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Vol: 1, Issue: 1

Journal of Pharma and Pharmaceutical Sciences

Juniper Extracts Induce Calcium signalling and Apoptosis in Neuroblastoma cells

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Article Type: Research, **Submission Date**: 12 March 2015, **Accepted Date:** 23 April 2015, **Published Date:** 22 May 2015.

Citation: Ingo Lange Ph.D, Julia Moschny, Vugar N Kerimov Ph.D, Manana Khutsishvili Ph.D, Daniel E Atha B.Sc, et al. (2015) Juniper Extracts Induce Calcium signalling and Apoptosis in Neuroblastoma cells. J.Pharm Pharm Scien 1(1): 5-11. doi: https://doi.org/10.24218/vjpps.2015.03.

Abstract

Despite the advances in modern medicine, effective neuroblastoma (NB) treatments are still limited and we are in need of new therapeutic strategies. In the current study, a library of 500 plant extracts was screened for anti-proliferative and cytotoxic effects in neuroblastoma cell lines. Cytotoxicity and induction of intracellular calcium signaling were determined in NB cells. Fluo4-AM (acetoxymethyl) staining and fluorescence microscopy were used while concurrently performing Sulforhodamine B (SRB) staining on NB cells for the screening of aqueous and organic extracts from plants. In addition to changes in NB cell viability (SRB) and intracellular free calcium (Fluo-4), mitochondrial calcium, and opening of the mitochondrial permeability transition pore (MPTP) were monitored. Finally, whole cell lysates were analyzed via western blot for cleavage of caspase 3 and PARP, markers for apoptosis.

Juniper has been widely used throughout history and employed by numerous cultures as food and medicine. In the current study, three dichloromethane extracts from *Juniperus oblonga* were identified as containing highly potent agents that significantly reduced cell survival and increased intracellular calcium in NB cells. Further analysis revealed that cell death induced by these extracts was associated with mitochondrial calcium overload, opening of the MPTP, and caspase 3- and PARP-cleavage in NB cells, suggesting that these extracts induced apoptosis via the mitochondrial pathway. Therefore, agents from *Juniperus oblonga* have the potential to lead to new chemotherapeutic drugs for NB.

Key words: Calcium; Channel; Neuroblastoma; Apoptosis.

Introduction

Neuroblastoma (NB) is an extra-cranial pediatric cancer. The options of effective neuroblastoma treatment are still limited, despite the advances in modern medicine, and we are in need for new therapeutic strategies [1-3]. The pool of potent chemotherapeutic drugs often diminishes during the course of treatment due to the development of drug resistance mechanisms [4]. As much as 50% of NB patients diagnosed with high-risk disease will be refractory to treatment or experience relapse after treatment [5]. As many drugs used in cancer chemotherapy is plant-derived or analogs based on naturally occurring compounds, a library of 500 plant extracts was screened for anti-proliferative and cytotoxic effects in neuroblastoma cell lines in order to identify new active substances for chemotherapy. After screening the library, we were able to identify three extracts from *Juniperus oblonga* as highly potent agents that significantly reduced cell survival and induced an increase of intracellular calcium in NB cells.

Junipers are coniferous shrub-like plants of the cypress family. There are 68 known species and 36 different varieties of juniper that are known. *Juniperus oblonga* is one of the most widespread juniper species and have been used throughout nearly every culture as food, medicine, for practical and gustatory purposes [6,7]. In the current study, three extracts from different parts of *Juniperus oblonga* were found to have potent anti-cancer effects. Current literature lack data on the cellular signalling pathways, in particular calcium and apoptosis signalling, regulated by *Juniperus* extracts. Therefore, the *Juniperus oblonga* extracts were further examined to elucidate their anti-cancer effects. Calcium is a second messenger that is involved in various cellular signaling pathways that control many cellular functions, including tumor progression and apoptosis. Therefore, we used Fluo4-AM staining and fluorescence microscopy while concurrently performing Sulforhodamine B (SRB) staining on NB cells in order to determine the effect of these extracts on intracellular calcium signaling and cell viability, respectively. Three dichloromethane extracts of *Juniperus oblonga* significantly increased intracellular

