CHARACTERIZATION OF BIOACTIVE GINSENOSIDES EXTRACTED FROM NATIVE
AND PROCESSED NORTH AMERICAN GINSENG PLANT COMPONENTS

by

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ABSTRACT

Ginseng root is one of the oldest and most utilized traditional herbal ingredients. Ginseng has been used to treat many diverse ailments. Compounds in ginseng known as ginsenosides, which are also known as steroidal saponins, are generally thought to be the most bioactive component. However, ginseng contains up to 30 different ginsenosides and the type and proportion of ginsenosides depends on the source of ginseng and portion of the plant material used. The overall objective was to identify, evaluate and recover sources of bioactive ginsenosides. In this thesis, the main ginsenosides that showed a propensity to inhibit cultured cancer cell viability were identified and a LC50 (concentration to inhibit 50% cell viability) was determined in three distinct cell lines. Generally, ginsenoside aglycones 20(S)-protopanaxadiol (PD) and 20(S)-protopanaxadiol (PT) and ginsenoside Rh2 were identified to have affected cell viability in all three cell lines. Specifically, the LC50 values for PD (13 µg/mL), ginsenoside Rh2 (15 µg/mL), PT (19 µg/mL) and ginsenoside Rh1 (210 µg/mL) were established in cultured leukemia cells (THP-1). In intestinal 407 cells (Int-407), a non malignant embryonic intestinal cell line established via HeLa cell contamination, the LC50 values were determined for PD (23 µg/mL), PT (26 µg/mL) and ginsenoside Rh2 (53 µg/mL). In comparison, LC50 for PD and PT were 24 µg/mL and Rh2 was 55 µg/mL in cultured Caco-2 cells, an adenocarcinoma intestinal cell line. Generally, cell cycle analysis showed that these specific ginsenosides which inhibited cell viability also resulted in a build up of sub-G1 cells, a characteristic of apoptosis. Furthermore, treatments that showed the greatest increase (P ≤ 0.05) in sub-G1 cells also had the largest (P ≤ 0.05) release of lactate dehydrogenase (LDH), a useful bio-marker for membrane integrity. It was concluded that specific structure-function relationships exist for bioactive
ginsenosides. The sources of rare bioactive ginsenosides, such as Rh2 have only been attributed to Asian red ginseng root. However, ginsenoside Rh2 was found in this study in North American ginseng leaf, an underutilized resource, as a product of applying thermal energy during extraction procedure. Furthermore, Rh2 formation was shown to be a function of heating time and a breakdown product of more abundant ginsenosides (e.g. Rb1 and Rd). Specific mechanistic studies with PD, PT, Rh2 and an enriched Rh2 North American ginseng leaf extract in THP-1 and Caco-2 cells showed that both ginsenoside concentration and exposure time were factors causing cytotoxicity. Generally, test ginsenosides increased both the buildup of apoptotic and necrotic cells while having a varying effect on Caspase-3 activity. In conclusion, the findings of this thesis indicate that variable bioactive response of ginsenosides may be explained on the basis of hydrophobic/hydrophilic balance of the compounds. Moreover, the bioactivity observed was more associated with non-specific changes in cell membrane function than specific trigger mechanism of programmed cell death.
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PREFACE

Portions of this thesis have been published or accepted for publication and are listed below. The principal author of these papers is David G. Popovich and the co-author is Dr. David D. Kitts (supervisor).

David G. Popovich

David D. Kitts


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David G. Popovich
CHAPTER I

Preface
Selected portions of Chapter I have been published in the following publication


1.1 GENERAL INTRODUCTION AND LITERATURE REVIEW

The use and acceptance of nutraceutical and herbal supplements have dramatically increased in recent years along with the general acceptance and use of alternative medicine (Yeh et al., 2002). A bounty of different disease states and afflictions have been suggested to be managed, prevented, or cured by individual herbs or herbal cocktails in traditional herbal folklore. The general public seems to have embraced herbs, nutraceutical products, and alternative medicine as natural alternatives to prevent and treat a diverse group of ailments. The herbal and nutraceutical market has responded to this interest, as reflected by the estimated worth of the United States market of over $4.2 billion (USD) in 2001 (Marcus and Grollman, 2002).

Ginseng is one of the oldest and most well known traditional herbal ingredients. Asian cultures have probably used ginseng for more than 5000 years, but the earliest written documentation about ginseng is in Interpretation of Creatures written by Shi You between 48 and 33 BC (Yun, 2001). Ginseng was thought to possess rejuvenating powers. Native North Americans have also used North American ginseng, as part of their traditional medicinal practice (Banthorpe, 1994).

Scientific research on ginseng started early; the first modern compositional study on ginseng was published in 1854 (Shoji, 1985). Currently, ginseng occupies a large portion of sales for an individual herb with sales of nearly $100 million (USD) in 1997, and with an estimated growth
of 26% per year in the United States (Brevoort, 1998). This has led to a rejuvenation of ginseng usage especially in North America. Ginseng is formulated into herbal supplements and nutraceuticals as capsules, powders, tinctures, tea, and as a beverage ingredient. These formulations are based on specific health expectations that focus on a broad range of effects such as, antioxidant, anti-diabetic, hypotensive, immune stimulatory and anti-cancer effects.

