THESIS

CHARACTERIZING THE CANCER PREVENTATIVE PROPERTIES OF ROSEMARY EXTRACT

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2020

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ABSTRACT

CHARACTERIZING THE CANCER PREVENTATIVE PROPERTIES OF ROSEMARY EXTRACT

Flavonoids have been established as antioxidants[5], with some demonstrating tumor suppression abilities; however, there has been little investigation into their abilities as selectively toxic agents, particularly in cancer cell lines. This investigation was carried out on three different cell lines: V79 a Chinese hamster lung cell, V-C8 a Breast Cancer type 2 (BRCA2) susceptibility protein mutant of V79[39], and a gene corrected variant of V-C8 containing the human chromosomes with intact BRCA2 gene. The latter two were used as off target cell lines to ensure only the BRCA2 deficient cells were negatively impacted and that intact human BRCA2 would be spared as well. A rosemary extract provided by Gifu University in Japan was tested for any potential cancer prevention abilities in BRCA2 deficient cell lines of breast cancer, due to inhibition of poly (ADP-ribose) polymerase (PARP) in the aforementioned three cell lines. Additional qualities of the rosemary extract were done *in vitro* using both the extract as a whole and the four known compounds within the extract, including the primary compounds of carnosic acid and carnosol. The four compounds were tested in pure form to better understand what part of the extract was causing any selective toxicity observed.

Using mammalian cell culture, we were able to prove selective cell toxicity of BRCA2 deficient cells in comparison to the two off target cell lines. Further investigation

revealed that rosemary extract acted as a PARP inhibitor, a quality that is associated with synthetic cell lethality in BRCA2 deficient cells[6,12,13]. Finally the antioxidant capability of this mixture was quantified against a known antioxidant, ascorbic acid. Although rosemary extract as a whole does possess some minor antioxidant capabilities, it is capable of some level of hydrogen donation at sub LD_{50} concentrations in healthy cells. Antioxidants, in particular ascorbic acid, has shown evidence of improving quality of life, survival rate[17], and decreasing the overall initial risk of developing breast cancer[19]. While there is not presently data in living systems, there is promise that due to selective cell toxicity and antioxidant abilities our rosemary extract will be able to act preventively against cancer developing in BRCA2 deficient cells.

ACKNOWLDGEMENTS

I would like to extend my thanks to my committee members, Dr. Takamistu Kato for guiding my research and introducing me to mammalian cell culture, Dr. Marie Legare for her encouragement and, when needed, frankness, and finally Dr. Gerrit Bouma, for his patience and enthusiasm, which at times helped me stay determined to finish my thesis. I would also like to thank my mother for being a brutal, but fair task master at points preventing me from being distracted.

DEDICATION

For Michael Earl Van Steenberg, my father who raised me to question everything.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Chemicals

1.1.1 Flavonoids

Flavonoids are a class of plant metabolites that are found in a wide variety of consumables including fruits, vegetables and teas[10]. As flavonoids are common in a person's diet[10] it is not surprising there has been extensive research on this broad group of compounds, examining their anti-inflammatory abilities, antioxidant capabilities[5, 35] and, effects on the fetal brain[4]. Despite investigations into potential medicinal properties, limited information reported as to the possible cancer preventative properties that these compounds may possess. However; one study of note, a report by Huang et al (1994) indicated that flavonoids, carnosic acid in particular, can inhibit tumorigenesis caused by benzo[a]pyrene exposure[17]. This gap in knowledge invites investigation into

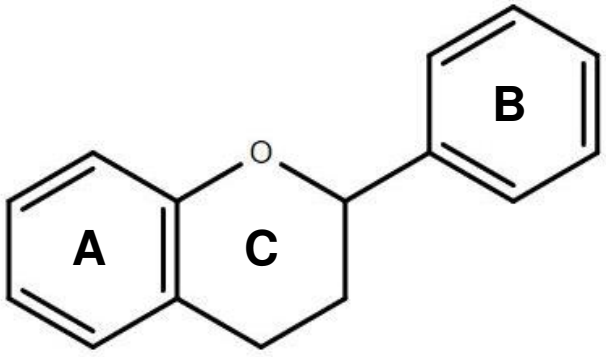

Figure 1: The majority of flavonoids come from a similar backbone in this figure above. The back bone is composed of three aromatic rings with one containing an oxygen.

potential cancer preventative properties of flavonoids[3].

Ass mentioned in a previous paragraph, a number of flavonoids express anti-oxidant capabilities. While cancer preventative attributes have not extensively investigated, the anti-oxidant properties have been characterized more completely, at least *in vitro*. The structure of the flavonoid does affect the efficiency of anti-oxidant properties, in addition to which mechanism of action is primarily used. Each flavonoid has a similar benzo-γ-pyrone molecular backbone with each variation in oxidization and substitution grouped together into one of six groups.

1.1.2 Rosemary and Rosemary Extract

Common rosemary, scientific name *Rosemarinus officinalis,* is an evergreen herb native to Europe in the Mediterranean area and a member of the mint family, *Lamaiceae.* There are a number of other plants species in the *Rosemarinus* genus, but for our purposes we are focusing on an extract from *R. officinalis.* While rosemary is primarily used as a cooking herb it has demonstrated anti-inflammatory, and anti-oxidant properties[29,31].

From a gardening standpoint, rosemary is an herb that does well in well-draining soil with full sun exposure. Additionally, rosemary does not require much in the way of fertilizer and can live for more than two years, classifying it as a perennial. Over the course of those two or more years the plant can grow up to three meters (about 9’10”) and will have small blueish-purple flowers. For culinary purposes rosemary keeps much of its flavor even when dried making it particularly useful in seasonings year-round. Historically, rosemary has been associated with remembering the dead and was scattered around graves. During the 15th through the 17th century the herb was commonly found at weddings, as it was associated with fidelity[28,29,31].

Table 1: Basic information about each of the four known compounds in our rosemary extract. Shown above is the specific class of compound, pKa, and plants other than *R. officinalis* that contain notable amounts of the respective compound.

Compound Name	**Family of Compounds**	**pKa**	**Other Sources**
Carnosol	Phenolic diterpene	9.19	Mountain Sage (*Salvia pachyphylla),* and Greek sage (*Salvia triloba)*
Carsonic Acid	Benzenediol abietane diterpene	4.29	Common Sage (*Salvia officinalis*)
Gallic Acid	Trihydroxybenzoic acid	4.5	Gallnuts, sumac, witch hazel, tea leaves, and oak bark
Rosemarnic Acid	Caffeic acid ester	3.13	Basil (*Ocimum basilicum)*, holy basil (*Ocimum tenuiforum*), Lemon Balm (*Melissa officinalis)*, Marjoram (*Origanum majorana),* and common sage (*Salvia officinalis)*

While it is difficult to pin down the exact amount of rosemary produced on a national, let alone global scale, at least in part due to private growing of the herb in family gardens. Another unfortunate wrinkle is that the herb is used both as a seasoning and is reduced into an “essential oil”, with multiple markets it becomes even more difficult to find an accurate number. However, based on the average yield per unit area and the acreage of one of the largest plantations for rosemary it’s possible estimate how much *R. officinalis* is produced, at least in the commercial market. Annually 200-300 metric tons of rosemary oil is produced[31]. Given the average kg yield of oil per hectare of land is 80-100 kg/ha[29], and each hectare produces between 12 and 13 metric tons[29] of rosemary an annual crop of about 3.5 thousand metric tons can be estimated globally.

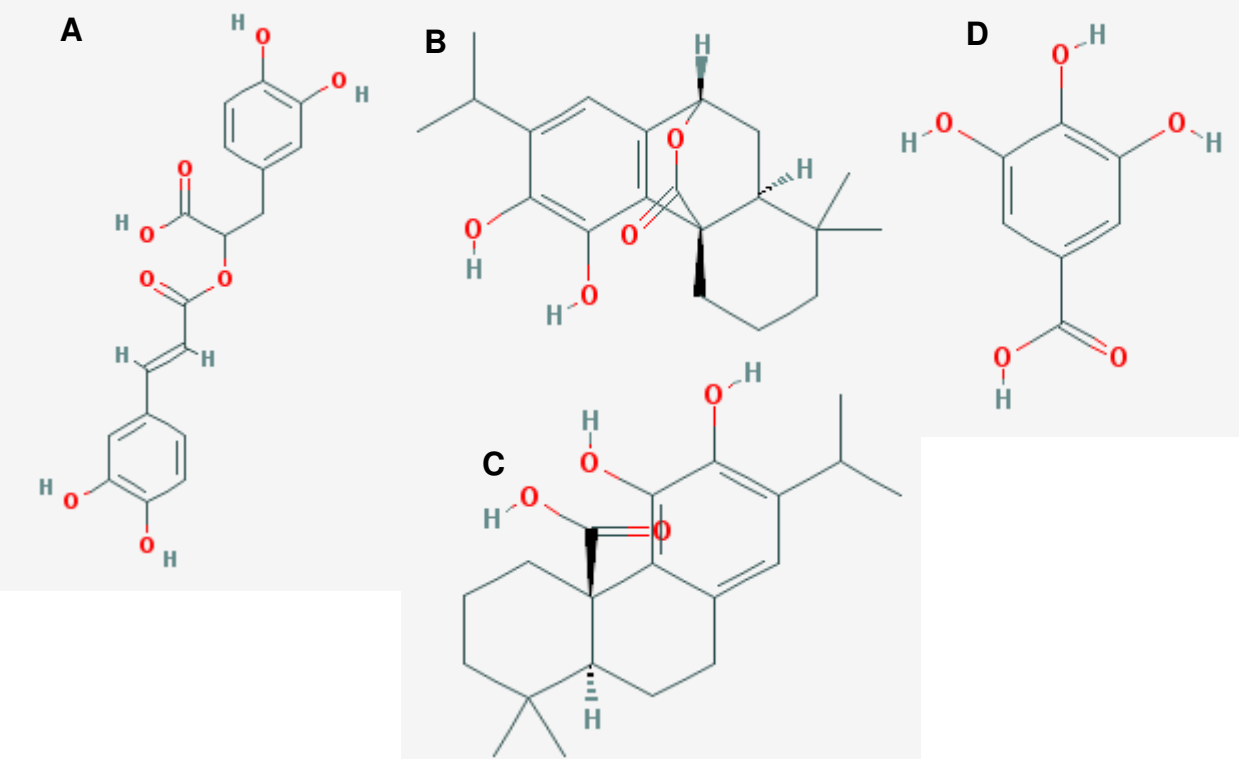

Figure 2: Rosemarinic acid (A) is the largest of the four compounds tested with a molecular weight of 360.3 $\frac{g}{mol}$ and is soluble both in polar and non-polar solvents. Carnosol (B) and carsonic acid (C) have similar properties as one another with carnosol having a bound between the carboxyl group and the hydrogen across the central cyclohexane. Carnosol is extremely soluble in non-polar solvents in comparison to the other 3 compounds that were investigated. Gallic acid (D) is the smallest of the four compounds with a molecular weight of 170.1 $\frac{g}{mol}$ and has similar solubility in non-polar solvents and polar solvents.

Images acquired from PubChem

Rosemary extract is a mixture of different compounds including flavonoids derived from *R. officinalis.* The two most common compounds, carnosic acid and carnosol, have been identified along with two other present compounds, gallic acid and rosmarinic acid. As these are known compounds in the extract these were the primary compounds investigated, both individually and in the form of the extract itself. Within these compounds, carnosic acid has demonstrated slowing cancer cell line growth, and

inhibiting tumorigenesis, in theory by preventing the binding of benzo[a]pyrene to DNA[17].

Given that rosemary extract is derived a plant and contains flavonoids, it is easy to conclude that it could act as an antioxidant, which means the question becomes how effective an antioxidant rosemary extract is. Two different methods of reducing a free radical were investigated, how readily a hydrogen is donated and how easily single electron transfer occurs. While there are other routes of reducing free radicals, these two were the least complex to test and two of the more common types of anti-oxidant activity.

1.2 DNA Repair Mechanisms

1.2.1 Poly (ADP-Ribose) Polymerase (PARP)

The first publications to mention PARP was “On the Formation of a Novel Adenylic Compound by Enzymatic Extracts of Liver Nuclei” by Chambon et al. in 1966 as a follow up to their publication in 1963, “Nicotinamide Mononucleotide Activation of a New DNA-Dependent Polyadenlic Acid Synthesizing Nuclear Enzyme.” In these publications Chambon et al., PARP was isolated from the nuclei of liver cells from hens, though it is found in multiple animal species and across different tissue types. As of the present there are seventeen proteins that are members of the PARP family, but our focus is was strictly on inhibition of PARP-1 due to its connection with DNA repair mechanisms and apoptosis. This particular type of PARP protein is 116 kDa and structurally is made up of two homologous zinc fingers domains, both that bind to DNA. The second zinc finger has a higher affinity, bit the first is the one essential for proper PARP-1 function both *in vivo* and *in vitro*.