free calcium levels and reduced NB cell viability. The IC_{50} for extracts from the roots, leaves & fruits, and stems were 8.3, 3.9, and 4.0 µg/mL respectively. In addition, the mitochondrial transition pore assay and western blot analysis revealed that cell death is associated with mitochondrial calcium overload, opening of the MPTP, and Caspase-3 and PARP cleavage in NB cells. The results from the current study suggest that extracts derived from *Juniperus oblonga* effectively kill NB cells by inducing apoptosis via the mitochondrial pathway. Further purification is necessary to isolate and identify the active ingredient so it can be tested in vitro and in animal models for its suitability for clinical use. This study creates a need to understand the cellular impact of these extracts as an alternative strategy for treating NB, and could lead to the development of more effective chemotherapy for the treatment of NB.

Methods

Cell culture

MYCN2 cells, a tetracycline inducible MycN over-expression NB cell line was authenticated by by the cell Line Authentication testing Services at Genetica DNA laboratories (USA) using STR DNA typing to verify each cell line and verify pure cells (no contamination). The cells were maintained in RPMI-1640 (Mediatech, Inc., Manassas, VA, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), and grown at 37°C, 5% CO_2, in a 95% humidity.

Chemicals

General chemicals were from VWR (West Chester, PA). Doxorubicin and ionomycin were from Calbiochem (Gibbstown, NJ). The Library of extracts, which included the *Junipers oblonga* extracts, was obtained from Dr. Robert Borris.

Preparation of Juniperus extracts

Samples of the roots , stems, leaves and fruit of *Juniperus oblonga* M.Bieb (Cupressaceae) were individually collected near Kish Village in the Sheki District of Azerbaijan in September 2006. Herbarium samples documenting the collection (Kerimov 57) have been deposited in the herbaria of the Institute of Botany, Azerbaijan National Academy of Sciences, and the New York Botanical Garden. Fresh samples were air dried and then milled to a coarse powder. A 1 kg (dry weight) portion of each sample was extracted with methanol (3 x 4L) and the solvent removed *in vacuo* to afford viscous oil. The resulting oil was dispersed in 1L of methanol : water (9:1) and extracted with n-hexane (3 x 1L). The depleted hydroalcoholic phase was freed of methanol, dispersed in distilled water (1L) and extracted sequentially with dichloromethane (3 x 1L) and water-saturated n-butanol (3 x 1L). The resulting solvent-soluble fractions were individually evaporated to dryness *in vacuo*, while the residual aqueous phase was freed of solvent and then lyophilized. Extracts and fractions were maintained at -20°C until needed for use.

Calcium assay

MYCN2 cells were washed and incubated with 1 µM Fluo-4 AM, the acetoxymethyl ester form of Fluo-4 (Molecular Probes, Eugene, OR, USA), for 30 minutes at 37°C in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 2 (or 0), $MgCl_2$ 2, glucose 10, Hepes·NaOH 10, pH 7.4, 330 mOsm. For nominally calcium free experiments 1mM EGTA was added to the external solution and calcium chloride was omitted. Cells were transferred to 96-well plates at 10,000 cells/well and stimulated as indicated. Epifluorescent measurements were performed using an Operetta High Content Imaging System (PerkinElmer, Santa Clara, CA, USA). Fluorescence intensity was quantified using Harmony (PerkinElmer, Santa Clara, CA, USA).

Fluorescence measurements

MYCN2 cells were incubated in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 2 (or 0), $MgCl_2$ 2, glucose 10, Hepes·NaOH 10, pH 7.4, 330 mOsm. For nominally calcium free experiments, 1mM EGTA was added to the external solution and calcium chloride was omitted. Cells were loaded with fura-2 AM, the acetoxymethyl ester form of fura-2 (Molecular Probes, Eugene, OR, USA). Cells were perfused with external solutions containing *Juniperus oblonga* extract, and cytosolic calcium was measured in individual cells using a Zeiss microscope and monochromatic light source tuned to excite fura-2 fluorescence at 360 and 390 nm for 20 milliseconds each. Emission was detedted at 450-550 nm using a photomultiplier.