1.1.1 History of use

Ginseng refers to different herbs found from the family Araliaceae and the genus Panax. The term Panax is derived from a Greek word meaning “cure-all”. Ginseng is indigenous to China and Korea (Panax ginseng C.A. Meyer), the Himalayas (Panax pseudo-ginseng), Vietnam (Panax vietnamensis), Japan (Panax japonicus) and North America (Panax quinquefolius). The majority of scientific studies have focused on two species of ginseng Panax ginseng and Panax quinquefolius. Ginseng is commonly referred to as either Asian ginseng (from China and Korea) or North American ginseng in addition wild ginseng, such as sanchi ginseng, is found in India, Nepal and Myanmar and have long growing periods, which make them very desirable (Tran et al., 2003). Siberian or Russian ginseng (Eleutherococcus senticosus) is from an entirely different plant than ginseng and should not be confused with true ginseng. Although known as “Siberian ginseng”, the active compounds are different than those found in ginseng. Ginseng’s main active compounds are a group of triterpene saponins commonly referred to as ginsenosides. Ginseng is regarded as an adaptogenic, stimulant and a herb with aphrodisiac properties (Gillis, 1997; Lui and Xiao, 1992). Adaptogens can be defined as offering a general protection against stressors (Davydov and Krikorian, 2000).

Ginseng is a primary ingredient in traditional Chinese medicine where it is used in combination with other herbs to restore vital energy, especially in the elderly and individuals that
are weakened by illness. Ginseng root resembles a human body shape with roots forming arms, legs, and main body shape (Figure 1.1) and therefore historically valued for treatment for all body health problems (Dixon, 1976). Recent reports focusing on ginsenosides, have shown a propensity to promote general health, well-being and assist with the prevention of disease. This is the basis for the role of ginseng to stimulate the “yin” (e.g. the cooling or negative force) and the “yang” (e.g. the hot or positive force), an ancient concept from Asian cultures that explains the balance in body cells and systems required to promote general health and well being. Achieving the correct balance between opposing forces allegedly gives vitality and good health (Dixon, 1976). Traditional Chinese medicinal uses of Asian ginseng are for its ability to stimulate the “yang”, which heats and energizes the body. Chinese traditional medicine practitioners have prescribed Asian ginseng to counter the effects of ageing, cold climates, stress, and hormonal changes for the purpose of alleviating such conditions as depression, asthma, and heart, liver, nervous system, digestive and circulatory system problems (Banthorpe, 1994; Li et al., 1996). Gradual use is recommended and is thought to result in stronger overall health. Conversely, North American ginseng is thought to have the opposite effect of Asian ginseng by stimulating the “yin” and thereby having a cooling effect on the body. North American ginseng is thought to be useful for those in warmer climates, the young, elderly and those with high blood pressure, diabetes, heart and lung problems. (Li et al., 1996).

In North America, Father Lafitau, a Jesuit priest was the first non-aboriginal person to recognize and identify the growth of wild ginseng in Canada in the 1700’s. Later he spawned a large trade market with China. When dried, North American ginseng is often confused with Asian ginseng (Dixon, 1976).
Figure 1.1. *Panax* ginseng root and plant material, adapted from Court (2000).
1.1.2 Current usage

Ginseng is listed in many pharmacopoeias from such countries as Austria, China, France, Japan, Russia and Switzerland. A growing number of products containing ginseng are available to the market. Standardized ginseng extracts are available as capsules and herb powders. Ginseng extracts have also been combined with tea leaves, fruit beverages, coffee, soft drinks and beer. Other important food usages of ginseng include the traditional practice of adding ginseng along with other herbs to soups (Kitts and Popovich, 2003).

1.1.3 Asian white and red ginseng

Typically, roots from Asian ginseng (*Panax ginseng* C.A. Meyer) are commonly divided into either white or red ginseng based on the drying technique used to ensure preservation. Asian ginseng usually refers to the root of *Panax ginseng* C.A. Meyer that has been air-dried. Red ginseng is usually implied to be Korean red ginseng that has been subject to a steam heating process before drying and processing; steaming ginseng will produce a red tinge due to caramelization of sugars when compared to the beige color of air-dried root. However, red ginseng is not solely a product of Korea; red ginseng is also produced in China. Compositional studies indicate that there are minor differences in content between red and white ginseng, with red ginseng typically believed to have the stronger potency (Kim et al., 2000).

1.1.4 North American ginseng

North American ginseng plant (*Panax quinquefolius*) is virtually identical in shape and physical characteristics to Asian ginseng. North American ginseng is typically grown in the eastern United States and in the Canadian provinces of Ontario and British Columbia. The compositional differences of ginsenosides are not great enough to differentiate between North
American ginseng and Asian ginseng. Ginsenoside Rf seems to be present in only Asian ginseng, and not in North American, but it is often misidentified during measurement (see Section 1.2.3). Ginsenoside ratios such as (Rb1/Rb2) have been used to confirm North American ginseng (Hu and Kitts, 2001) but it is not always apparent. At present, there is no simple way to differentiate between Asian and North American ginseng and a reliance on morphological characteristics is also not reliable (Cui et al., 2003). Furthermore, in Hong Kong, the major trade market for ginseng, North American ginseng is 5 to 10 times more expensive than Asian ginseng (Wang et al., 2001) which may result in the substitution of less expensive ginseng. Classification of different varieties by genetic markers have been reported (Cui et al., 2003; Wang et al., 2001), and are essentially the only definitive method to identify authentic ginseng samples.