PARP-1 is a protein associated, but not directly involved, with BER and SSBR. PARP-1 binds to DNA at single strand breaks and turns NAD^+ into PAR[18] after undergoing a structural change. PAR then signals for the cell's DNA repair mechanisms, including LigIII, polβ and XRCC1 before PAR is degraded by PARG, preventing PAR build up which would signal the cell to undergo apoptosis. LigIII, polβ and XRCC1 then repair the break itself after PARP leaves the DNA. PARP plays a virtually identical role in SSBR. The primary difference between these two repair mechanisms is that in BER, base lesions are detected by a Gly and then removed via APE which then creates the single strand break the PARP then binds to, while in SSBR there is already a break present in one of the strand of DNA. Additionally, in regards to BER, a certain model has inhibited PARP-1 acting as a wrench in the works, preventing the ligation process.

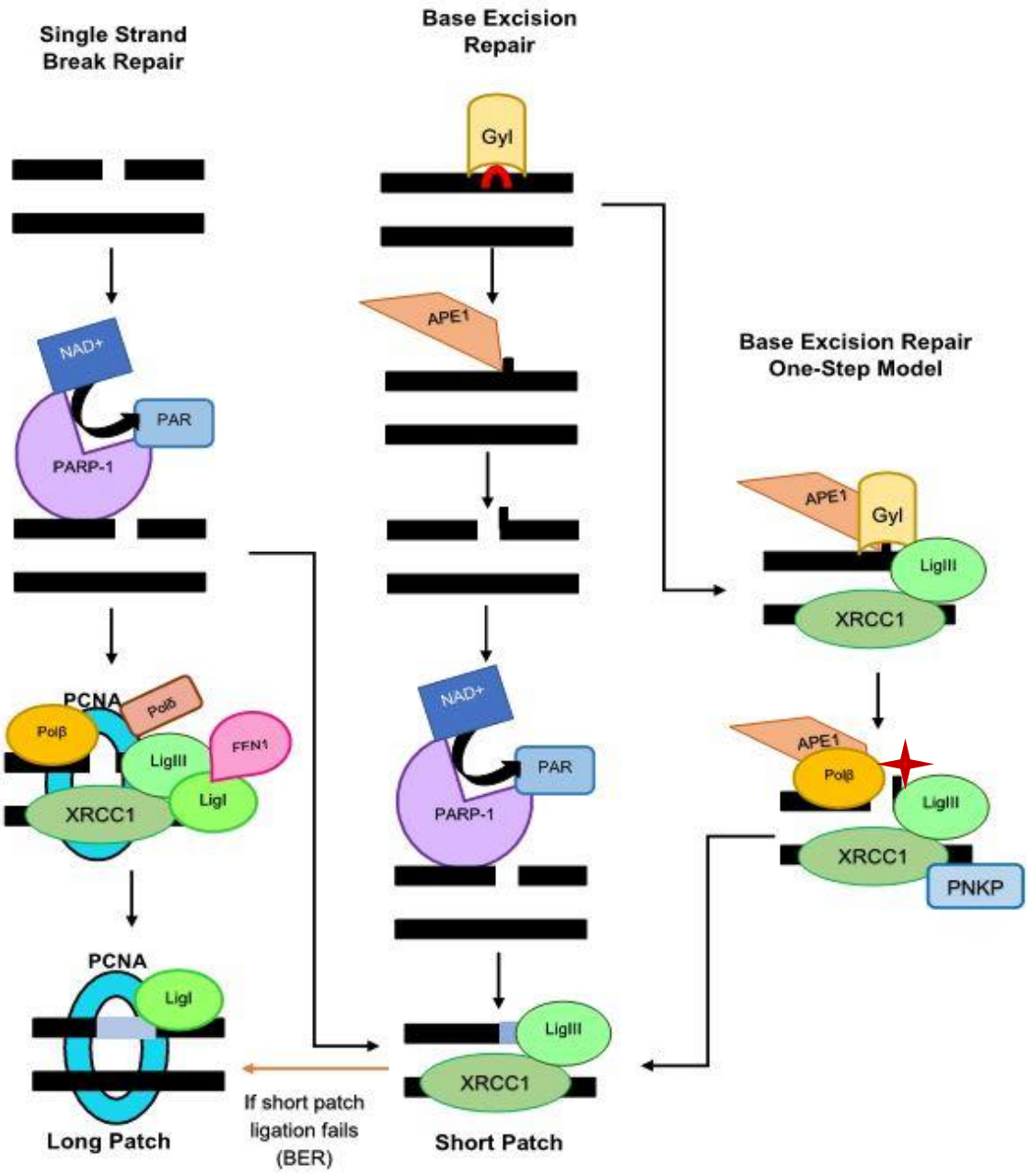

Figure 3: The above figure depicts two different ways repair can occur in a single strand DNA error with one having two possible models. The process on the left is single strand break repair in which there is already a base missing in one of the DNA strands. PARP-1 attracts repair mechanisms to the site of the break and either short patch or long patch ligation occurs. Base excision repair has an incorrect base removed and marked before repair can take place. In the two-step model PARP-1 then recruits DNA repair mechanisms in a similar fashion to single strand break repair. However, in the one-step model PARP-1 is not needed for recruitment and instead can act as a jam during the ligation step if it is inhibited (marked by the red four-pointed star).

1.2.2 BRCA2

Estimates of the number of people affected by BRCA2 heterozygous mutations are 1 in 400 to 1 in 800[11]. Homozygous negative BRCA2 can only occur in an after birth random mutation as complete loss of BRCA2 results in death of the embryo. Although this is not a common risk, given the 2014 United States of America census that would be between 400 thousand and 800 thousand people in the United States of America alone that would be at a greatly increased risk of developing breast cancer along with a slight increase in other cancers including ovarian cancer and melanoma. BRCA2 is a 348.2 kDa protein located on the long arm of chromosome 13 at position 12.3 and a tumor suppressor gene that interacts directly with RAD51 and DNA during homologous recombination (HR). In addition to the connection of BRCA2 deficiency to an increase risk in developing breast cancer and other cancers. BRCA2 +/- cells are a concern because reduced expression of BRCA2 leads to an increase in HR as there is less BRCA2 present to inhibit that type of double strand break repair, which is more error prone than NHEJ. Due to an uptick of errors during DNA repair, it is more likely for the cells to become BRCA2 -/- preventing NHEJ from occurring at all and leading to yet more errors resulting potentially in the cells turning cancerous.

As mentioned before, BRCA2 is involved in HR, a double strand break repair mechanism. In particular, BRCA2 is associated with a rate limiting step in this repair process by directly binding to both DNA and RAD51[18,27]. BRCA2 binds to a single strand of DNA at the c-terminus and RAD51 in the BRC repeat region, which has been theorized to “provide and assembly line of RAD51” [27]. Without BRCA2 present, the HR process becomes increasingly error prone and is unable to adequately repair double

strand breaks. Another double strand break repair mechanism is NHEJ. Without properly functioning BRCA2 or BRCA1, the cell is forced to undergo NHEJ more frequently, which is unfortunately an error prone DNA repair mechanism[18, 27, 38]. NHEJ is error prone in comparison to other repair mechanisms and directly ligates each end of the break together without the need for homogeneity between the strands. While if the overhangs exactly match up, errors rarely occur with NHEJ, if there are non-compatible overhangs, a more common case, this process forces them together leading to loss of bases or another wise imperfect repair of the double strand break, potentially resulting in the loss of function of tumor suppressing genes. When coupled with inhibition of another two of the six total DNA repair mechanisms, the cell is limited in how accurately it can repair damaged DNA.

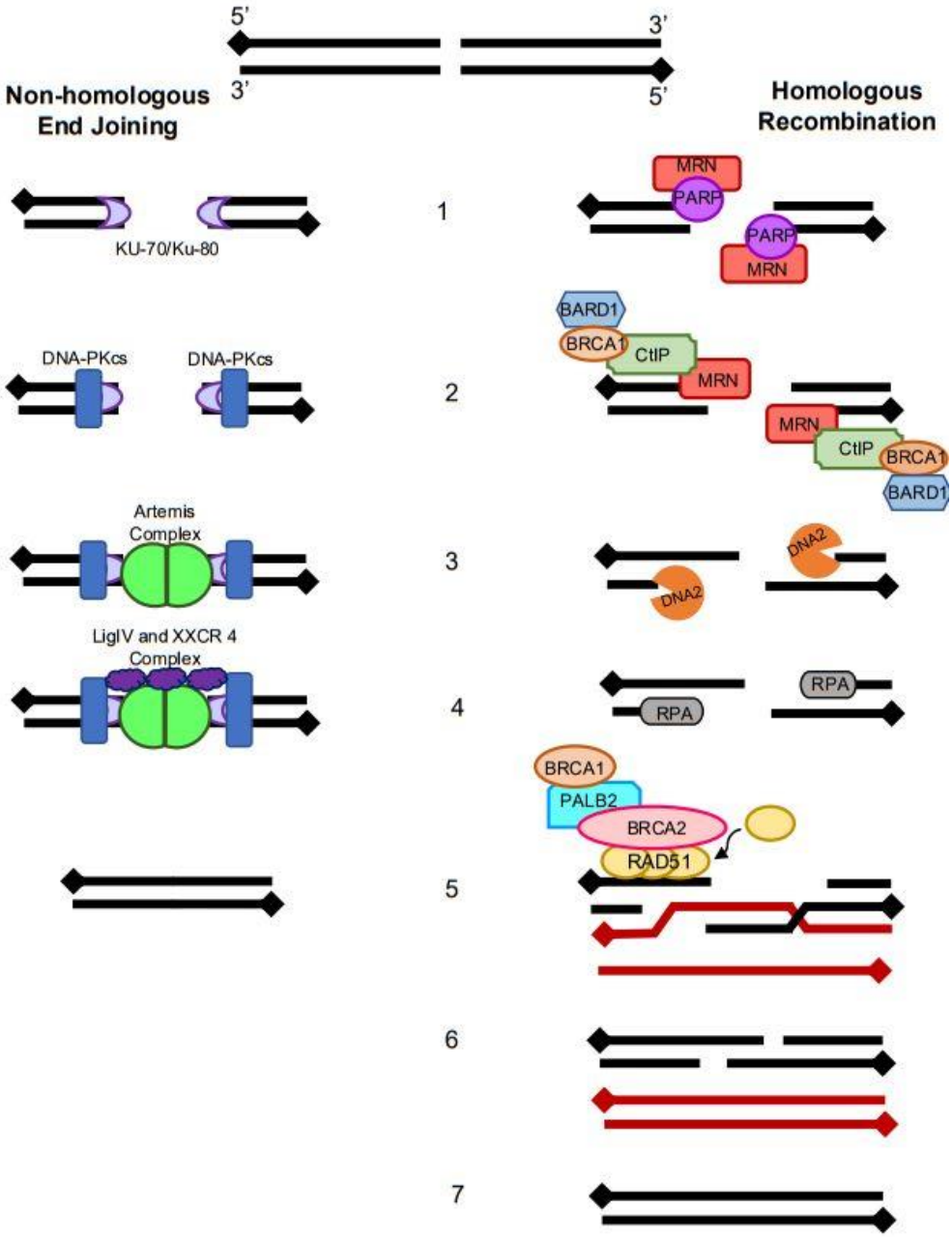

Figure 4: In homologous recombination the break is recognized, and then each end of the break is processed by removal of part of the 5' end closest to the break on each set of strands to create a 3' overhang on each side. RAD51 and its paralogs for complexes to stabilize the single stand DNA and to recruit other proteins necessary for break repair. BRCA2 assists, alongside the paralogs, to have RAD51 loaded correctly on to the single strand of DNA. BRCA2 in particular binds between the DNA and RAD51. After RAD51 is in place it helps to stabilize the intermediate while the 3' over hang finds a matching piece of unbroken DNA and "invades" in order to ensure no bases are missing. Then the two over hangs match their base pairs and the breaks are fixed via ligation. In non-homologous end joining, there are no checks for homology between the two stands, the breaks are ligated back together as is regardless if there may be a base missing

Table 2: A summary of the four different DNA repair mechanisms that will be discussed in this thesis, single strand break repair, base excision repair, homologous recombination and non-homologous end joining. The roles of both PARP and BRCA2 are listed for each mechanism along with the qualitative error rate and the type of DNA break the mechanism repairs.

Repair Mechanism	**Single Strand or Double Strand Break**	**Rate of Errors**	**PARP Involvement**	**BRCA2 Involvement**
Single Strand Break Repair	Single Strand	Low	Recruitment of XRCC1, Polβ, and LigIII	None currently known
Base Excision Repair	Single Strand	Low	None, or recruitment of XRCC1, Polβ, and LigIII	None currently known
Homologous Recombination	Double Strand	Low	Marks the break on the 5' end of each strand along with MRN	Binds to DNA and c-term of RAD51
Non-homologous End Joining	Double Strand	Moderate	PAR creation to inhibit	BRCA2 inhibits

1.2.3 Synthetic Lethality

Unlike *BRCA2*, a loss of both PARP alleles does not result in death of the developing embryo. There are PARP knockout mice that have little to no negative effect as a result of the loss. Interestingly when PARP is inhibited in cell lacking an effective homologous recombination pathway, cell death occurs. Additionally, inhibition of XRCC 1, a protein recruited by PARP-1, does not result in cell death. A potential explanation for this is that PARP-1 is involved in more than SSBR and BER, and that when inhibited PARP-1 can become jammed in the BER process and prevent the ligation step from occurring. The primary focus in this investigation has been on PARP-1 as the assays used specifically focused on this member of the PARP family as it is the one most

involved in SSBR and BER along with being the most frequent member inhibited. Due to the synthetic lethality that is caused by BRCA2 deficient cells being exposed to a PARP, PARP inhibitors[6] have become an attractive option for preventative treatment in those with the heterozygous *BRCA2* mutation.