Sulforhodamine B assay

The SRB colorimetric assay was used to determine cell proliferation following the protocol previously described [8]. Briefly, cells were seeded at a density of 10,000 cells/ well on a transparent, flat-bottom, 96-well plate and allowed to settle overnight. At the initiation of each experiment (t = 0), and after drug treatments, 100 µL of 10% (w/v) TCA were added to each well, incubated for 1 h at 4°C, washed with deionized water, and dried at room temperature. One hundred microliters of 0.057% (w/v) SRB solution were added to each well, incubated for 30 min at room temperature, rinsed four times with 1% (v/v) acetic acid, and allowed to dry at room temperature. Finally, 200 µL of 10 mM Tris base solution (pH 10.5) were added to each well, and after shaking for 5 min at room temperature, the absorbance was measured at 510 nm in a microplate reader. The absorbance at t = 0 was compared with the absorbance at the end of the experiment to determine cell growth in treated cells compared with control cells.

Mitochondrial calcium assay

Treated and untreated NB cells were washed twice in modified Hanks' Balanced Salt Solution (HBSS: sodium bicarbonate, calcium, and magnesium that also included 10 mM HEPES, 2 mM L-glutamine and 100 µM succinate), then labeled with 1.0 µM calcein AM, 200 nM MitoTracker Red CMXRos, 1 µM Hoechst 33342 dye and 1.0 µM $CoCl_2$. Cells were incubated for 15 minutes at 37°C, 5% CO_2, in 95% humidity, then washed in modified HBSS. 1 µM Ionomycin was used as a positive control for calcium-mediated pore opening. Epifluorescent measurements were performed using an Operetta High Content Imaging System (PerkinElmer, Santa Clara, CA, USA). Fluorescence

intensity was quantified using Harmony software (PerkinElmer, Santa Clara, CA, USA).

Western Blot Analysis

Cell lysates were prepared in radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 7.5), 0.1% (w/v) sodium lauryl sulfate, 0.5% (w/v) sodium deoxycholate, 135 mmol/L NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mmol/L EDTA] supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals) and phosphatase inhibitors sodium fluoride (20 mmol/L) and sodium vanadate (0.27 mmol/L). Western blot analysis was done as previously described (20). The total protein concentration was determined using the protein assay dye reagent from Bio-Rad Laboratories. Cell lysates in SDS-sample buffer were boiled for 5 min and equal amounts of total protein were analyzed by 10% SDS-PAGE and Western blotting. The antibodies used in this study are mouse monoclonal p53 (1:250) from Santa Cruz Biotechnology; rabbit polyclonal cleaved caspase-3 (1:1,000), rabbit polyclonal cleaved PARP (1:1,000), mouse monoclonal GAPDH (1:1,000), and mouse monoclonal PCNA (1:1000) from Cell Signaling Technology. Proteins were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NB, USA) and analyzed with Licor Image Studio 2.0 acquisition and analysis software.

Analysis

Results are shown as the mean ± standard deviation. Statistical significance was determined based on a two-way analysis of variance (Student's t-test). Adjacent to data points in the respective graphs, significant differences were recorded as follows: single asterisk, $p < 0.05$; double asterisk, $p < 0.01$; triple asterisk, $p < 0.001$; no symbol, $p > 0.05$. SRB and FLuo-4 experiments are all *n* of at least 3, in triplicates. All other experiments are all *n* of at least 3.

Results

Juniperus oblonga extracts reduce NB cell viability

To examine the anti-cancer effects of the library of 500 plant extracts, NB cells were treated with the extracts or left untreated, and the cell viability was measured using the SRB assay. The results clearly show that the dichloromethane extracts of *Juniperus oblonga* roots (Rt), leaves and flowers (Lv/Fl) and stems (St) significantly decreased cell viability, compared to untreated MYCN2 cells. In NB cells with non-amplified *MYCN* gene (NAM), Rt, Lv/Fl, and St (at a concentration of 8ug/mL) reduced cell viability of NB cells by 98.8%, 98.9% and 98.6%, respectively, compared to the viability of untreated control cells (Figure 1). In NB cells with over-expression of MycN (MOE), Rt, Lv/Fl, and St extracts reduced cell viability by 99.1%, 98.7% and 98.9%, respectively (Figure 1). The cytotoxic effects were more potent than that of doxorubicin, which reduced cell viability of NAM and MOE by 78.3% and 86.3%, respectively (Figure 1). The IC_{50} of these extracts were determined using the SRB assay. The IC_{50} of Rt, Lv/Fl, and St in NAM cells were 8.3 µg/mL, 3.9 µg/mL and 4.0 µg/mL, respectively (Table 1). The IC_{50} of Rt, Lv/Fl, and St in MOE cells were 7.9 µg/mL, 3.4 µg/mL and 3.2 µg/mL, respectively (Table 1). The data suggests that the dichloromethane extracts from *Juniperus oblonga* roots (Rt), leaf and flowers (Lv/Fl) and stems (St) exhibits cytotoxic effects on NB cells.