1.2 GINSENG INGREDIENTS

Chemical analysis of North American ginseng has shown the presence of many different components. Greater than 200 different compounds have so far been identified and in various amounts (Duke, 1992). The compounds include vitamins and minerals (vitamins A, B-12, calcium, niacin, folic acid, zinc), sugars (glucose, fructose), inorganic salts (sodium, magnesium and trace elements vanadium, selenium and fluorine) simple organic acids (acetic acid), to more complex constituents such as sterols (e.g. \( \beta \)-sitosterol, campesterol, stigmasterol, saikosaponin-A,B), oligopeptides (peptidoglycans), polysaccharides, volatile oils and many different types of saponins and many more yet unidentified compounds.
1.2.1 Ginsenosides

The primary active components of ginseng are generally recognized to be a group of 30 different triterpene saponins, also referred to as ginsenosides or dammarane triterpenoids. Ginsenosides share a similar basic structure consisting of a gonane steroid nucleus having 17 carbon atoms arranged in four rings (Figure 1.2). Differences in structure, which include the type, position and number of sugar moieties attached by glycosidic bonds at positions C-3 and C-6 determine the classification of ginsenosides. Ginsenosides that have sugar moieties attached at C-3 are referred to as protopanaxadiol ginsenosides and attachment at position C-6 is referred to as protopanaxatriol ginsenosides. Only one oleanolic acid type saponin, ginsenoside-Ro, has been identified from ginseng (Tang and Eisenbran, 1992). Ginseng composition varies according to many factors, which include the growing region, varieties, age of ginseng, light levels (Fournier et al., 2003; Li and Wardle, 2002) among other factors. Ginsenoside composition is usually expressed as percentage of six ginsenosides typically found in all ginseng types (Tang and Eisenbran, 1992). These reference ginsenosides are Rb1, Rb2, Rc, Rd, Re and Rg1. However, the bioactive properties of ginsenosides differ with respect to individual ginsenosides and biological system (Popovich and Kitts, 2002). Furthermore, increased levels of the reference ginsenosides do not generally refer to the bioactive potential of ginseng or products.
Figure 1.2. A selection of the ginsenosides of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol classifications. Ginsenoside basic structure consists of gonane steroid nucleus having 17 carbon atoms arranged in four rings. Individual ginsenosides differ by attachments of molecules at regions R1-R3 respectively. Ap- arabinopyranose, Af-arabinofuranose, G-glucopyranose, R-rhamnopyranose, X-xylopyranose (Kitts and Popovich 2003).
1.2.2 Functions in plant

A general explanation of the function of saponins in plants is that they are thought to be involved in the defensive systems of plants, to deter an attack by a predator (Sen et al., 1998). Many saponins have been suggested to have multiple effects on animal cells, fungi and bacteria, and an ability to inhibit mold (Francis et al., 2002).

1.2.3 Detection and analysis

Ginseng extracts can be prepared in many different ways. Extraction methods may employ solvents such as ethanol, hot water or a mixture such as 75% ethanol. Extraction techniques have been found to influence the concentrations and the proportions of ginsenosides in the final extract. Furthermore, ginseng root, leaf, flower, stem and seed all contain various levels of ginsenosides, adding to the overall complexity (Tang and Eisenbran, 1992). Techniques used for isolation and detection of ginsenosides vary. For the quantitative analysis of ginsenosides, total ginsenosides can be measured colourimetrically (Hiai et al., 1975a; Hiai et al., 1975b; Lui and Staba, 1980) and identified by TLC, HPLC, LC/MS.

Thin layer chromatography (TLC) is the standard method to analyze many different plant components and it is especially true for ginsenosides. Ginsenosides are effectively named according to the retention times or distances migrated on a TLC plate. A number of different solvent systems have been used to separate ginsenosides; using a solvent system of CHCl₃/MeOH /H₂O (65:35:10 lower phase) for TLC analysis showed that ginsenosides generally migrate according to their polarity, and the naming convention of Rx generally applies. For example, ginsenoside Rb1 is found closer to the origin of the TLC plate than Rb2 and Rc end
up further along and so on (Figure 1.3). However, ginsenosides Re and Rd are the exceptions, and can reverse their order of migration when using CHCl$_3$/MeOH/$\text{H}_2\text{O}$ solvents.

Figure 1.3. TLC separation of selected ginsenosides and aglycones (PD and PT). Ginsenosides were plated on Fischerbrand silica gel G glass backed plates (20 x 20 cm, 250 microns) (Fisher Scientific, Chicago, IL). The solvent system used to separate ginsenosides was chloroform-methanol-water (65:35:10 v/v/v) lower phase and visualized by spraying sulfuric acid (10% H$_2$SO$_4$) in ethanol, heated and photographed under long range UV light, adapted from (Popovich and Kitts, 2003).
A reversed phase HPLC system using a C$_{18}$ column allows the separation of the major and minor ginsenosides (Meier et al., 1985). Typically, the solvent combination of acetonitrile-water is used for separation, with acetonitrile concentration increasing with time. However, other types of columns and solvent systems have been used (Shibata et al., 1985), (Corthout et al., 1999; Park et al., 2002; Yip et al., 1985). After the extraction of ginsenosides from ginseng, a solid phase extraction (SPE) step is often used to remove interfering components before HPLC analysis (Corthout et al., 1999; Li et al., 1996), but appropriate fractioning by different solvents can also be successful (Kwon et al., 2001). It is possible, with an appropriate solvent control program to adequately separate the main reference ginsenosides such as Rb1, Rb2, Rc, Rd, Re, and Rg1, from the rare ginsenosides Rh1, Rg3, Rh2 and the aglycones. Furthermore, ginsenosides have been successfully characterized by using LC/MS (Chan et al., 2000) and LC/MS/MS (Haijiang et al., 2003; Ji et al., 2001). A LC/MS/MS method has been developed to distinguish Asian ginseng from North American ginseng based on identification of ginsenoside Rf and pseudo-ginsenoside F11 (Li et al., 2000). Scanning samples by MS electrospray ionization (ESI-MS) in positive (Chan et al., 2000; Wang et al., 1999) and negative ionization (Fuzzati et al., 1999; Haijiang et al., 2003; Kite et al., 2003) mode have been reported. A sample of the reported literature values of HPLC quantification of the ginsenoside content of selected North American ginseng and Asian white and red root and leaf are listed in Table 1.1. Ginsenoside Rh2 has, for the most part, been only reliably reported to be found in Korean red ginseng.
Table 1.1 Reported ginsenoside composition of North American and Asian ginseng root and leaf.