While PARP inhibition alone does not cause cell death, as shown in PARP deficient rats, where an increase in single strand breaks were noted, with minimal adverse health effects[18]. However, when PARP inhibition is combined with BRCA2 deficiency increased cell death does occur, with sparing of other cells which do not have this lethal combination[6, 12, 13]. This particular kind of cell toxicity is referred to as synthetic lethality. Synthetic lethality occurs when two effects or mutations that on their own do not result in cell death, cause cell killing when combined[6, 12, 13]. For example when a heterozygous BRCA2 cell mutates to a homozygous BRCA2 negative cell, the cell still functions; however, when PARP is inhibited in the same cell, the cell dies, even though loss of PARP function does not cause cell death on its own.

Although there are different members of the PARP family, PARP-1 is associated with SSBR in DNA. While PARP-1 does not directly repair the DNA, it does recruit the repair mechanisms for both SSBR and BER. While the exact mechanism of action is still not completely known, it has been theorized that “PARP trapping” occurs with PARP-1[18]. With this PARP-1 is unable to create poly (ADP-ribosylation), preventing PARP-1 from being able to detach from its location next to the break in the strand[18]. Without PARP-1 leaving the damaged DNA, DNA repair mechanisms are unable to fix the break[18].

With PARP inhibition, PARP-1 tends to be inhibited, but, PARP-2 is another common target along with PARP-3, though the latter is less frequently affected. PARP-2 assists in poly (ADP-ribosylation) reactions alongside PARP-1[18]. Less involved in SSBR is PARP-3, found primarily in daughter centrioles during cell division and has a role in inhibiting non-homologous end joining (NHEJ)[38]. Since PARP inhibitors tend to be non-specific, in that if the compound inhibits PARP-1 or PARP-2, our experiment took place in a controlled environment. As only PARP-1 was used in the assay any inhibition that took place would have to be of PARP-1. As inhibition of PARP-1 is the most closely associated with the synthetic lethality BRCA-2 deficient cells[6, 12, 13], it was decided this should be the focus of testing. This however does not mean that inhibition of PARP-2 or PARP-3 does not occur, just that there is evidence of PARP-1 inhibition.

1.3 Cell Survival

1.3.1 Cell Death

Apoptosis and Necrosis are two main forms of cell death[16]. Apoptosis is also referred to as cell suicide and is instigated by the cell itself and can occur naturally without the cell undergoing an attack by a toxic agent, and is frequently used during development for things such as the formation of digits[15,16]. Necrosis, on the other hand, is not directed by the cell and is instead usually due to a sudden and irrecoverable damage done to the cell[16]. For the overall health of the tissue, apoptosis is highly preferable as the cell that dies limits its damage to surrounding cells. As necrosis is not mediated by the cell, when the cell dies it does cause damage to the surrounding cells, potentially forcing the previously healthy cells to die as well[16]. Both of these processes

can be distinguished either by morphological changes or by looking at certain biochemical markers[2,15,16].

Apoptotic cells condense themselves and tightly package their cleaved DNA without inducing swelling in any of the organelles, in a way they implode, keeping cytosolic and lysosome enzymes separate from the surrounding cells in the tissue[16]. Macrophages then are signaled to consume the apoptotic cell, disposing of the cell and helping to prevent an inflammatory response. When looking for evidence of apoptosis, shrinking of the cell, formation of small pockets of the cell membrane, referred to as "blebs"[2], micronuclei, and presence of compounds like cytochrome C, or activated caspases 2,3, and 7 through 9 in the tissue[2,15].

Necrosis, as previously mentioned is more damaging to the surrounding tissue due to the nature of this process of cell death[15,16]. While the cell does not initiate necrosis, there are still several pathways involved, including proinflammatory pathways that cause an inflammation response at the site of the cell in the tissue[2,16]. Similar to apoptosis, there are changes in cell morphology that can be used to distinguish necrosis from apoptosis along with biochemical markers that can be found in the tissue[2,15,16]. Cells undergoing necrosis swell, as do the organelles, and while budding does occur, similar to apoptosis, it is not limited or controlled as the apoptotic blebs are[2,16]. Additionally, HMGB1, a proinflammatory molecule, is produced and released by cells undergoing necrosis, and can be used as a marker for necrosis as opposed to apoptosis[2,15,16].

Table 3 : Summary of the two different types of cell deaths. Included are ways to identify both, key enzymes involved, common situations that lead to one over the other, and effect on the surrounding cells in the tissue.

Aspect of Cell Death	**Apoptosis**	**Necrosis**
Cell Mediated	Yes	No
Effect on Surrounding Tissue	Minimal to none as the cell remains tightly packaged waiting to be disposed of	Release of cytoplasmic enzymes that can damage surrounding cells
Example of Common Instance	Formation of digits during utero	Hepatic necrosis to acetaminophen or iron over dose
Type of Triggering Situation	Normal cell cycling due to lack of essential processes	Irrecoverable, sudden damage or exposure to a toxin
Marker Enzyme	Cytochrome C	HMGB1
Visual Appearance	Small compact ball with blebs	Overly swollen cell with blebs

1.3.2 Population Doubling Time

Colony doubling time assays quantify the effects of a compound on the speed at which the cell lines double. A compound lengthening the time it takes the average cell in a cell line to divide does not necessarily indicate that the cell cycle has been stalled or disrupted in some way. The assay used in our investigation focuses on cell count after a given time, then the doubling time of the treated cells is calculated based on these results. The typical doubling time observed in our lab conditions is shown in table 2, and are 11.8 hours for the off-target V-79, 15.1 hours for the target V-C8, and 13.8 hours for the gene corrected cell lines.

1.3.3 Colony Forming Ability

The clonogenic assay was initially created by T.T Puck and Phillip I. Marcus from the University of Colorado in 1955 and is frequently used in cancer research to determine the effects of a treatment on cancer cell lines. Colony survival investigates the ability of a compound to either kill or reduce the ability of cell lines to successfully form colonies after exposure during plating. The success of a colony, or lack thereof, does not demonstrate cell death as the compound could be reducing the cells ability to divide, or in other ways reducing the ability of the cells to form successful colonies over the course of the incubation period. Colony survival is useful to observe longer term effects, in our case of a single initial dose, on the cell lines that can produce different results than those of cell doubling.

1.4 Anti-oxidants and Cancer

Antioxidants have been well characterized as a cancer preventative[14, 16], thus, in the exploration of rosemary extract it was important to determine if there is any antioxidant ability. In order to qualify the effectiveness of rosemary extract, ascorbic acid was used as a known. An additional benefit of using ascorbic acid is that it has been well researched especially in regards to cancer prevention and care. Ascorbic acid in plasma in particular has been shown to reduce the risk of breast cancer in case-controlled studies and increasing intake of vitamin C after diagnosis lowers the risk of death from cancer in general, and especially breast cancer[17,19].

While these are important discoveries, there are holes left in the characterization of carnosic acid and flavonoids in general. As previously mentioned, the primary focus of research has been on the antioxidant capacity and anti-inflammatory effects of

flavonoids[5, 35]; however, the evidence of some flavonoids being cancer preventative agents is convincing enough to investigate further[3].

1.4.1 Total Antioxidant Capacity

The Total Antioxidant Capacity (TAC) Assay, while less specific than the DPPH assay, it's a technique that is used to generally establish the antioxidant abilities of a compound. Given the TAC assay used involved the reduction of Copper (II) to Copper (I) via single electron transfer it tests a different aspect of rosemary extract's antioxidant properties. Just as with the DPPH assay, there is a color change that occurs indicating the reduction of the copper; however, the high absorbance means more of the reduced copper than the unreduced with a low absorbance indicating that the copper is largely un-effected.

1.4.2 DPPH Assay

The 2,2-diphenylpicrylhydrazyl (DPPH) scavenging assay take advantage of the stable free radical with in DPPH. When DPPH comes in contact with an antioxidant it gains an electron in the form of a single hydrogen. After the addition of the hydrogen a color change occurs making it possible to detect using a spectrometer with a large absorbance indicating little to no reduction of DPPH, and a small absorbance the opposite. For this assay, along with the total antioxidant capacity (TAC) assay, ascorbic acid is used as a point of comparison given the well-established antioxidant qualities of the compound. Additionally, in the case of ascorbic acid, it has reasonably "fast" kinetics, meaning that within the time of the assay, 30 minutes, and concentrations at or above 10 µM (5.68 $\frac{\mu g}{mL}$) are capable of reducing at least half of the available DPPH[28].

CHAPTER 2: CHARACTERIZING THE CANCER PREVENTATIVE PROPERTIES OF ROSEMARY EXTRACT

2.1 Introduction

In order to properly characterize our rosemary extract, cell culture was used as necessary first step. In therapeutic research this is usually used to determine the capacity to have a desired effect without having to use animal models. In the instance specific cell lines derived from Chinese hamster lung cell containing normal and truncated BRCA2 genes. BRCA2 deficient cells in particular were used based on the gene's connection to the development of breast cancer. Traditional cell culture techniques were used and are explained in greater detail in later paragraphs. Additionally, chemical based antioxidant assays were done on both the compound as a whole and on three of the four primary molecules found in this particular rosemary extract.

2.2 Hypothesis

Rosemary extract has anti-cancer effect against BRCA2 cancers. Rosemary extract possess PARP inhibitory effect and can kill BRCA2 deficient cells selectively. Moreover, rosemary extract has antioxidant activity which provides additional antitumor effect.

2.3 Specific Aims

In general, our goal was to characterize the possible positive qualities that could make this rosemary extract a viable BRCA2 -/- cancer preventative supplement. These qualities are including but not limited to a mechanism of action and any antioxidant activity.

2.3.1 Determine Selective Cell Toxicity

Critical to the entire investigation is that selective cell toxicity occurs after exposure to chemicals within rosemary extract, or more significantly a difference in survival rates between target (V-C8) and off target (V79 and gene corrected) cell lines which will help determine the exact molecular mechanisms by which rosemary extracts exerts its effects

2.3.2 Investigate Whether PARP Inhibition is the Mechanism by which Rosemary Extract Exerts its Selective Cell Toxicity

By utilizing a V-79 and V-C8, it's complimentary BRCA2 deficient cell line, we will investigate the possibility that rosemary extract is causing an increase in cell line specific toxicity via a combination of PARP inhibition in combination with the BRCA2 deficiency. A gene corrected variant of V-C8 was used to ensure that there would be no negative effects against normal human BRCA2 and only effect cells lacking functional BRCA2.

2.3.3 Establish Additional Cancer Preventative Properties

As rosemary extract is composed primarily of flavonoids, we will investigate whether the extract can act as an antioxidant in a similar fashion to other flavonoids.

Specifically, we will test the ability of rosemary extract and the four afore mentioned compounds for their ability to donate hydrogens and preform single electron transfer.

2.4 Materials and Methods

2.4.1 Rosemary Extract

The rosemary extract used in our investigation was provided by Gifu University in Gifu, Japan and was dissolved in a non-polar solvent. The solution has a yellow brown color, though had a limited effect on the UV spectra taken in the antioxidant assays due to the low concentrations used.

2.4.2 Other Compounds

We were informed about four different primary molecules found in this rosemary extract, carnosol, carsonic acid, gallic acid, and rosemarnic acid. All four of these compounds were tested for PARP inhibition, by Gifu University, and three of the compounds had their UV spectra imaged along with the extract as a whole. Carnosol was not used in all of assays as it has been documented as having similar properties as carsonic acid has a pKa of 4.29 making it deprotonated at physiological pH.

2.4.3 Cell Lines

For the purposes of testing selective cell toxicity of rosemary extract V-C8 cells were used as the target cell line. V-C8 cells are a truncated BRCA2 negative mutant of the V79 cell line[35], which is derived from Chinese hamster lung cells. Additionally, a gene corrected mutant version of the V-C8 cell line was also used as check for any non-specific negative effect against intact human BRCA2. V79 cells in particular have a history of use in mutagen studies and due to their overall hardiness and ease of growth were used along with the two mutants derived from it. All cells were maintained in minimum essential medium alpha (MEMα), and supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics and antimycotics in a humidified incubator at 37°C and 5% CO_2. All experiments were done in a minimum of triplicate,

Table 4: Pertinent information regarding each cell line investigated, the two off target cell lines, V79, and gene corrected, along with the cell line containing the targeted BRCA2 deficiency. Each cell line's origin, doubling time, number of chromosomes, average doubling time based on our controls from the doubling time experiment and the state of the cell line's BRCA2 are recorded above.

Cell Line	**# of Chromosomes**	**BRCA2 Status**	**Doubling Time**	**Target**	**Origin**
V79	22	Normal Hamster	11.8 hrs.	No	Chinese Hamster Lung Fibroblast
V-C8	22	Truncated Hamster	15.1 hrs.	Yes	Mutated V79
Gene Corrected	24	Truncated Hamster Normal Human	13.8 hrs.	No	V-C8 with human chromosome pair

and the figures provided are based on an average of this data collected, with bars representing the standard error of the mean, and curves of best fit are also included for each graph.