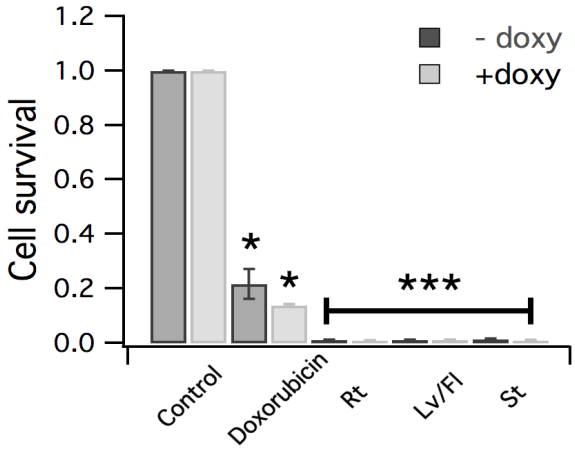

Figure 1: *Juniperus oblonga* extracts reduce NB cell viability. Cell proliferation was assessed using Sulforhodamine B (SRB) assay in 96 well formats. MYCN2 cells were grown with and without induction of MYCN overexpression for 96 hours. Cells were treated with 500 nM doxorubicin and 8ug/mL of extracts (Rt, Lv/Fl, St) for 48 hours. Growth inhibition was normalized to untreated cells. The experiments are all *n* = 3-4, in triplicates. *, $p < 0.05$; ***$p < 0.001$; no symbol, $p > 0.05$.

Table 1: IC_{50} of dichloromethane extracts from Juniper Rt, Lv/Fl and St in NAM and MOE NB cells.

IC50	Rt	Lv/Fl	St
NAM	8.3 µg/mL	3.9 µg/mL	4 µg/mL
MOE	7.9 µg/mL	3.4 µg/mL	3.2 µg/mL

Juniperus oblonga extracts increase intracellular calcium in NB cells

Calcium signalling plays a role in many fundamental cellular processes, including cell proliferation, motility and survival. Importantly, aberrant calcium signalling may lead to mitochondrial calcium overload, opening of the MPTP and apoptosis. Therefore, in order to determine the effect of the *Juniperus oblonga* extracts on calcium, intracellular free calcium was measured in NB cells treated with *Juniperus oblonga* extracts and compared to untreated cells. NB cells were treated with Rt, Lv/Fl, and St extracts for 24 hours. Cells were loaded with Fluo-4, and epifluorescent measurements were performed using an Operetta High Content Imaging System, in order to determine the intracellular calcium levels in these cells. Figure 2A shows that there was a 63, 51 and 49 fold increase in intracellular free calcium when NAM NB cells were exposed to Rt, Lv/Fl, and St extracts, respectively, compared to untreated control. This was a significant increase in intracellular free calcium, as doxorubicin increased intracellular by 2.8 fold. In addition, there was a 71, 55 and 90 fold increase in intracellular free calcium when MOE NB cells were exposed to Rt, Lv/Fl, and St extracts, respectively, compared to untreated control. This was a significant increase in intracellular free calcium, as doxorubicin increased intracellular by 3.1 fold. Further examination of the calcium inducing effects of these extracts showed that external application of extracts to MOE NB cells increased intracellular free calcium after a slight

