol*. 2008;3:157–188.
27. Longo R, Cacciamani F, Naso G, et al. Pancreatic cancer: from molecular signature to target therapy. *Crit Rev Oncol Hematol*. 2008;68:197–211.
28. Sigstedt SC, Hooten CJ, Callewaert MC, et al. Evaluation of aqueous extracts of *Taraxacum officinale* on growth and invasion of breast and prostate cancer cells. *Int J Oncol*. 2008;32(5): 1085–1090.
29. Hasel C, Rau B, Perner S, et al. Differential and mutually exclusive expression of CD95 and CD95 ligand in epithelia of normal pancreas and chronic pancreatitis. *Lab Invest*. 2001;81(3):317–326.
30. Siegmund D, Klose S, Zhou D, et al. Role of caspases in CD95L- and TRAIL-induced non-apoptotic signalling in pancreatic tumour cells. *Cell Signal*. 2007;19(6):1172–1184.
31. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006;25:4798–4811.
32. Foster BC, Arnason JT, Briggs CJ. Natural health products and drug disposition. *Annu Rev Pharmacol Toxicol*. 2005;45: 203–226.

AQ9

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