2.4.4 Spectroscopy

The data was collected in the lab using a nanodrop using the UV-Vis setting. As none of the three compounds or the extract are fully soluble in water DMSO was used to dilute a stock solution to 25-50 $\frac{\mu g}{mL}$. The goal was to have the peak absorbance values between 1.0 and 1.5, the data was then normalized for each compound with the absorbance value at the λ max treated as one and all of the other absorbance values adjusted by the λ max so that all of the spectrums could be compared consistently regardless of concentration used.

2.4.5 Selective Cell Toxicity

Cell doubling time was done using 10,000 trypsinized cells from the 3 different cell lines were plated into 12-well cell culture plate. Cells were treated with select amounts of rosemary extract were added before the 12-well plates were returned to the incubator. Every 24, 48, 72, and 96 hours cells were counted using the Coulter Counter Z1 from Beckman Coulter. The data was adjusted to account for differences of normal doubling time which has been normalized to one across all cell lines. The average of each cell lines' doubling times originated from the doubling time of the control cultures of each cell line in each trial.

Colony survival fractions were determined through exponentially growing cell cultures were trypsinized and approximately 300 cells were placed in cell culture dishes, and treated immediately with varying concentrations of rosemary extract and chemicals before being allowed to grow up, undisturbed in an incubator for one week. Afterwards the cells were fixed with 100% ethanol and stained with 0.1% crystal violet and the stained colonies were counted. A colony having more than 50 cells was considered to be a survivor as this is standard in our lab. This was done in triplicate for each of the concentrations in each of the cell lines. IC_{50} values (50% inhibitory concentration doses) were derived by fitting dose response curves using a sigmoidal dose response equation obtained by GraphPad Prism 7.

As rosemary extract is not exceedingly soluble in water DMSO had to be used. As DMSO itself is toxic to cells no more than 1% of the total media for the cells could be made up of DMSO, along with thorough mixing of the cells before they could adhere to the plate. A control plate was used with the same concentration of DMSO to ensure the effects observed were from the rosemary extract and not from the DMSO.

2.4.6 PARP Inhibition

A HT Universal Colorimetric PARP Assay Kit (Trevigen) was used to assess the capacity of rosemary extract as a PARP inhibitor. Solutions were prepared per directions provided with the kit from the manufacturer and 3-Aminobenzamide(3-AB) was used as a known to compare rosemary extract's PARP inhibition levels against. The histone coated strip wells were rehydrated using 50 µL of a 1X PARP buffer and leaving the strips at room temperature before emptying the wells and adding 5µL of the desired dilutions of rosemary extract and 3-AB, then 7.5 µL the PARP enzyme (0.5 $\frac{unit}{well}$)

solution. This was allowed to sit at room temperature for 10 minutes before the addition of 12.5 µL of the PARP cocktail, the wells were left at room temperature for 1 hour before washed toughly with PBS and PBS with 0.1% Triton X-100. After washing 25 µL of prepared Strep-HRP was added to each well. Again, the wells were allowed to sit at room temperature for 1 hour before repeating the washing process. After the wells were washed and dried, by patting on top of a paper towel, 50 µL of TACS-Sapphire was added and the wells were placed inside a drawer at room temperature for 15 minutes before adding 50 µL of 0.2 M hydrochloric acid to quench the reaction.

As these are the compounds individually as opposed to the extract that contains them in some quantity, even rosemary extract at the same concentration of 100 µM may not have the exact same fraction of PARP inhibition. Gifu University had tested each compound in a similar fashion to how the experiments conducted to determine PARP inhibition by rosemary extract as a whole, making it likely to be a PARP-1 inhibition assay.

2.4.7 Antioxidant Capabilities

The total antioxidant capacity of the rosemary extract was measured with a Sigma Total antioxidant activity kit (Sigma, MAK 187A) as manufacture's instruction. Ascorbic Acid was prepared in a similar way and was used as a positive control. The Copper (II) reagent was diluted 50-fold in the provided diluent and 5 µL was added to 5 µL of the diluted test compound. The solution was incubated at room temperature for 90 minutes before the absorbance at 570 nm was read using a Nanodrop (Thermofisher). All values used double distilled water as a blank, and each concentration of each compound was repeated in triplicate. The points reported in figure 16 is the mean of

these triplicates and contains error bars based on the data collected based on calculations done by GraphPad Prism 7 (GraphPad software, Inc.).

The efficiency of the rosemary extract as a free radical scavenger was tested with a DPPH assay. Ascorbic acid was used as a reference compound to help better quantify the results from the assay. All dilutions were done in 100% ethanol for both the compounds and the dissolving of DPPH to make a 500 µM stock, of which 40 µL was added to each well except the negative control. Varying amounts of the compounds were added in order to make a concentration range from 0.1 $\frac{\mu g}{mL}$ to 10 $\frac{mg}{mL}$. The total volume of each well was 200 µL with ethanol making up the majority of the volume. A VersaMax ELISA microplate reader was used to measure the absorbance of 517 nm after the solution had incubated at room temperature for 30 minutes. The data is presented in figure 17 as fraction of the remaining free radical based on the positive control of 100 µM DPPH in ethanol as all of the free radical being available. All data was collected in at least triplicate and error bars shown are calculated based on the data gathered. Additionally, due to the natural golden-brown color of rosemary extract, background absorbance was taken in triplicate and accounted for in the reported data.

A similar protocol was used to test the four known components using the TAC assay and the DPPH assay. The primary difference between the experimentation done on the rosemary extract and the four compounds is that the four compounds were tested at lower concentrations.

2.5 Statistical Analysis

All of the following statistical analysis was done using Graphpad Prisim 7 unless otherwise noted. In some cases, the concentrations used were not constant across test

compounds in which case some test were more difficult to do and the best alternative was used instead in order to accurately represent the results.

CHAPTER 3: RESULTS

3.1 Spectroscopy

In order to properly characterize the rosemary extract, we will utilize several different techniques in mammalian cell culture, and chemical assays. As this is a preliminary investigation, animal models would not be practical at this time. The main focus will be to determine if rosemary extract is capable of selectively killing targeted cancer cells, if selective toxicity of BRCA2 deficient cells is observed, what is the mechanism of actions, and any addition qualities associated with cancer prevention, specifically antioxidant activity.

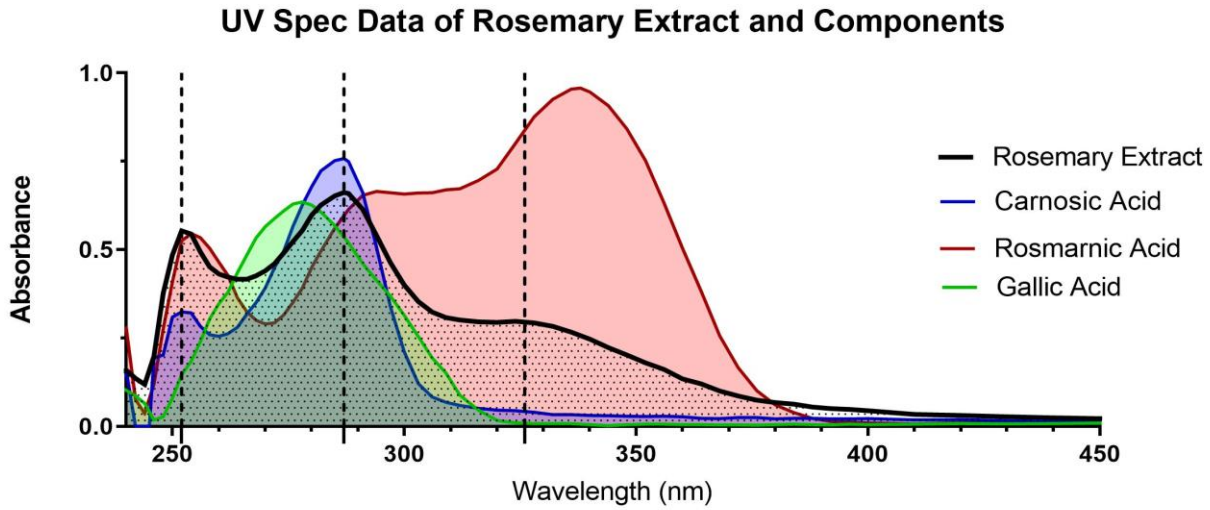

Figure 5: UV-Vis spectra of the extract (black line with black dot fill) and three of the known components, carnosic acid (blue), rosemarnic acid (red), and gallic acid (green) all compounds were diluted with DMSO and blanked as such. Peaks for rosemary extract are at 252, 287, and 326 nm and marked with a dashed line.

Table 5: Relevant information about each one of the four known compounds, carnosol, carnosic acid, gallic acid and rosemarnic acid, including the lambda max as established in literature and the lambda max values found in UV-Vis spectra in the fig. 5, their solubility in DMSO (primary solvent used in our experiments), and the compounds molecular weight.

Compound	Molecular Formula	Lambda Max (nm)	Recorded λ Max (nm)	Solubility In DMSO (mg/mL)	Molecular Weight (g/mol)
Rosemarinic Acid	$C_{18}H_{16}O_8$	290 330	294 338	~25	360.318
Gallic Acid	$C_7H_6O_5$	272.5	278	~16	170.12
Carnosol	$C_{20}H_{26}O_4$	284	N/A	~250	330.424
Carnosic Acid	$C_{20}H_{28}O_4$	284	287	~30	334.44

3.2 Selective Cell Toxicity

We examined ability of rosemary extract to selectively target cells. Using changes in cell doubling time and colony formation to assess toxic effects on the cells. For both of these tests the same cell lines were used, V-C8, V79, and a gene corrected variant of V-C8 containing human BRCA2, and the same medium in order to ensure that any differences were due to the rosemary extract as opposed differences in media or origin of cell. All experiments were done in a minimum of triplicate and the figures provided are based on an average of this data collected, standard error of the mean values were plotted as error bars and curves of best fit are also included for each graph. Changes in cell doubling time can indicate a compound has an effect on the cell cycle, while it may not convey which part of the cell cycle is interrupted, it will determine if addition investigation into cell cycle effects are warranted. Cell survival test the compound's

ability to reduce colony formation though is unable to determine if cell death does occur and if so whether it is necrosis or apoptosis.

3.2.1 Cell Doubling Time

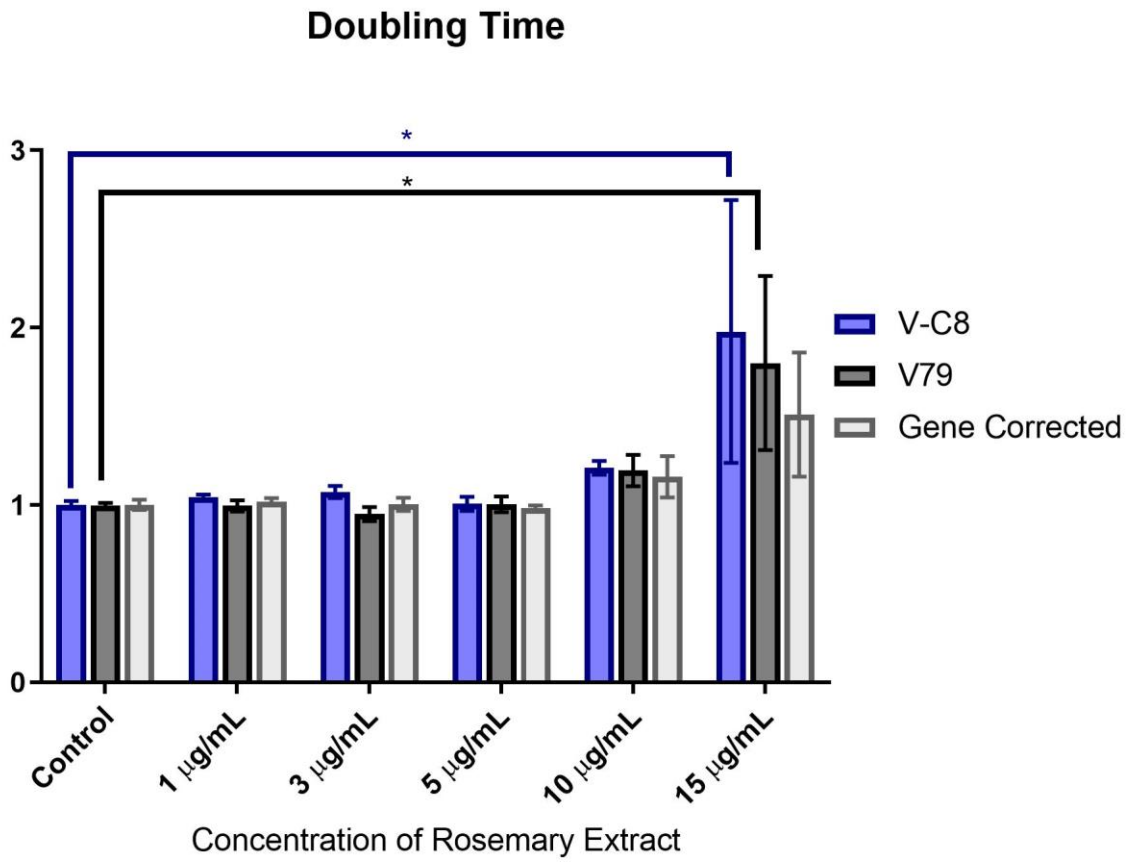

Figure 6: The ratio of observed cell doubling time to the normal, control doubling time of the cell. No statistical significance in doubling across the three cell lines, V79 (black), gene corrected (gray), and the target cell line V-C8 (blue).