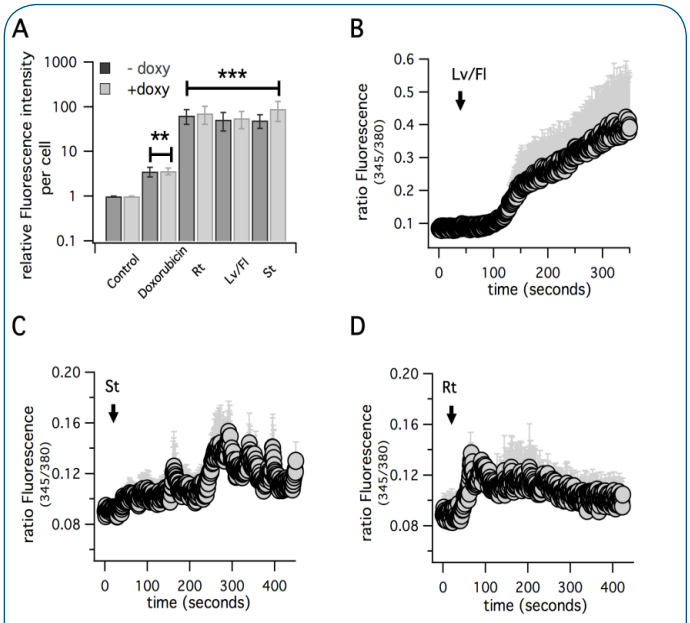

Figure 2: *Juniperus oblonga* extracts increase intracellular calcium in NB cells. MYCN2 cells were grown with and without induction of MYCN overexpression for 96 hours. The last 24 hours of the experiment cells were treated with 500 nM doxorubicin and 8ug/mL of extracts (Rt, Lv/Fl, St). Global intracellular calcium levels intensity was measured using Fluo-4-am and Perkin Elmer Operetta and normalized per cell (A). For time resolved acquisition of calcium signaling MYCN2 cells were loaded with Fura-2-am. Extracts (Rt, Lv/Fl, St) at 8ug/mL in ringer solution was applied at 40 seconds into experiment indicated by black arrow (B-D). The experiments are all $n = 3$-4, in triplicates. **, $p < 0.01$; ***, $p < 0.001$; no symbol, $p > 0.05$.

delay of approximately 30-45 seconds, whereas no signal was induced by vehicle control (Figure 2B, C and D). Interestingly, extracts Rt and St induced calcium oscillations (Figure 2B and D), while Lv/Fl induced a marked increase in calcium followed by a sustained increase in calcium levels (Figure 2C). These results clearly show that the *juniperus* dichlormethane extracts had significant and distinct effects on calcium signalling in NB cells.

Juniperus oblonga extracts induce mitochondrial calcium and opening of MPTP in NB cells

To examine the effect of the *juniperus* dichloromethane extracts on mitochondrial calcium, mitochondria were stained with calcein AM and mitochondrial dye, and epifluorescent measurements were performed using an Operetta High Content Imaging System. The calcium levels in the mitochondria were quantified using the Harmony software. Figure 3 shows that treatment of NAM cells with Rt, Lv/Fl, and St increased mitochondrial calcium levels by ~62.5, ~37.1 and ~39.0 fold, respectively, compared to control. Ionomycin increased mitochondrial calcium levels by 1.6 fold compared to control. In addition, treatment of MOE cels with Rt, Lv/Fl, and St increased mitochondrial calcium levels by 39.4, 64.6 and 27.9 fold, respectively, compared to control. Ionomycin increased mitochondrial calcium levels by 1.9 fold compared to control. Interestingly, the mitochondrial calcium levels were consistently greater in treated and untreated MOE cells compared to NAM cells. These results suggest that the *juniperus* extracts increase intracellular calcium which induces mitochondrial calcium overload.

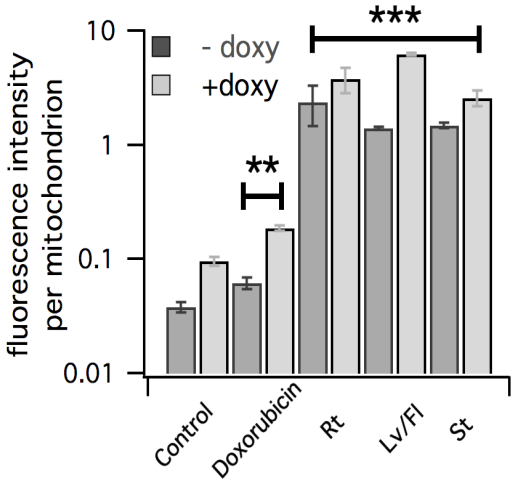

Figure 3: *Juniperus oblonga* extracts induce mitochondrial calcium and opening of MPTP in NB cells. MYCN2 cells were grown with and without induction of MYCN overexpression for 96 hours. The last 24 hours of the experiment cells were treated with 500 nM doxorubicin and 8ug/mL of extracts (Rt, Lv/Fl, St). Mitochondrial calcium levels were measured using MitoTracker red. Mitochondrial calcium levels were normalized through single cells recognition using Hoechst 33324 nuclear stain. The experiments are all $n = 3$, in triplicates. **, $p < 0.01$; ***, $p < 0.001$; no symbol, $p > 0.05$.