<table>
<thead>
<tr>
<th>Classification</th>
<th>N.A. Ginseng (1)</th>
<th>Asian White Ginseng (1)</th>
<th>Asian Red Ginseng (1)</th>
<th>N.A. Ginseng leaf (2)</th>
<th>Asian Ginseng leaf (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protopanaxadiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb1</td>
<td>1.8</td>
<td>0.5</td>
<td>0.4</td>
<td>1.22</td>
<td>0.184</td>
</tr>
<tr>
<td>Rb2</td>
<td>0.03</td>
<td>0.2</td>
<td>0.2</td>
<td>0.02</td>
<td>0.553</td>
</tr>
<tr>
<td>Rc</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.18</td>
<td>0.736</td>
</tr>
<tr>
<td>Rd</td>
<td>0.5</td>
<td>0.2</td>
<td>0.036</td>
<td>0.29</td>
<td>1.113</td>
</tr>
<tr>
<td>Rg3</td>
<td>ND</td>
<td>0.0003</td>
<td>0.015 (20S)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rh2</td>
<td>ND</td>
<td>ND</td>
<td>0.001</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Protopanaxatriol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re</td>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
<td>1.10</td>
<td>1.524</td>
</tr>
<tr>
<td>Rf</td>
<td>ND</td>
<td>0.05</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rg1</td>
<td>1.9</td>
<td>0.2</td>
<td>0.3</td>
<td>0.18</td>
<td>1.078</td>
</tr>
<tr>
<td>Rg2</td>
<td>0.008</td>
<td>0.014</td>
<td>0.02 (20S)</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>Rh1</td>
<td>ND</td>
<td>0.0015</td>
<td>0.006 (20S)</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Data are expressed as a percentage of total analyzed ginsenoside standards. (1) adapted from (Shibata, 2001), (2) adapted from (Li et al., 1996) mature leaf average (% dry weight), (3) adapted from (Tanaka and Kasai, 1984), ND-not detected, NR-not reported, N.A.-North American.
1.3 DIVERSE EFFECTS ATTRIBUTED TO GINSENG

1.3.1 Antioxidant properties

Antioxidant properties such as free radical scavenging activity of ginseng extracts have been demonstrated from a number of *in vitro* studies but antioxidant properties attributed to specific ginsenosides have not been clearly established. These reports showed activity of ginseng extracts in scavenging stable free radical (e.g. 1,1-diphenyl-2-picrylhydrazyl (DPPH)) (Kim et al., 2002b; Kitts et al., 2000) and carbon-centred free radicals (2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Hu and Kitts, 2001; Kim et al., 2002b). North American ginseng extracts have been shown to be effective at both chelation of metal ion and scavenging of free radicals in lipid and aqueous mediums (Kitts et al., 2000). North American ginseng was reported to have higher affinity to scavenge free radicals compared to Asian ginseng (Hu and Kitts, 2001). Both Asian and North American ginseng protected low-density lipoproteins and supercoiled DNA against cupric ion oxidation and DNA breakage, respectively (Hu and Kitts, 2001). Alternatively, aqueous extracts from Korean red ginseng, prepared with and without heat, did not significantly differ in free radical scavenging ability and was overall not a strong scavenger (Kim et al., 2002b). Methanol extract of heat-treated Asian ginseng reduced lipid peroxidation in rat brain homogenates, which was induced with ferric ion and ferric ion plus ascorbic acid, and the extract protected against supercoiled DNA strand scission (Keum et al., 2000). In transfected hepatoma cells, ginsenosides Rb2 induced the transcription of Cu, Zn-superoxide dismutase gene (*SOD1*) to a greater extent than ginsenoside Rb1 and total saponins did not have an effect (Kim et al., 1996). Superoxide dismutase is a key enzyme involved in the removal of superoxide radicals. Ginseng extracts have also been shown to affect
the antioxidant defense mechanism by increasing the hepatic glutathione peroxidase activity, and superoxide dismutase in rats receiving ginseng extract for three months (Voces et al., 1999). Pretreatment of endothelial cells with crude saponins of Korean red ginseng, reduced NADPH-driven superoxide generation (Kim et al., 2002a). Artificial digestion (in vitro) of a standardized ginseng extract (G115), which can modify the ginsenoside composition resulted in a greater effect on pulmonary vasodilation and protection from free radical injury compared to ginsenoside standards Rb1 and Rg1 (Rimar et al., 1996). This effect on vasodilation and protection from free radicals may be mediated through the production of nitric oxide (NO). The antioxidant potential of individual ginsenosides were ordered by the ability to induced free radical (AAPH) hemolysis of human erythrocytes (Liu et al., 2003). The relative order of antioxidant ability to be in the following decreasing order: Rc, Rb1, Re, Rd, Rg1 and Rb3 (Liu et al., 2003). Rg3, Rd and Rh2 were suggested to possess pro-oxidant characteristics in this erythrocyte hemolysis model (Liu et al., 2002); these results tend to suggest that larger ginsenosides, those with more attached sugar molecules such as Rc and Rb1 have greater antioxidant potential compared to smaller molecules such as Rh2.