After the cell doubling time had been normalized no statistical difference was found between the three cell lines that were tested using a P-value of <0.05. With no statistically significant difference, it is unlikely that the primary mechanism of action for selective cell death is connected to cell cycle arrest. However, a statistically significant difference was found between the control and the 15 $\frac{\mu g}{mL}$ concentration for both V79 and

V-C8 with a p-value of 0.0205 and 0.0207 respectively. Although this may not be the mechanism of action for selective cell toxicity it is important to note that at sufficiently high enough doses there was non-specific cell toxicity observed.

A two-way analysis of variance (ANOVA) was done for the data set to determine if there was a factor, either cell line or concentration that caused statistically significant changes in the data. The two-way ANOVA indicated that concentration was the only statistically significant factor effecting the doubling time with a p-value of 0.0002. Cell line was calculated to not be a contributing factor to the changes in doubling time even if a 90% confidence interval (CI) was used as opposed to the standard 95% CI used for all of the other analysis. As there was a confirmed factor effecting doubling time, a follow up test was used to determine the exact points that were statistically significant. In the case of cell doubling time a Dunnett's multiple comparison test was used. Two different pairs of data were discovered, each between the control and the maximum concentration of 15 $\frac{\mu g}{mL}$. The cell lines V79 and V-C8 had p-values of 0.0205 and 0.0207 respectively between those two concentrations.

3.2.2 Colony Survival

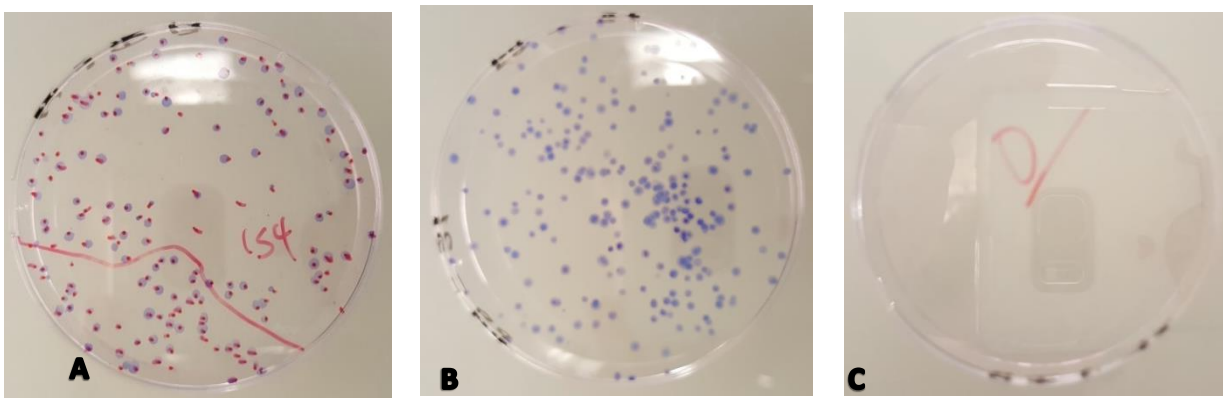

Figure 7: Images of typical plates with colonies used in colony survival experiments in the Kato Lab, including a counted plate (A), a yet to be counted plate (B) both with varying colony sizes and shaped present, and a plate with no colonies meeting the minimum cell count of 50 cells per colony in order to count as a surviving colony (C).

While the cell doubling time was not statistically significant across the cells lines there was a noticeable and statistically significant differences were found between V79 and V-C8 at both 20 $\frac{\mu g}{mL}$ (P-value < 0.001) and 25 $\frac{\mu g}{mL}$ (P-value < 0.0001) and between the gene corrected and V-C8 at 20 $\frac{\mu g}{mL}$ (P-value < 0.01) and V79 at 25 $\frac{\mu g}{mL}$ (P-value < 0.01). This suggests that while the cell cycle may not be disrupted there is some form of selective cell toxicity occurring, additionally that this selective cell toxicity is in some way tied to the BRCA2 deficiency in the target cells. Similar to what was done to analyze if there were, and if so which, factors that contributed to the change in colony survival a two-way ANOVA was done, and then followed up with a post-hoc test to determine where the statistically significant variation occurred in the data. The Turkey's test determined four points of statistically significant variances in the data.

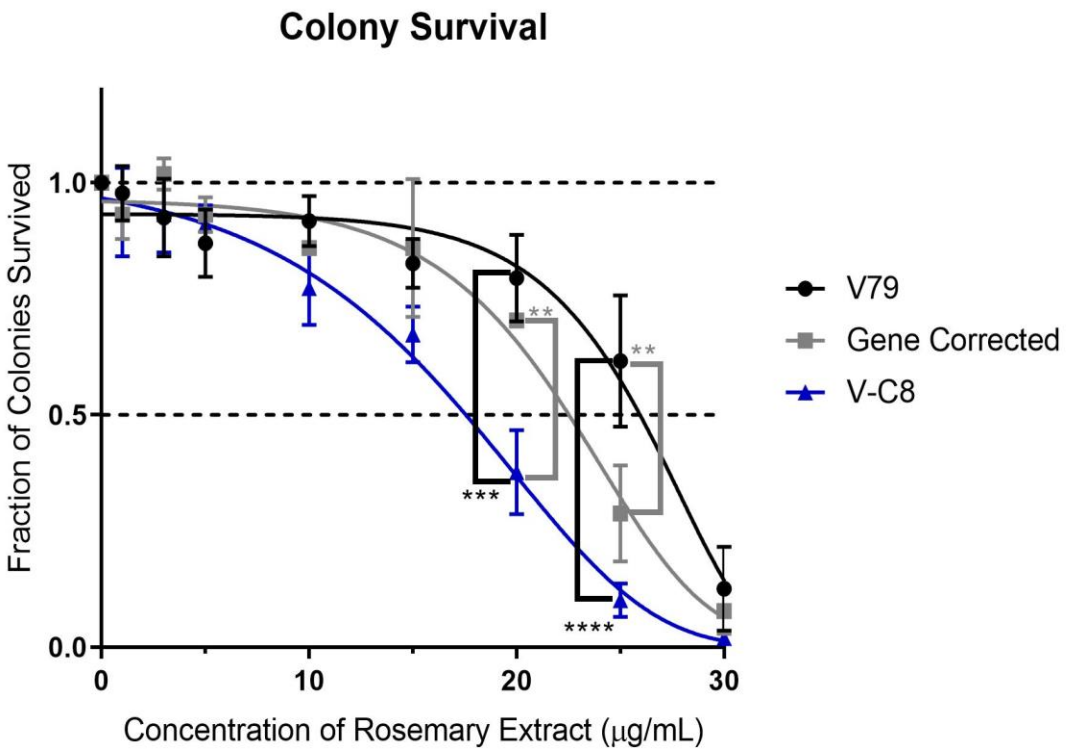

Figure 8: The fraction of surviving colonies at 1 $\frac{\mu g}{mL}$, 3 $\frac{\mu g}{mL}$, 5 $\frac{\mu g}{mL}$, 10 $\frac{\mu g}{mL}$, 15 $\frac{\mu g}{mL}$, 20 $\frac{\mu g}{mL}$, 25 $\frac{\mu g}{mL}$, and 30 $\frac{\mu g}{mL}$ of rosemary extract vs. the respective control. As with the cell doubling time, three cell lines were used, V79 (black), gene corrected (gray), and the target cell line V-C8 (blue). Standard error of the mean has been plotted on the graph as the error bar value.

These points were between V79 and V-C8 at both 20 $\frac{\mu g}{mL}$ (P-value < 0.001) and 25 $\frac{\mu g}{mL}$ (P-value < While the cell doubling time was not statistically significant across the cells lines there was a noticeable and statistically significant differences were found between V79 and V-C8 at both 20 $\frac{\mu g}{mL}$ (P-value < 0.001) and 25 $\frac{\mu g}{mL}$ (P-value < 0.0001) and between the gene corrected and V-C8 at 20 $\frac{\mu g}{mL}$ (P-value < 0.01) and V79 at 25 $\frac{\mu g}{mL}$ (P-value < 0.01). This suggests that while the cell cycle may not be disrupted there is some form of selective cell toxicity occurring, additionally that this selective cell toxicity is in some way tied to the BRCA2 deficiency in the target cells. Similar to what was

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3.2 PARP Inhibition

As the target cell line of V-C8 are BRCA2 deficient, PARP inhibition is a likely candidate for the primary mechanism of action in selective cell killing of only the target cell line. PARP inhibition is fairly easy to test for and does not require cell culture in order to do so. If no PARP inhibition is observed than another pathway must be the culprit in the case of selective cell killing. Additionally, testing inhibition outside of the cellular system would show that rosemary extract primary mechanism is not affecting upstream processes in PARP production, but inhibiting PARP itself.

3.2.1 Rosemary Extract

Despite the difference in the two curves, there is evidence of there being PARP-1 inhibition occurring. There is approximately 20% PARP-1 inhibition in sub LD_{50} doses in off target cells. As mentioned before, it is critical to keep in mind that this data was gathered from an assay that tests PARP-1 activity in a non-living setting. It is still possible those other members of the PARP family are inhibited or that there are additional routes of inhibition that rosemary extract is capable of within the cellular environment. There were overlapping 95% CI calculated between the Y_0 (fraction of PARP-1 activity at concentration=0) and the plateau (fraction of PARP-1 at concentration= ∞) which makes sense as absence of the inhibitor should be the same regardless of the inhibitor, and at a high enough concentration all PARP-1 would be inhibited, even by a weak inhibitor, though it is important to note that unlike non-

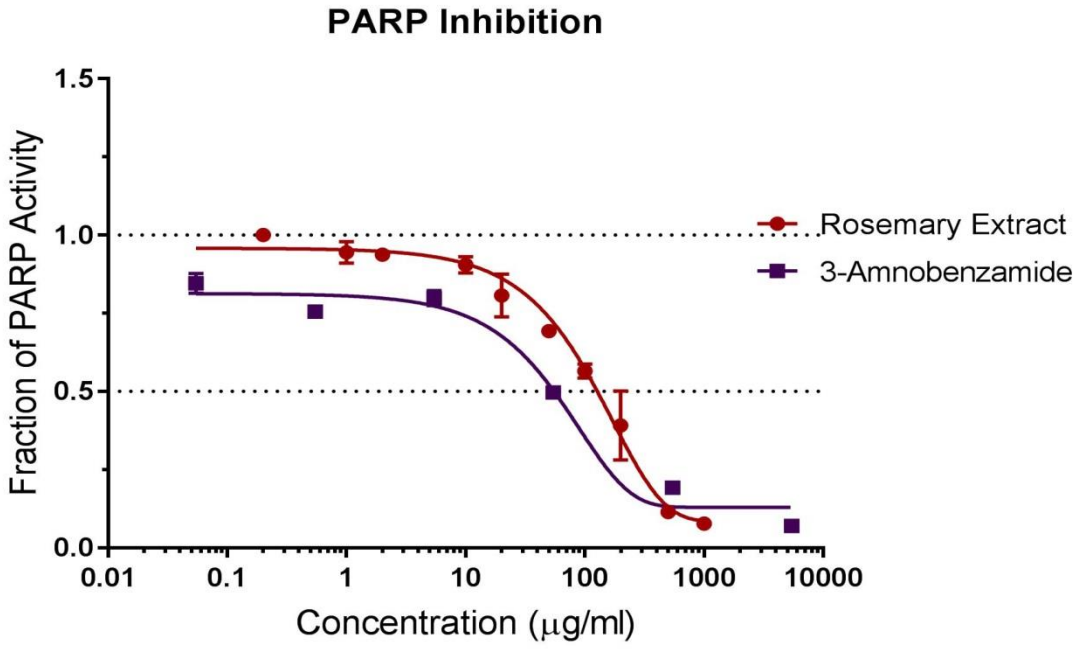

Figure 9: The fraction of PARP-1 inhibited by rosemary extract (red) and known PARP inhibitor 3-AB (purple), standard error of the mean has been plotted on the graph as the error bar value.

overlapping CI's, overlapping CI's do not mean that there is or is not a statistically significance at that CI. The IC_{50} (concentration at which 50% of PARP-1 is inhibited), tau (is a concentration constant and reciprocal of K), and K (rate constant). Based on this information, both the rate at which concentration effects PARP-1 activity and the necessary dose required to have half of PARP-1 inhibited are statistically different from one another at the 95%CI. 3-AB is the more efficient and in turn requiring a lower concentration to inhibit half of PARP-1

When comparing the one phase decay curves of best fit there is a statistically difference in the curves calculated for the two data sets ($P<0.0001$). The plateau, K, Tau, CI_{50} and Y_0 were used as points of comparison between the two curves. Based on the calculated 95% CI there were only two factors which had overlapping ranges, Y_0 and the plateau. The three parameters that do not have overlapping CI are to be considered at statistically significant with a P-value<0.05. The R square for rosemary extract and 3-AB is 0.9422 and 0.9633 respectively.