Juniperus oblonga extracts induce apoptosis in NB cells

To determine whether the *juniperus* extracts indeed induced mitochondrial calcium overload and subsequently promoted apoptosis, whole cell lysates were prepared from NB cells treated with the extracts or left untreated, and the lysates were analysed by western blot for detection of apoptotic markers, cleaved caspase 3 and cleaved PARP. Figure 4A and B shows that Rt, Lv/Fl, and St increases cleavage of caspase 3 and PARP. However, there was no effect on total caspase expression. In addition, there was no effect on proliferation marker, proliferating cell nuclear antigen (PCNA). The band density in each lane of the western blot was quantified, and the level of cleaved caspase 3 and cleaved PARP were normalized to GAPDH expression. The quantification of cleaved caspase 3 showed that the Rt, Lv/Fl, and St extracts significantly increased caspase 3 cleavage by 8.3, 8.8 and 9.3 fold, respectively, compared to control (Figure 4C). The quantification of cleaved PARP showed that the Rt, Lv/Fl, and St extracts significantly increased PARP cleavage by 14.4, 12.9 and 12.8 fold, respectively, compared to control (Figure 4D). These results suggest that the dichloromethane juniperus extracts induce mitochondrial calcium overload, opening of the mitochondrial permeability transition pore and promotes apoptosis.

Discussion and Conclusion

Junipers are coniferous shrub-like plants of the cypress family that have been used in nearly every culture as food, medicine, for practical and gustatory purposes. *Juniperus oblonga* is one of the most widespread juniper species [6]. However, the medicinal properties of this juniper species have not been examined in neuroblastoma. In the current study, three extracts from different parts of *Juniperus oblonga* were examined and found to have potent anti-cancer effects in neuroblastoma cells. The