1.3.2 Anti-diabetic effects

In primary rabbit renal proximal tubules, exposure to protopanaxatriol saponins from Korean red ginseng (> 50 mg/mL) inhibited the Na\(^{+}\)-dependent \(\alpha\)-methyl-D-glucopyranoside (\(\alpha\)-MG) uptake compared to propanaxadiol saponins (Han et al., 1999); PT inhibition of \(\alpha\)-MG was related to an increase in arachidonic acid release (Han et al., 1999). Rb1 was reported to stimulate glucose transport in sheep erythrocytes at concentration of 1 \(\mu\)M but not at 10 \(\mu\)M and an Asian ginseng extract also stimulated glucose uptake dose-dependently (Hasegawa et al.,
Two ginsenosides Rg3 and Rh2 were reported to have variable effects on 2-deoxy-D-glucose (2-DG), a non-metabolized analogue of glucose, uptake in Erlich ascites tumor cells (Hasegawa et al., 1994b). The difference was observed for epimers [20(S) and 20(R)]. The aglycones (PD and PT) increased 2-DG uptake at 10 μM but PT decreased uptake at 100 μM. 20(S)-Rg3 decreased uptake at concentrations of 50 and 100 μM whereas 20(R)-Rg3 did not and 20(R)-Rh2 reduced 2-DG uptake at 100 μM and 20(S)-Rh2 did not. These effects may in part be due to an interaction with the cellular membranes (Hasegawa et al., 1994b).

In mice, four week oral administration of ginseng extracts derived from the white ginseng root (radix alba) and rootlet (radix palva) resulted in reduction of fasting blood glucose levels by 40% and 37%, respectively compared to untreated group (Chung et al., 2001). Specifically, ginseng root was reported to block intestinal glucose absorption and inhibited hepatic glucose-6-phosphatase (Chung et al., 2001). In two diabetic animal models, male KK-CAy mice and alloxan-diabetic mice, the water extract of ginseng significantly reduced fasting glucose by 76% and 62% after intraperitoneal injection (90 mg/kg) (Kimura et al., 1999).

1.3.3 Immune-stimulating properties

The immune stimulatory properties of ginseng have long been touted as one of the main biological effects of ginseng (Scaglione et al., 1996). The results from in vitro immune stimulation studies are variable. For example, a North American ginseng extract (200 μg/mL) has been reported to stimulate polymorphonuclear leukocytes (human white blood cells) in vitro and treatment stimulated human tumor necrosis factor α (TNF-α) release after 6 hours (h) (Zhou and Kitts, 2002). TNF-α is an important marker in early immune response. Specific ginsenosides Rb1, Rb2 and Rc were found to inhibit TNF-α dose dependently after
lipopolysaccharide (LPS) stimulation of murine cells (RAWE264.7) and differentiated
human macrophage cells (U937) (Cho et al., 2001a). Asian ginseng increased cytokine (IL-12)
from mouse macrophage cells (J774A.1) at the protein level and mRNA expression but no
change was reported for IL-1β, IL-15, TNF-α (Wang et al., 2003). Cellular immunity was
enhanced by exposure to ginseng extracts in normal, chronic fatigue syndrome, and AIDS
subjects assessed by in vitro natural killer-function in isolated peripheral blood mononuclear
cells (See et al., 1997).

The effect of ginseng extracts in healthy human volunteers is reported to enhance cellular
immunity (Scaglione et al., 1990). Capsules of the extract (100 mg) taken for 12 weeks with an
influenza vaccination reduced the frequency of influenza from 42 cases for the placebo group
(vaccination only) to 15 for the ginseng treated group (Scaglione et al., 1996). Administration
of Korean red ginseng to HIV-1-infected patients was reported to delay the resistance to active
anti-retroviral drug therapy. Resistance greatly reduces the success of drug therapy, zidovudine
(ZDV, also known as AZT), in the treatment of HIV infection (Cho et al., 2001b). Treatment
with Korean red ginseng in combination with ZDV therapy resulted in significantly longer time
(e.g. 34 months) to develop drug-resistant mutant strains compared to control patients.
Interleukin-8, a cytokine which exerts chemotaxis on neutrophils, T-cells and basophils can be
induced by ginseng root, an induction which is accompanied by increased IL-8 mRNA
expression (Sonoda et al., 1998).

Asian ginseng was shown to enhance antibody production in pigs when vaccinated
against porcine parvovirus and Erysipelothrix rhusiopathiae (Rivera et al., 2003). Vaccines were
administered with ginseng (2 mg /dose) and aluminum hydroxide as a co-adjuvant. Greater titres
of antibodies were found when ginseng was administered in combination and it resulted in a
switch from the production of IgG1 to IgG2 antibodies, with no reported side effects (Rivera et al., 2003). Further studies with ginsenoside Rb1 in dairy cattle immunized against *Staphylococcus aureus*, increased the production of antibodies and stimulated proliferation of lymphocytes after mitogen stimulation (Hu et al., 2003).

The general ability of saponins to stimulate the immune system may in part be explained by the ability to affect the cells of the gastrointestinal tract and alter permeability. Enhanced permeability would create a gut environment that would likely allow increased uptake of antigens from the gut (Francis et al., 2002) and thus a broad stimulation of the immune system. Oral administration of a boiled Asian ginseng extract to mice was reported to enhance dose-dependently the antibody formation to either a primary or a secondary challenge with sheep red cells. Specifically, at a dose of 250 mg/kg, the primary IgM response was increased by 50% and the secondary IgG and IgM responses were increased by 50 and 100% (Jie et al., 1984). Although the precise mechanism of saponins or ginsenosides ability to impact gastrointestinal tract cell membranes have not been fully clarified, they are however, able to interact with the gastrointestinal cell environment (Francis et al., 2002).