3.2.2 Components

As is shown by our own data, the primary compounds are capable of PARP inhibition, especially in the case of carnosol, carsonic acid and gallic acid. More importantly at this concentration, 69.1%, 70.3%, 70.4%, and 35.4% of PARP is inhibited on average by carnosol, carsonic acid, gallic acid, and rosemarnic acid respectively. Finally, given these concentrations are in µM per liter while the rest of the experiments have concentrations in µg per milliliter. The 100 µM concentrations of carnosol, carnosic acid, rosemarnic acid, and gallic acid converted to $\frac{\mu g}{mL}$ would be 33.042 $\frac{\mu g}{mL}$, 33.240 $\frac{\mu g}{mL}$, 17.012 $\frac{\mu g}{mL}$, and 36.030 $\frac{\mu g}{mL}$. Additionally, Gifu University looked at the individual primary

compounds and determined the level of PARP inhibition at a set concentration (100 µM) and repeating the measurements for a total of four trials per compound. An ordinary one-way ANOVA with Turkey's test follow up showed no statistically significant differences between the four compounds at the 95% CI, though there are statistically significant differences between rosmarinic acid compared to the other three compounds if a 90% CI was used. As all other data sets use a 95% CI it was decided to not make an exception with this data set.

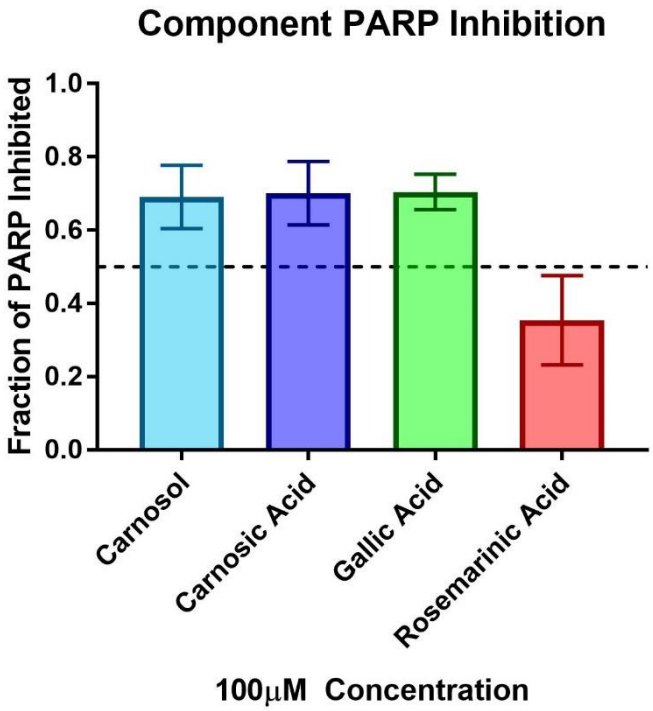

Figure 10: The fraction of PARP-1 inhibited by each of the four known components in rosemary extract, carnosol (teal), carnosic acid (blue), gallic acid (green) and rosemarnic acid (red) at a concentration of 100 µM standard error of the mean has been plotted on the graph as the error bar value. Each of the concentrations of the compounds in $\frac{\mu g}{mL}$ are as follows (from left to right), 33.042 $\frac{\mu g}{mL}$, 33.240 $\frac{\mu g}{mL}$, 17.012 $\frac{\mu g}{mL}$, and 36.030 $\frac{\mu g}{mL}$.
Provided by Gifu University, Gifu Japan

3.3 Anti-Oxidant Capabilities

Based on the current knowledge of flavonoids, at least some compounds with in rosemary extract probably act as anti-oxidants. While there are a few different pathways for reducing free radicals, testing hydrogen donation and single electron transfer are well defined and common pathways for antioxidants. DPPH assays and TAC assays are well known and simple tests that determine the effectiveness of an anti-oxidant's ability to donate hydrogen and to transfer a single election. While this is not directly related to the selective toxicity to BRCA2 deficient cells, antioxidant activity is associated with improving quality of life, reducing risk of cancer developing[17], and increasing chance of survival[19].

3.3.1 TAC Assay: Rosemary Extract

A statistically significant difference was observed between ascorbic acid and rosemary extract at two different concentrations, 1,000 $\frac{\mu g}{mL}$ (P-value =0.0265) and at $100\frac{\mu g}{mL}$ (P-value < 0.0001). For analysis of the TAC results two-way ANOVA was used to determine if the two compounds deferred in a statistically significant way, followed up by a post hoc test to determine which point was causing the variance. The two-way ANOVA showed that the ascorbic acid and the rosemary extract have statistically significant difference when it comes to TAC assay results with a P value of <0.0001. The follow up Sidak's test discovered a statistically significant difference was observed between ascorbic acid and rosemary extract at two different concentrations, 1,000 $\frac{\mu g}{mL}$ (P-value =0.0265) and at $100\frac{\mu g}{mL}$ (P-value < 0.0001). The IC_{50} that was used was found via the half-life for the one phase decay model used for the curve of best fit of both

rosemary extract and ascorbic acid were found to be 131.2 $\frac{\mu g}{mL}$ and 27.04 $\frac{\mu g}{mL}$ respectively.

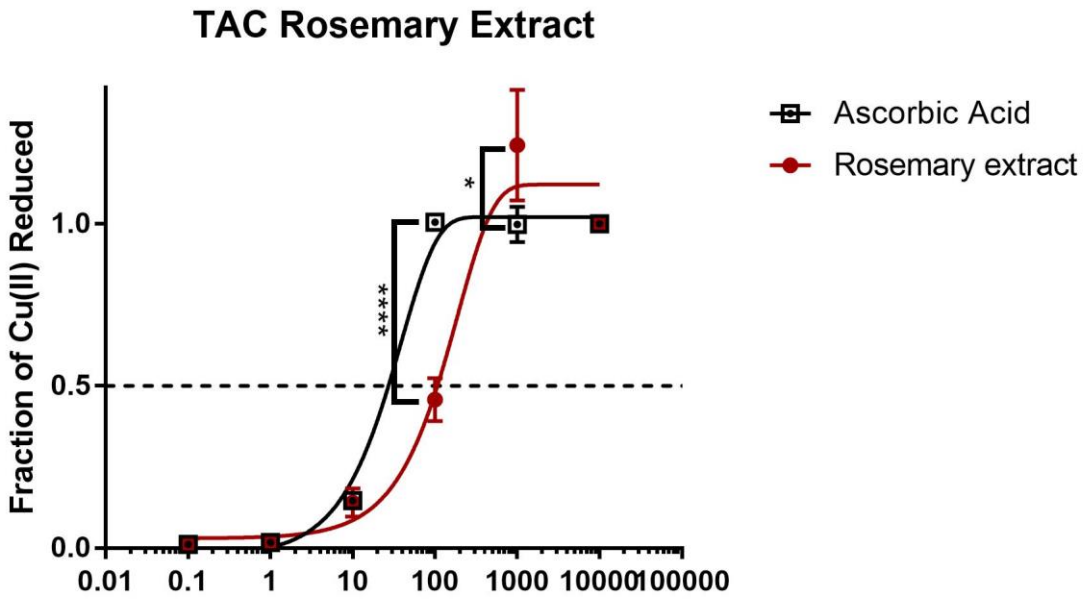

Figure 11: Fraction of Copper (II) reduced to Copper (I) by rosemary extract (red) and ascorbic acid (black). Standard error of the mean has been plotted as the error bars for this graph. The approximate concentration at which 50% of the Cu(II) has been reduced is 27.04 $\frac{\mu g}{mL}$ for ascorbic acid and 131.2$\frac{\mu g}{mL}$ for rosemary extract.

3.3.2 DPPH Assay: Rosemary Extract

Based on the DPPH assay data collected lines of best fit were created based on a sigmoidal four parameter logistic regression (4PL) model. The statistical analysis showed statistical difference between the two models with a P-value of less than 0.0001. Then to compare the two data sets, as the concentrations were not the same cross the different compounds the two curves had to be compared based on their top, bottom, and logIC_{50}. Finally, the IC_{50} of the rosemary extract and the ascorbic acid were found to be 12.45 µg/mL and 0.9147 µg/mL respectively. In regards of goodness of fit, the R squared was 0.9401 for rosemary extract and 0.9147 for ascorbic acid.

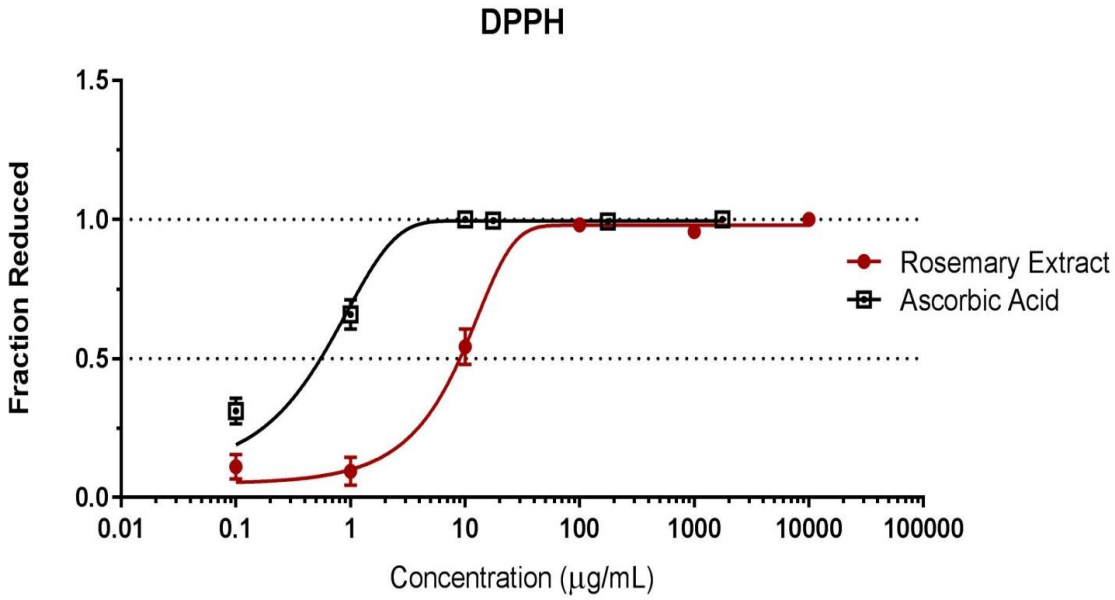

Figure 12: The fraction reduced of the stable free radical DPPH, rosemary extract (red) reaches 50% reduction at 8.90 µg/mL while ascorbic acid (black) reaches the same level of reduction at 0.54 µg/mL standard error of the mean has been plotted on the graph as the error bar value.

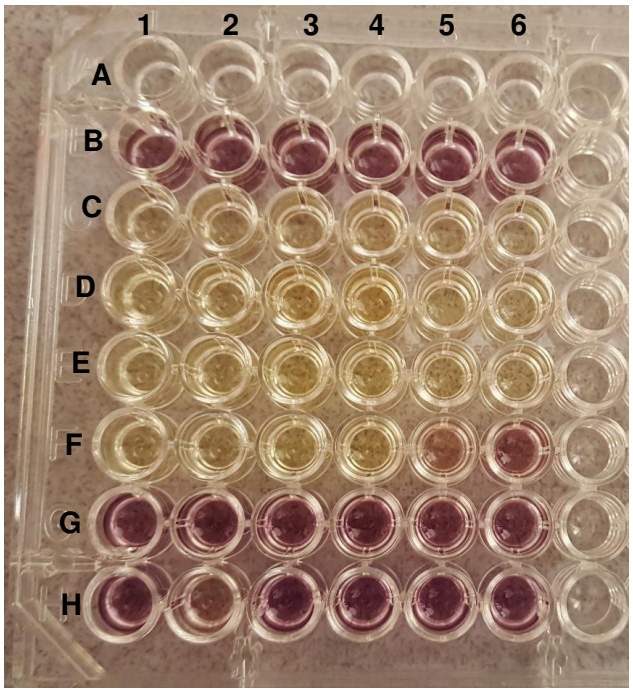

Figure 13: A complete DPPH assay well plate with ethanol blank (**A**), negative control (**B**), positive control 10 mM ascorbic acid (**C**), 500 $\frac{\mu g}{mL}$ of test compound (**D**), 50 $\frac{\mu g}{mL}$ of test compound (**E**), 5 $\frac{\mu g}{mL}$ of test compound (**F**), 0.5 $\frac{\mu g}{mL}$ of test compound (**G**), and 0.05 $\frac{\mu g}{mL}$ of test compound (**H**). Column **1** and **2** test carsonic acid, **3** and **4** test gallic acid, and **5** and **6** test rosemarinic acid. As DPPH is reduced the solutions shifts from purple to yellow.

CHAPTER 4: DISCUSION

The primary goal is to discover if the rosemary extract provided by Gifu University in Gifu, Japan is capable of killing only target cells while sparing off target cells. Using cell culture to test different cell lines in an accepted and safe way to start initial investigations in to the cell toxicity. Although animal models are preferred for determining the potency and possibly adverse effects in a biological system, the lack of information about rosemary extract in mammalian cell culture let alone animal models makes them impractical at this point. Once the ability for selective cell toxicity is established, the mechanism of action can be determined.