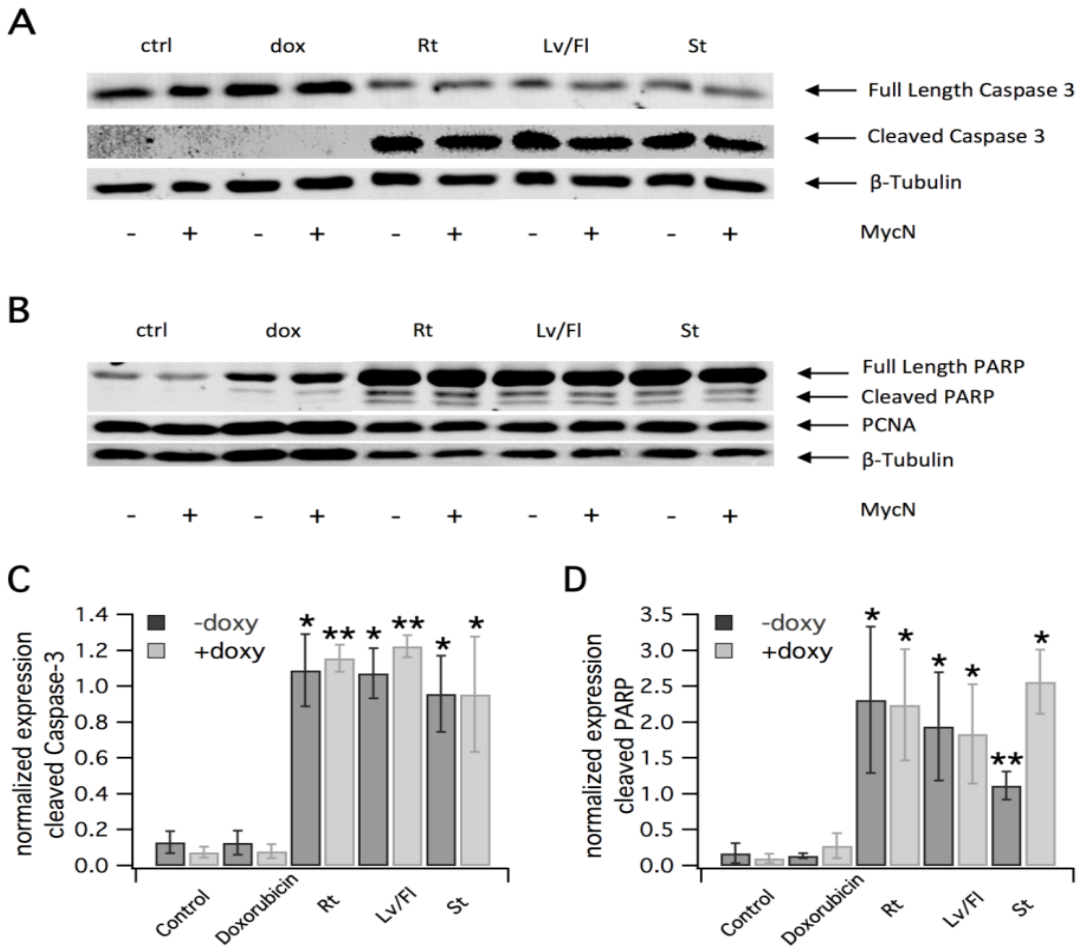

Figure 4: *Juniperus oblonga* extracts induce apoptosis in NB cells. MYCN2 cells were grown with and without induction of MYCN overexpression for 96 hours and were treated the last 24 hours of the experiment with 500 nM doxorubicin and 8ug/mL of extracts (Rt, Lv/Fl, St). Whole cell lysates were analyzed by western blot to determine protein level of cleavage of caspase-3 (A) and PARP (B) as well as PCNA. For quantification of caspase-3 protein levels were normalized on beta-tubulin levels (C). For quantification of PARP cleavage fragments were normalized on beta-tubulin and cleaved PARP fragment was normalized on beta-tubulin (D). The experiments are all $n = 3$. *, $p < 0.05$; **, $p < 0.01$; no symbol, $p > 0.05$.

juniperus dichloromethane extracts significantly decreased cell viability of NB cells with and without MycN over-expression. MycN is a transcription factor and *MYCN* gene amplification is a negative prognostic indicator of NB. *MYCN* amplification is associated with high-risk and advanced stage NB and poor prognosis, as well as relapsed and refractory disease [2,9]. In addition, MYCN amplification may alter the anti-cancer effects of chemotherapeutic drugs and/or confer drug resistance [10]. Identification of alternative treatments with selectivity for *MYCN* amplified NB or NB cells with MycN over-expression would be beneficial for the treatment of advanced stage, high-risk, and relapsed or refractory NB. Therefore, this study examined the effect of the Juniper extracts in NB cells with and without MycN over-expression. Interestingly, the IC_{50} of the extracts were lower in NB cells MycN over-expression compared to non-amplified MYCN (Table 1). This suggests that components of the extracts are effective in NB cells regardless of MycN status. Only ~25-30% of NB patients present with *MYCN* amplification but these patients often do not respond adequately to chemotherapy. Therefore, the anti-cancer effect of extracts in NB also has clinical relevance, as there may be components in the extracts that could also treat all NB patients, with and without *MYCN* amplification.

The Juniperus extracts also altered intracellular calcium regulation. Interestingly, application of the extracts induced immediate changes in intracellular calcium. Rt and St extracts exhibited calcium levels that oscillated between 0.1-0.16 (fluorescence 345/380). The calcium oscillations induced by Rt and St suggests that these extracts may alter intracellular signalling via intracellular calcium stores (e.g. endoplasmic reticulum, mitochondria). However, unlike the St extract, the Rt extract induced a rapid calcium release within seconds after application of the extract, while St extract exhibited low amplitude calcium oscillations that slowly increased in amplitude. Interestingly, Lv/Fl extracts induced a slow increase in calcium which was initiated more than 50 seconds after the application of the extract, followed by a sustained increase that peaked at about 0.4 (fluorescence 345/380). The variations in calcium signalling induced by these extracts suggest that the roots, stems and leaves and flowers of the juniper may contain different components that induce these distinct effects on calcium regulation. Interestingly, while the root extract had the highest IC_{50} of the dichloromethane Juniper extracts, it had a greater effect on intracellular free calcium levels, mitochondria calcium and PARP cleavage. The root extract may contain different components than the other extracts, and the calcium release induced by the root extract, which was not observed in the Lv/Fl and St extracts, may initiate a different cascade of events that leads to higher mitochondria calcium and