1.3.4 Bioavailability

The extent of bioavailability and absorption of ginsenosides in the gastrointestinal tract has been not been adequately established. In rats, the oral bioavailability data are variable. One report suggests ginsenosides Rb1 and Rg1 were 4.35% and 18.4% absorbed respectively (Xu et al., 2003). An earlier study reported 23% absorption of ginsenoside Rb1 after a period of 2.5 h (Takino et al., 1982). Recovery of Rb1 was noted in the liver (0.25% dose), heart (< 0.1% dose) and with the majority of the material recovered in the small intestine. Very little of the original material was recovered in the fecal material (< 1% dose). Rg1 administered orally (100 mg/kg)
to rats deprived of food resulted in a maximum level after 30 minutes (min) of 0.9 μg/mL in the serum (Odani et al., 1983). Rg1 was detected in the stomach and small intestine at 42% and 36% of the dose, respectively, after 15 min and 57% in the small intestine after 30 min. After 6 h, no Rg1 was found in the small intestine and 52% remained in the large intestine. Furthermore, decomposition or biotransformation of Rg1 was noted in the stomach and large intestine (Odani et al., 1983). Acid hydrolysis of Rb1 and Rb2 by 0.1N HCl (stomach acid) resulted in the formation of breakdown products 20(S, R)-Rg3 (Karikura et al., 1991).

Human bacteria collected from fecal material have been reported to hydrolyze ginsenosides Rb1 and Rb2 to specific metabolites (Hasegawa et al., 1997). Biotransformation of ginsenosides was reported to be quickest for Rb1 followed by ginsenoside Rg1, Rb2, Rc and Re (Hasegawa et al., 1996). These biotransformed ginsenosides may be at least in part absorbed. Administration of a ginseng extract to human volunteers resulted in the detection of biotransformed intestinal metabolites in the urine, but none of the original material was detected (Hasegawa et al., 1996). In the rat, both intestinal metabolites and original ginsenosides Rb2, Rc and Rd were found (Hasegawa et al., 1996). Bacterial metabolites of ginseng have also been reported to have antigenotoxic properties (Lee et al., 1998a). Compound IH-901 is formed after Rb1 is inoculated with human fecal bacteria (Lee et al., 2000a), and IH-901 has specific reported effects on cultured leukemia cells (HL-60) such as induction of apoptosis by activation of caspase-3 protease. In a pilot study of two volunteers, ginsenoside Rb1 was detected by LC-MS analysis in the urine and plasma; hydrolysis products and the main intestinal bacterial metabolite were also detected (Tawab et al., 2003). Alcoholic tinctures of ginseng may provide relatively greater bioactivity of active components than powder preparations, due to the potentially improved solubility and thus availability from cell wall structure components of the plant.
Post-operative stage III gastric cancer patients treated with chemotherapy and Korean red ginseng (4.5 g/day for six months) were reported to have an overall greater survival rate and disease free rate after 5 years compared to placebo treated chemotherapy group (Suh et al., 2002). This report suggests at least some oral bioavailability potential of ginseng.

1.4 CELL GROWTH

Normal growth and development of cells is a balance between cell proliferation, differentiation and cell death. The balance between cell growth and cell death is strictly regulated and normally controlled (Hortelano et al., 1999; Surh, 1999). In a disease state, this balance may either be shifted in favour of proliferation (carcinogenesis) or cell death; altered rates of cell death are central to many types of disease states (Watson et al., 2000), whereas, death suppression can lead to proliferation of cancer cells (Ahmad et al., 1997; Wright et al., 1994). Normal cells grow and divide according to cell cycle events. Internal controls called checkpoints prevent the formation of genetically abnormal cells (Fotedar et al., 1997; King and Cidlowski, 1998). When DNA damage is detected, the cells can be repaired or eliminated preventing potentially mutagenic cells from proliferating. The checkpoints determine if a cell will progress, repair or be eliminated. Loss of checkpoint control may lead to proliferation of cancerous cells and consequently triggering and increasing checkpoint blockage is an important factor to inhibit the progression of cancer cells (King and Cidlowski, 1998).

A cell has a number of vulnerable areas that when damaged will compromise the cell survival; vulnerable sites include the plasma membrane, the mitochondria, lysosomes, endoplasmic reticulum and the nucleus (Kanduc et al., 2002). Two modes of cell death can be distinguished and are categorized as apoptosis and necrosis (Figure 1.4).
Figure 1.4. Typical routes available for cells, adapted from Proskuryakov et al. (2003).
1.4.1 Apoptosis

Apoptosis or “programmed cell death” is an active, controlled and physiological mode of cell death where a cell executes a set of programs that eventually lead to death (Darzynkiewicz et al., 1997). Apoptosis can be induced by a number of physiological events and pharmacological agents (Kanduc et al., 2002). Many chemo-preventive agents exert an effect by triggering apoptotic cell death (Watts et al., 1999). Apoptosis is part of the natural defense mechanisms that removes damaged, mutated or infected cells. Morphological changes during apoptosis generally can be divided into overlapping phases. The chromatin condenses resulting in a reduction in nuclear size, disintegration of nucleoli shrinkage of total cell volume; this is further followed by blebbing of the membrane, and constriction of the cytoplasm (Ramachandra and Studzinski, 1995). The cellular material is eventually packaged into enclosed membrane bound apoptotic bodies consisting of oligonucleosomal DNA fragments (Proskuryakov et al., 2003). The apoptotic bodies can also be phagocytosed by neighbouring cells.