Based on our experiments rosemary extract and specific components did exhibit both PARP inhibition and mild antioxidant properties. PARP inhibition by rosemary extract against a known (fig 9) and, PARP inhibition by component at a given concentration (fig 10) show that rosemary extract can act as PARP inhibition and that the primary components involved in this property are carnosol, carsonic acid and gallic acid with some contribution from rosemarnic acid. Additionally, both DPPH (fig 12) and TAC (fig 11) assays of the extract as a whole demonstrated, while not on par with ascorbic acid, hydrogen donation at a low enough concentration to be beneficial in a medicinal capacity. Additionally, we know that selective targeting of the desired cell line does occur (fig 8) and can narrow the mechanism of action down due to the lack of a selective effect on doubling time based on cell line (fig 6). A potential issue that has

arisen from these results is that the concentration at which there are statistically significantly fewer colonies forming is higher than the concentration that starts effecting cell doubling time across cell lines.

4.1 Purposed Mechanisms of Action

The most interesting property of rosemary extract found in our experimentation was the apparent selective cell toxicity against BRCA2 deficient cells at low enough concentrations low enough to spare the off target “healthy” cell lines. With the extensive amount of research available regarding PARP inhibitors being toxic to cells that are BRCA2 deficient and our own results showing noticeable PARP inhibition *in vitro* when compared against a known PARP inhibitor, it is very likely the primary mechanism of action involved PARP inhibition. This mechanism of action would also explain the non-cell line specific effects on doubling time seen in fig. 6 as PARP is not integral to the cell cycle. However, this does mean that there are multiple mechanisms of actions in play, which should be expected with a plant-based extract. For our purposed we focused solely on the selective toxicity against the target cell line V-C8 displayed in fig. 8.

In regards to the demonstrated anti-oxidant properties, a proposed mechanism is significantly easier to determine based on both literature and results observed in our experimentation.

4.1.1 PARP Inhibition

PARP inhibition when coupled with BRCA2 deficiencies tends to cause four negative effects on the cell in regards to DNA repair. First, PARP-1 can become trapped, preventing the physical repair of the DNA from going on[18], like a wrench being

thrown into the gears of a machine (depicted in fig 3). Additionally, the cell's ability to do single strand break repair and other model of BER is inhibited as it is not possible for XRCC1 to be recruited to the point of the break and interact with DNA ligase III[18]. This occurs due to the lack of PAR production by the PARP-1 preventing the PARP from detaching from the DNA[18, 38]. The lower levels of PAR also mean less inhibition of NHEJ as not enough PAR is available to bind to proteins involved with NHEJ and prevent the

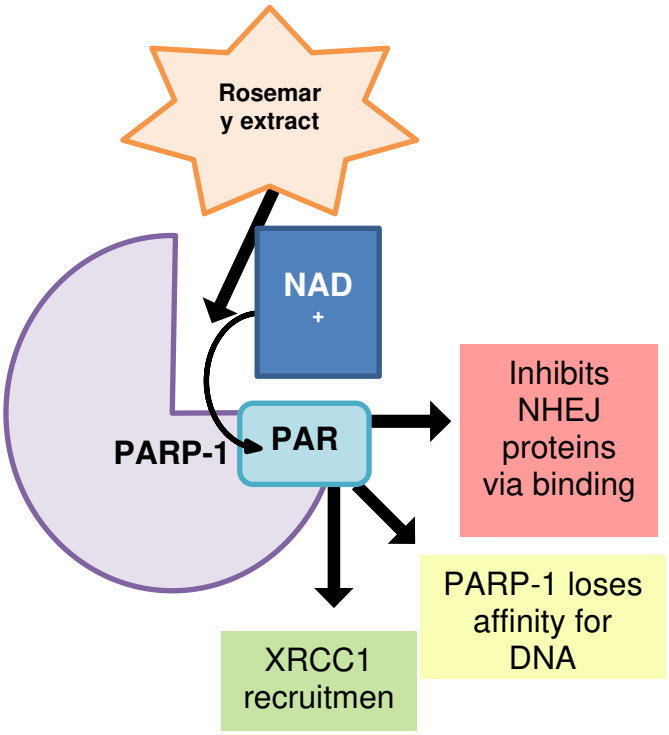

Figure 14: Graphic depicting rosemary extract inhibition of PARP-1 by in some fashion preventing the binding of NAD^+ or by preventing the conformational change that is needed for PARP-1 from detaching from the DNA by inhibiting the NAD^+ production of PAR. Additionally it is speculated that PARP-1 can act as a wrench in the works in the single step model of BER preventing ligation from occurring.

NHEJ proteins from binding from the DNA[18, 38]. This coupled with the BRCA2 deficient cell's diminished ability to use HR forces an increased reliance on NHEJ[38].

PARP-1, as mentioned earlier is involved with but does not physically repair damage to DNA. PARP-1 does a similar job in both SSBR and the two-step BER model

in recruiting ligation proteins, including XRCC1. In both cases PARP-1 does leave the DNA before ligation can occur, and in the on-step BER model, PARP-1 is not involved in a correctly functioning model[18]. This being said, when a cell is exposed to PARP inhibitors the cell loses the ability to attract XRCC1 to the site of the single strand breaks and, in the case of the one-step BER model, PARP-1 can end up jamming the ligation step, preventing both the short and long patch repairs.

While there may be other processes that lead to the observed selective cell killing of BRCA2 deficient cells, the lipophilic nature of the components in rosemary extract, and the demonstrated PARP inhibition both by the extract as a whole and the primary components strongly suggested that PARP inhibition plays a role in selective cell death of the BRCA2 deficient cells[6, 12, 13]. We have shown in multiple experiments that our rosemary extract does inhibit PARP-1 (fig 9 and fig 10), and acts in such a way to selectively be toxic to BRCA2 deficient cells (fig. 8). This coupled with the non-polar nature of the primary four compounds strongly suggests that rosemary extract is entering the cells in culture and inhibiting PARP-1 in a sufficient quantity to present as statistically significantly fewer colonies forming in the on-target cell line than the two off target cell lines.

In regards specifically to how the PARP-1 is inhibited it is likely in a similar fashion to PARP inhibitors currently undergoing clinical trials, olaparib, veliparib, and, niraparib, for example[1]. These PARP inhibitors act as competitive antagonists of NAD^+, preventing the NAD^+ from binding to PARP and thus preventing the creation of PAR. As carnosol, carnosic acid, and, gallic acid have expressed the highest levels of PARP inhibition it is likely that those flavonoids are what are acting as the antagonist and

ultimately leading to the death of the BRCA2 deficient cells. While there is another way for PARP to be inhibited it involves interaction with caspases, which none of the previously mention three compounds are known to be.

By continuing to examine the specific mechanisms of cell death in BRCA2 deficient cells, it would be prudent to compare both primary BRCA2 deficient cells along with a normal, off target primary cell line rather than continue on in transformed cell lines. V-C8 and V79 cells are both immortal cell lines isolated originally from Chinese hamster lung cells, which although it gives us initial data could potentially be problematic in elucidating the actual cause of pathology with flavonoids, and rosemary extract in particular. While immortality makes the cells easy to use for cell culture, they inherently have mutations that genetically make them different from primary cells. Additionally, these are also hamster cells, which do have genetic differences from human cells, such as hamster cells having 22 chromosomes vs 46 in humans.

4.1.2 Antioxidant

As shown in figures 11 and 12 rosemary extract as a whole is capable of both hydrogen donation (DPPH assay) and electron donation (TAC assay). As mentioned before the DPPH assay is test the compound's ability to donate a hydrogen to the stable free radical DPPH. This particular ability of a compound should not be confused with other methods of antioxidant activity. In regards to ascorbic acid, it is often considered a middle of the road antioxidant so a compound with a similar IC_{50} in theory should be approximately equal in strength. With there being a 16.5-fold difference between the two compounds it is safe to consider rosemary extract as not a strong electron antioxidant

by way of hydrogen donation. This does not mean that there are no antioxidant qualities, just that they are not a primary benefit of rosemary extract.

Similar to with the DPPH assay it is crucial to remember that the TAC assay only tests one aspect of a compound's antioxidant capabilities. In the case of the particular TAC assay the single electron donation attribute was tested, using ascorbic acid as a point of reference for the sake of consistency. Once again, the IC_{50} of each compound is compared and only a 4.76-fold difference was found between the two. However, it is important to note that both ascorbic acid and rosemary extract underwent a 50.93 and 14.71-fold increase in concentration respectively from the DPPH assay. While rosemary extract may behave more similarly to ascorbic acid in regards to how efficient it is at single electron transfer, they both appear to be far better at hydrogen donation.

Based on literature available, the most likely of the known compounds to be engaging in anti-oxidant activity would be carnosol and carsonic acid[23]. As far as the specifics go for the mechanism of action, as mentioned above based on our data in fig. 11 and 12 and data available from other sources hydrogen donation seems the most likely the primary method.

4.2 Overall Summary

Cancer preventatives are an evolving field of medicine, especially for those with a genetic predisposition for the disease. As genetic mutations that result in an increased risk of cancer have few treatments and cannot be avoided in the same way that environmental or life style risks can be, even though a smaller part of the global population is affected by this it worthwhile to investigate. Breast cancer in particular is associated with a mutation in the *BRCA2* gene that results in either lower levels or lack

of the protein by the same name. BRCA2 deficient cells are sensitive to PARP inhibitors[6, 12, 13] and have a tendency to die as a result of exposure to them. Due to the connection of BRCA2 deficiencies and breast cancer along with a clear, well characterized interaction that leads to the death of BRCA2 deficient cells, V-C8 was selected as the on-target cell line to test for selective killing in cells exposed to rosemary extract.

In an effort to determine if the rosemary extract provides was capable of working as a cancer preventative supplement for persons with a BRCA2 mutation, we utilized three different groups of experiments. The first experiments were done in target and off target cell lines to determine if rosemary extract could affect the BRCA2 deficient cells preferentially and spare the off-target cells that lack the mutation. Results here showed an increased sensitivity in the target cells to rosemary extract in colony formation, with no truly difference in effect across the cell lines when cell doubling time was examined. The next group was in order to narrow down the mechanism of action. A colorimetric assay indicated that there was indeed PARP inhibition by rosemary extract, while a probe at PARG inhibition showed not apparent effect. These results suggested that an effect directly on PARP, whose inhibition has been well characterized as toxic to BRCA2 deficient cells[5,12,13], was the cause of the observed selective cell killing. Finally, possible additional cancer preventative properties were assessed. Given the primary compound, this has been confirmed by Gifu University, is a flavonoid, a natural exploration in this topic would be to test for rosemary extract's ability to act as an antioxidant. While there was definite evidence of antioxidant activity in both assays used, the results did not support rosemary extract being a strong antioxidant.

While the contents of rosemary extract may not be completely known, some of the extract's compounds have been characterized. Carnosol, carnosic acid, gallic acid, and rosemarinic acid, are known to be parts of the extract, with the first two being confirmed as primary compounds. As a way to allow for comparison to other rosemary extracts, a UV-Vis spectrum was taken of the rosemary extract. The peaks of the 3 compounds tested were similar to established values with only a few nanometers different from the accepted values. These differences may be due to DMSO used as the solvent, or variances in the Nanodrop used.

Once a "finger print" was created for the rosemary extract, testing of the potential of the extract as a cancer preventative was conducted. Any effects to cell cycle were examined by observing the effects to cell doubling time with varying exposure concentration. After analyzing the data collected, no significant difference between cell lines was observed due to increases in concentration of rosemary extract, though there was an over all, non-specific effect across cell lines at 15 $\frac{\mu g}{mL}$. As the difference in the doubling time increases, there is unlikely to be an effect on the cell cycle that could be result in cell death, at least not specifically. The three cell lines were then tested for selective cell toxicity, and more importantly to see if any selective toxicity would be focused on the on-target cell line of V-C8 when exposed to rosemary extract. Interestingly selective cell toxicity was observed, V-C8 dishes formed fewer colonies at a statistically significant ratio when compared at the same concentration than the off target V79 and gene corrected cell lines. Although the gap between curves is smaller than would be preferred, there is proven selective cell toxicity.

Since the ability of rosemary extract to selectively kill BRCA2 deficient cells has been observed (fig 8), determining the mechanism of action is an important next step. As mentioned, several times before, BRCA2 deficient cells are particularly susceptible to PARP inhibition, enough PARP inhibition will result in synthetic lethality within the deficient cells[6, 12, 13]. Given this information, PARP inhibition appears to be a likely candidate for the cause of the selective cell toxicity. In order to observe the capacity to which rosemary extract can inhibit PARP, an assay was conducted outside of the cellular environment. As the primary testing components are lipophilic, and some are known to be able to cross the blood brain barrier, and the blood placental barrier, it is likely they are capable of entering a cell with minimal difficulties. In the PARP assay, 3-aminobenzamide, a known PARP inhibitor, was used as a point of comparison, and while there is a statistically significant difference between the two, sub LD_{50} doses of the extract do act as PARP inhibitors. Gifu University also conducted a similar test in which they looked at the PARP inhibition qualities of each of the known components of rosemary extract. All of the compounds expressed some level of PARP inhibition with the two primary compounds expressing the largest percentage of PARP inhibition. Based on what other PARP inhibitors currently undergoing clinical trials have as their specific interaction with PARP, it is likely that carnosol, carnosic acid, and/or gallic acid competitively bind and block the NAD^+ binding domain on PARP[1]. In doing this PARP is un-able to detach from the DNA or to create pADPr[1,18]. While there is only approximately 20% inhibition at the LD_{50} point for the off-target cells, BRCA2 deficient cells are particularly susceptible to PARP inhibition making sub 20% inhibition

potentially enough to kill the on-target V-C8 cells while sparing the off-target cell lines of V79 and the gene corrected variant.