more PARP cleavage. Interestingly, full-length PARP expression increases with doxorubicin and the dichloromethane Juniper extracts. Doxorubicin appears to induce caspase independent cell death, as shown by the lack of caspase cleavage, less PARP cleavage but increased PARP expression [11]. It is clear that the dichloromethane Juniper extracts contain multiple components, and some of the components may induce caspase dependent cell death as shown by the increase in caspase 3 cleavage and PARP cleavage. However, there may be some components in the extracts that also induce caspase-independent cell death, as there is an increase in full-length PARP expression [11]. Therefore, these extracts may induce their anti-cancer effects through both caspase-dependent and –independent mechanisms.

The effect of the Juniper extracts on calcium regulation also altered mitochondrial calcium to similar degrees. Regardless of the initial effect on calcium mobilization, calcium oscillations or sustained increase of calcium, the extracts eventually increased mitochondrial calcium levels. The western blot analysis showed that this increase in mitochondrial calcium consequently induced apoptosis as shown by the increase in both cleaved caspase 3 and cleaved PARP, apoptosis markers. However, the extracts did not affect proliferation, as levels of the [12] proliferation marker PCNA were unchanged when cells were treated with the extracts. Interestingly, the effects of the extracts on mitochondrial calcium and induction of apoptosis were similar in both NAM and MOE NB cells. Interestingly, mitochondria associated ER membranes facilitate the transfer of calcium from the ER to mitochondria, which is important for normal mitochondrial function. In addition to ATP production and lipid metabolism, mitochondria protect tumor cells by acting as a calcium sink to buffer increases in cytosolic calcium [13]. However, excessive calcium signaling at mitochondria associated ER membranes may facilitate mitochondrial calcium overload and opening of mitochondrial permeability transition pore (mPTP) [14-16]. The Fura-2 calcium measurements and mitochondria calcium measurements indicate that there may be multiple calcium signalling events occurring that may be involved in juniper-induced cell death. ER-mitochondria calcium signalling may play a role in this process.

Podophyllotoxin was previously extracted from *Juniperus communis*, and was shown to exhibit potent anti-cancer effects and induce apoptosis in cancer cells by inhibiting microtubule formation [17-19]. However, there are over 62 *Juniperus* species, and at least 12 species have been shown to contain podophyllotoxin [20]. However, there are no studies that show podophyllotoxin in *Juniperus oblonga*. In addition, the extraction method suggests that podophyllotoxin is not the active component in the Juniperus extracts examined in the current study. Previous studies have shown that extracts of fruits from the *Juniperus oblonga* may be composed mainly of alpha-pinene, sabinene, beta-myrcene, limonene, beta-elemene, germacrene-D, germacrene-D-4, bisabolol and linoleic acid, or various species of flavonoids, depending on the extraction method [7,21]. However, this is the first study to examine dichloromethane extracts of *Juniperus oblonga*. Triterpenoids are compounds found in plants that are commonly isolated from dichloromethane extracts [22]. The active component in the Juniperus extracts examined in the current study may include triterpenoids. However, while the isolation and identification of the active compounds are critical, it is beyond the scope of the current study.

In conclusion, the current study showed that Juniperus oblonga extracts have potent anti-cancer effects. These extracts induced distinct effects on calcium regulation that ultimately led to increased mitochondrial calcium and induction of apoptosis in NB cells with and without MycN over-expression. The distinct calcium regulatory effects suggest that there may be different compounds responsible for inducing the apoptotic effects in NB. Further investigation into the active compounds in the Juniperus extracts may yield potentially novel anti-cancer compounds for the treatment of advanced stage NB with and without MYCN amplification.

Acknowledgements

This study was supported by the Alex's Lemonade Stand Foundation Young Investigator Award 439744 (D.-L. Koomoa), the NIH grants from the National Cancer Institute Mentored Research Scientist Development Award (K01) CA154758, (D.-L. Koomoa), and the National Institutes of General Medical Sciences P20GM 103466. (D.-L. Koomoa).

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