1.4.2 Necrosis

The main features of necrosis involve organelle swelling, cellular disintegration, and clumping of the chromatin all of which are not as uniformed or defined as in apoptosis (Kerr and Harmon, 1991; Bonfoco et al., 1995). Swelling of the cell may be reversible during the early stages, and is thought to be due to a few factors such as direct membrane damage lesions, ATP depletion and disturbance or disruption of membrane pump activity; other causes of necrosis include hypoxia, ischemia and freezing and thawing of cells (Kerr and Harmon, 1991). A large increase in membrane permeability marks the irreversible stage of injured necrotic cells and loss of membrane barrier function results in the release of cellular constituents such as lysosomal
enzymes and is involved in cellular degradation and disappearance of chromatin (Kerr and Harmon, 1991) along with the release of proteins and nucleic acids (Shacter et al., 2000). Necrosis is thought to be an unregulated and passive form of cell death.

1.4.3 Distinguishing between apoptosis and necrosis

A potential overlap between apoptosis and necrosis can occur and mild injury (Kerr and Harmon, 1991) to the cell will result in both modes of cell death. Proskuryakov et al. (2003) suggested that as methods for identification and detection of cell death improves, differentiating between the two modes of cell death become more complex. For example, both biochemical and morphological characteristics of necrosis and apoptosis have been observed in the same cell. This overlap complicates a definitive classification of cell death.

Distinguishing between and measuring apoptotic and necrotic cells can be achieved by a few different measures; these include morphological assessment, DNA fragmentation (Ramachandra and Studzinski, 1995), flow cytometry cell cycle analysis of fixed cells (Darzynkiewicz et al., 1997), and flow cytometry analysis of unfixed cell by annexin-V-FITC and PI labeling (Vermes et al., 1995) (Table 1.2). Apoptosis can be measured by a variety of different techniques; a classical measurement of apoptosis is by DNA fragmentation visualized on agarose gels. Apoptotic cells are visualized by a ladder pattern of 180 bases pairs but this pattern is not always apparent in all cell systems (Ramachandra and Studzinski, 1995). The degradation of DNA is also apparent in cells undergoing necrosis which produce a smear pattern during electrophoresis (Kerr and Harmon, 1991; Proskuryakov et al., 2003). This smear pattern can potentially mask any underlying DNA fragmentation. Flow cytometry measures individual cells by scanning single cells flowing past excitation sources and is unique as it is able to provide rapid, quantitative and multi-parameter analysis on single living, or dead cells (Sharrow, 1996).
The hallmark and the earliest feature of apoptosis is the externalization of phosphatidylserine (Proskuryakov et al., 2003) which serves as a marker to identify the apoptotic cells and easily identified by annexin-V-fluroescein iостhiocyanate (FITC) and propidium iodide (PI) labeling.

Table 1.2. Summary of general features characteristic to apoptosis and necrosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cellular Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular changes</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Shrinking</td>
</tr>
<tr>
<td>Damage to organelles</td>
<td>Late determination</td>
</tr>
<tr>
<td>Release of lysosomal</td>
<td>Absent (until late)</td>
</tr>
<tr>
<td>enzymes</td>
<td></td>
</tr>
<tr>
<td>Nuclear changes</td>
<td>Condensation, segmentation, fragmentation</td>
</tr>
<tr>
<td></td>
<td>Formation of apoptotic bodies</td>
</tr>
<tr>
<td>Other positive</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>Measurements</td>
<td>Flow cytometry Sub-G1 (PI stained)</td>
</tr>
<tr>
<td></td>
<td>DNA Fragmentation (ladder pattern on agarose gel)</td>
</tr>
<tr>
<td></td>
<td>Annexin V-FITC</td>
</tr>
<tr>
<td></td>
<td>Annexin V-FITC-PI</td>
</tr>
<tr>
<td></td>
<td>Disappearance of nuclei, random clumping</td>
</tr>
<tr>
<td></td>
<td>Swelling and disintegration</td>
</tr>
<tr>
<td></td>
<td>Immune stimulation</td>
</tr>
<tr>
<td></td>
<td>LDH release</td>
</tr>
</tbody>
</table>
1.4.4 Cell lines

Three distinct cultured cell lines have been used in the following experiments: THP-1 cells are a human acute monocytic leukemia suspension cell line, and have been widely used as a model cell system of human monocytes, macrophages, and immune response (Klegeris et al., 1997; Lorton et al., 1996; Nakayama et al., 2000). THP-1 cells have a range of properties similar to monocytes and mature phagocytes. Intestine 407 (Int-407) cell line was initiated from the nonmalignant, small-intestine (jejunum and ileum) of a human embryo of approximately two months gestation and exhibits typical epithelial morphology and growth (Henle and Deinhardt, 1957). This cell line is used as a model of intestinal response, but, has been established via HeLa cell contamination. Caco-2 is a human colon adenocarcinoma monolayer cell line that has been utilized as a model of normal intestinal epithelia system (Barthe et al., 1999; Bestwick and Milne, 2000). This cell line has been also used to study intestinal absorption, transport mechanism of pharmaceutical and plant compounds and to assess potential toxic effects of formulations (Barthe et al., 1999).