Flavonoids almost universally can and do act as antioxidants[5], and when the effects of antioxidants are investigated in connection with improving the chances of survival and quality of life among cancer patients, assessing at least some of the antioxidant qualities of the rosemary extract. Once again, the compound was assessed as a whole along with the cell survival and cell doubling as this will be used as a total compound and not its parts. Both hydrogen generation and single electron transfer abilities were tested using a DPPH assay and TAC assay using Copper (II) respectively. Based on the concentrations required to achieve full reduction of both, rosemary extract is more efficient at hydrogen donation than single electron donation. Additionally, rosemary extract is not as good an antioxidant, via either of those two pathways, as ascorbic acid. Since ascorbic acid is considered to be more of a middle of the road type of antioxidant, rosemary extract is unlikely to be classified as a strong anti-oxidant, at least in regards to those two processes.

Carnosic acid has been shown in literature to have some interesting and favorable properties. All though there has been previous evidence to support the selective killing of cancer cell lines by carsonic acid, none of the cell lines tested were BRCA2 deficient. While MCF-7 is a breast cancer cell line and exposure to carsonic acid leads to cell death, this cell line lacks a BRCA2 mutation and has been used as a control for BRCA2 expression in some cases. In breast cancer, specifically, approximately 5-10%[11] of cases are due to genetic causes. While this may not be anywhere near a majority of breast cancer cases, as mentioned earlier it is difficult to

treat a genetic cause of cancer. As a *BRCA2* homozygous negative embryo has difficulty dividing[14], and thus becoming viable to begin with, the mutation more frequently present is heterozygous with a mutation occurring later on in life that leads to the loss of both alleles. The estimate of how prevalent the heterozygous mutation is between 1 in 400 and 1 in 800[11] and the only viable treatment at present for reducing one's rick of breast cancer in these patients is mastectomy[11]. There is a clear need for a potential alternative that can preemptively kill the *BRCA2* heterozygous cells. BRCA2 itself is associated with homologous recombination and interacts directly with RAD51 and the DNA during this process and assists in correctly positioning the RAD51 in the intermediate of the 3' overhang. Additionally, BRCA2 appears NHEJ by blocking binding of critical NHEJ proteins to the DNA.

In tests investigating potential neuroprotective effects of carnosol and carnosic acid it was found that both compounds act to reduce inflammation in the nervous system, at 5 µM by reducing the secretion of select proteins, such as IL-6, a pro-inflammatory cytokine[1]. Additionally, both compounds are able to protect the hippocampus in rats from undergoing apoptosis when exposed to some toxins[1] namely Amyloid-β 25-35. Additionally, there is evidence that carnosic acid may be helpful in assisting in weight loss by inhibiting gastric lipase[36], at least in rats when fed a diet with high levels of rosemary extract. Most importantly was the study done with carnosol and mice exposed to benzo[a]pyrene. After the exposure and subsequent promotion by 12-O-tetra decanoylphorbol-13-acetate (TPA), it was found that there was a decrease in tumors on the skin of the exposed mice[20]. When the mechanism of action was investigated, it was found that less benzo[a]pyrene was bound to the DNA in the mice

treated with a rosemary extract shortly before each benzo[a]pyrene exposure in comparison to the control group treated in a similar fashion with acetone[20]. Over all, carnosol and carsonic acid are promising compounds to assist in treating a wide vary of issues. As is gallic acid, although there is less information about this compound in literature than there is in regards to carsonic acid and carnosol. However, despite gallic acid having a molecular weight that is nearly half that of carnosol and carsonic acid, it is the least soluble out of the four tested compounds potentially making it harder for gallic acid to pass through the cell membrane despite its smaller size.

With positive results about rosemary extract acting as a PARP inhibitor outside of a cellular environment, selectively targeting of BRCA2 deficient cells and lack of strong antioxidant capabilities, PARP inhibition logically seems to be at least one of the primary mechanisms of action leading to the toxicity observed in of target cells at sufficiently high enough concentrations while at least partially sparing the off-target cell lines. As this was only a partial investigation, it is entirely possible that there are other mechanisms of action that affect both the off target and the target cell lines provided rosemary extract is in high enough concentrations. As mentioned earlier, unfortunately the complete breakdown of what compounds are within rosemary extract it is difficult to test of any and all interaction between compounds inside the rosemary extract. While the antioxidant abilities of rosemary extract are not great, it may still have an effect, especially since the known compounds have been documented passing through stringent barriers in the body such as the blood brain barrier.

Cells used in our experiments were derived from a Chinese hamster lung tumor, thus selecting a rodent in future in vivo experimentation would be a good potential

animal model as successful experimental data has already been gathered in vitro. Presently there already exist BRCA2 deficient mice[14], eliminating the large and time-consuming step of creating a new line of mice with the needed BRCA2 deficiency. As these models have been refined over the years in order to observe the effect of the BRCA2 and BRCA1 mutations in humans[14], they would more accurate as a model for rosemary extract's ability to act as a selective toxic cancer preventative. While rats would be preferable over mice as rat livers are more physiologically similar to a human liver than a mouse liver, any animal model would give better insight into how best to deliver the rosemary extract at sufficient concentrations to cancerous tissue without harming the animal themselves. Ideally a decrease in the number of tumors formed would be observed in an animal model, although even a decrease in the size of the tumors or an increase in time before tumors were observed would be a positive outcome.

Above all, the most critical attribute for rosemary extract to possess is the ability to enter cells. As the target based on the perceived mechanism of action resides inside the cell, even the strongest PARP inhibitor would be unable to effect BRCA2 deficient cells if it was unable to enter the cell and interact with PARP. While exact values are difficult to find, current PARP inhibitors that are undergoing clinical trials are being administer in 400 mg twice a day dose[1], with positive increases in survival rate observed. At the present moment, there does not seem to be a benefit of oral over topical route for treatment. Delivering rosemary extract via injection of any kind is likely unnecessary and further investigation into this particular aspect of rosemary extract would seem to be better suited as either oral as a supplement or a food additive or as a

topical application taking advantage of the natural non-polar properties of the known compounds in the extract. The disadvantage to topical involves how long it would take for the compounds to be absorbed and be delivered to the target site in the breast tissues, while oral administering of rosemary extract does run into the potential issue of biotransformation via the liver as the blood from gastrointestinal area cycles through the portal veins before going to the heart and delivered to breast tissue. Additionally, the dose would have to be large enough for there to still be a high enough concentration at the desired site after it has cycled through at least part of the body, especially as a non-polar compound that seems to easily pass through membranes and barriers. If the concentration of 10 µg/mL is applied to the human body, with a density of approximately 985 kg/m^3 and the average weight of a human based on the FDA is 60 kg, the average person would need to have 610 mg per day provided all of the rosemary extract was evenly distributed and the pharmokinetics allow the rosemary extract to stay active and available in the body for a 24-hour period at sufficient concentrations. This is not quite a reasonable consideration due to the likelihood of rosemary extract tending diffuse out of the plasma and into tissues, potentially making it hard for a sufficient dose to be available within the breast tissues themselves. Surprisingly, at least for oral ingestion 610 mg, even in one tablet, is a dose that could be consumed in a realistic scenario.

If this line of inquiry were to continue a few things would be logical next steps to better characterize this rosemary extract. First would be determining what the majority of the extract is made up of other than carnosol and carnosic acid, and potentially how many other compounds are in the extract. Knowing what components make up rosemary extract would open up the possibility of testing for any compound interactions

and possibly aid in refining the extract to make it more potent. Next would be testing in primary cells as immortal cell lines do have their own inherent issues that are a result of multiple splits and the genetic mutations that make those cell lines immortal. Finally, provided that primary cell trials go well, testing in an animal model to prove cancer prevention via rosemary extract treatment is a possibility would provide a method to test for any issues with biotransformation or method of administration whether oral or topical.

With regards to the extract itself, as opposed to its capabilities, identifying how much of which compounds are in the rosemary extract would be important. At the moment only four compounds are known with absolute certainty to be in the extract, rosemarnic acid, gallic acid, carnosol, and carnosic acid, with the last two being primary compounds in the extract. While a UV-Vis spectrum was taken of rosemary extract, gas chromatography would assist in determining the quantities of these compounds which are known to be present if the compounds can be volatilized. Mass spectrometry would assist in determining the makeup of the entire extract as well as the amounts of these compounds present within the rosemary extract. The potential problem with this line of inquiry is that the company through which Gifu University has received the rosemary extract has been reclined to impart this information.

Finally, some investigation has been done into the possible poly (ADP-ribose) glycohyrdolase (PARG) inhibition by rosemary extract. The preliminary data did not suggest that there was inhibition; however. PARG inhibition does result in cell death; PAR a product of PARP does act as a signal for cell death if it is not degraded by PARG[18]. As no evidence of inhibition was found using a PARG inhibition assay perhaps

a different method of quantifying PARG inhibition could be essential to rule out the possibility of PARG inhibition being a primary mechanism of action.

CHAPTER 5: CONCLUSION

As rosemary extract is a product of plants, it was logical that some of the compounds in the extract would be flavonoids. Flavonoids have been investigated previously for some anti-tumor capabilities with minimal information on the mechanisms involved with cell death. The most direct connection being the inhibition of tumorigenesis triggered by exposure to benzo[a]pyrene[20] with some flavonoids, carsonic acid most importantly, can selectively kill particular cancer cell lines including MCF-7, a breast cancer cell line. Additionally, flavonoids have been well characterized as antioxidants[5], a trait that has been proven to help reduce the risk of cancer. As carsonic acid is a primary component in the rosemary extract, information regarding previous research proved to be invaluable to better understanding the attributes of rosemary extract and determining methods to test new qualities of carsonic acid and of the extract as a whole.

In the end, this particular rosemary extract, that has a high concentration of carnosol and carsonic acid along with measurable contributions to the makeup by gallic acid and rosemarnic acid, has shown promise moving forward as a potential cancer preventative in heterozygous *BRCA2* patients. This is only a preliminary investigation meant to determine if this extract warranted further exploration and based on the data collected further investigation should be done. The dose in the tissues is a realistic amount, potentially along with the amount needing to be consumed provided that the

body's pharmokinetics are favorable to the compounds within the extract. Even if extensive biotransformation occurs when rosemary extract is administered orally, topical application at the target site may be a viable alternative, whether in the form of a patch or a cream. Additionally, what is currently understood as the primary mechanism of action would mostly spare off target cells at concentrations that are selectively toxic to the BRCA2 deficient cells, making this favorable in the field of cancer prevention.

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LIST OF ABBREVIATIONS

3-AB……...3-Aminobenzamide
4PL……Four Parameter Logistic Regression
ADP……Adenosine Diphosphate
ANOVA……..……....….Analysis of Variance
APE1……..…….. AP-endonuclease 1
BER……Base Excision Repair
BARD1……..….BRCA1-associated RING domain protein 1
BRCA1……..……Breast Cancer 1
BRCA2……..……Breast Cancer 2
CI……Confidence Interval
CtIP……..……Retinoblastoma-binding protein 8
DMSO……..……Dimethyl Sulfoxide
DPPH……..2,2-diphenylpicrylhydrazyl
DNA……...…...……Deoxyribonucleic Acid
FBS……...……Fetal Bovine Serum
FEN1……Flap Endonuclease 1
HMGB1……...……...High Mobility Group Box 1
HR……..Homologous Recombination
Gly……Glycosylase
LigI……..…..... Deoxyribonucleic Acid Ligase 1
LigIII……..……..….Deoxyribonucleic Acid Ligase 3
LigVI……....Deoxyribonucleic Acid Ligase 4
MEMα……...Minimal Essential Medium alpha
MRN Complex……...……..…Mre11, Rad50, and Nbs1 Complex
NAD^+…….. ……..……..Nicotinamide adenine dinucleotide
NHEJ……..……...…….Non-homologous End Joining
PALB2……... Partner and locator of BRCA2
PAR……...……..……..…Poly-Adenosine Diphosphate Ribose
PARG……..…….Poly-Adenosine Diphosphate Ribose Glycohydrolase
PARP……....…….... Poly-Adenosine Diphosphate Ribose Polymerase
PCNA……..……..….Proliferating Cell Nuclear Antigen
PNKP……..…….…. Polynucleotide Kinase 3'-Phosphatase
Polβ……..……Deoxyribonucleic Acid Polymerase beta
Polδ……Deoxyribonucleic Acid Polymerase delta
RAD51.....RAD51 recombinase
RING……..Really interesting new gene
RNA…… Ribonucleic Acid

RPA…….Replication protein A
SSBR……...Single Strand Break Repair
TAC……..…….Total Antioxidant Capacity
TPA……..12-O-tetra decanoylphorbol-13-acetate
XRCC1…….……X-ray cross-complementing gene 1