1.5 BIOACTIVITY OF SPECIFIC GINSENOSES

1.5.1 Effect on membranes

Saponins, such as ginsenosides, may exert an effect on membrane function in a general non-specific way (Foulkes, 1998). A non-specific effect can be exhibited directly at the membrane levels, by the disruption of the electric charge, transmembrane potential, voltage gated channels, altering the influx or efflux of ions and may influence membrane composition (Foulkes, 1998). Steroids have similar structural characteristics to ginsenosides, and ginsenosides have been reported to be a functional ligand of the glucocorticoid receptor (Lee et al., 1997),
potentially producing steroid-like effects. A classical view of steroids was that they primarily affect protein synthesis, which requires time to produce, and to relocate in the cell (Wehling, 1997). However, rapid effect of steroids have been reported and these effects are likely not genomic. Steroids have been reported to affect cells in a non-specific, non-genomic way, as a result of a change in membrane physiochemical properties (Falkenstein et al., 2000). Two mechanisms proposed for nongenomic effects, include, an interaction with specific receptors (membrane), or an interaction with membrane lipids (Shivaji and Jagannadham, 1992). Some ginsenosides, such as Rh2 and Rg3 can influence cellular viability but the mechanism or mechanisms of action are unclear. Two types of mechanisms have been proposed in the literature for the effect of ginseng on cultured cells; the first mechanism is a genomic one which modulates the cell cycle, and the second mechanism involves an effect on the cellular membrane. It is not clear if ginsenosides are able to modulate membrane properties first followed by a genomic effect or in fact have a combined effect.

1.5.2 Membrane channel and ion activation

Ginsenosides were reported to inhibit the catecholamine secretion in acetylcholine stimulated bovine adrenal chromaffin cells, a model of autonomic nervous system or sympathetic neurons. Generally, the inhibitory effects might be related to ginsenoside structure; the effects were greater for the protopanaxatriol group compared to protopanaxadiol ginsenosides (Kudo et al., 1998). In the protopanaxatriol classification of ginsenosides, Rg2 was found to have greatest effect followed by Rf, Rg3, Re and Rh1. Ginsenosides Rg3 and Rh2 reduced secretion dose-dependently (Kudo et al., 1998) compared to other protopanaxadiol ginsenosides (Kudo et al., 1998). Rg3 has been reported to affect nicotinic acetylcholine receptor operated cation channels in bovine adrenal chromaffin cells (Tachikawa et al., 2001). Acetylcholine binding to the
nicotinic receptor results in depolarization of the cell membrane and an influx of Na⁺ through receptor operated cation channels, and an influx of Ca²⁺ through voltage sensitive Ca²⁺ channels. This eventually results in catecholamine secretion (Tachikawa et al., 1999). The non-competitive effect of ginsenoside Rg3 with nicotine has been suggested to block the Na⁺ influx in cells through cation channels (Tachikawa et al., 2001). This effect may be due in part to a reduction of membrane integrity or fluidity. At a concentration of 30 μM, Rg3 increased the membrane microviscosity measured by a fluorescence probe (DPH) (Tachikawa et al., 2001). DPH penetrates the plasma membrane embedding at the hydrophobic membrane core. Furthermore, membrane effects such as loss of fluidity will generally affect plasma membrane receptor properties and likely leading to ion activation and permeability.

A characterized ginsenoside mixture containing 11 ginsenosides was reported to reversibly inhibit the inward Ca²⁺ current in rat adrenal chromaffin cells. Specifically, ginsenosides Rb1, Rc, Re, Rf and Re had an effect (Kim et al., 1998a). A reduction in membrane capacitance, after activation of voltage-dependent Ca²⁺ channels and Ca²⁺ may play a role in ginsenoside effect on catecholamine regulation. An increase in membrane capacitance after depolarization is an indirect measure of secretion of neurotransmitters or hormones (Kim et al., 1998a). Specifically, ginsenosides were also shown to selectively regulate three of the five types of calcium channels in bovine chromaffin cells (Choi et al., 2001a). Ginsenosides were found to interact with membrane components of Xenopus oocytes (frog) leading to Ca²⁺ activated Cl⁻ channel opening (Choi et al., 2001b). The relative potency of signal transduction was reported to be greatest for Rf followed by Ro, Rb1 and Rb2, Rg2 and Rc. Ginsenosides Rd, Re and Rg1 had no effect (Choi et al., 2001b). In transfected tsA201 cells (human embryonic kidney cells) with α subunit brain2a Na⁺ channel, North American ginseng extract reversibly
blocked the channel and ginsenoside Rb1 was primarily responsible for the effect (Liu et al., 2001). Total saponins prepared from Asian ginseng stimulated the influx of Ca\textsuperscript{2+} into Ehrlich ascites tumor cells, which might affect mobilization of Ca\textsuperscript{2+} (Abdrasilov et al., 1996). Also, *Panax notoginseng* extract was reported to block Ca\textsuperscript{2+} uptake in synaptosomes prepared from rat cortex (Tachikawa et al., 2001). Other saponins such as digitonin, at low concentrations, are suggested to interact with the plasma membrane by forming complexes with cholesterol. A decrease in membrane fluidity of cardiac cells was found with an increase in contractility and a rise in intracellular Ca\textsuperscript{2+} (Ishida et al., 1993). After exposure to digitonin, these effects implicate a leaky membrane as a result of the formation of membrane holes (Ishida et al., 1993).

### 1.5.3 Effect on hemolysis and membranes

The hemolytic activity of saponins has been known for some time and was measured by the degree of rupture of erythrocytes (Hosettman, 1995). Using a free-radical induced human erythrocyte hemolysis model, ginsenosides exhibit different effects. The majority of ginsenosides have a protective effect on hemolysis even after exposure to free radical induced hemolysis, however, certain ginsenoside such as Rg3 and Rh2 increased the extent of free radical induced hemolysis (Liu et al., 2002). These effects were based on structural classifications. For example, Rg3, Rh2 and Rg2 have sugar moieties attached at position C-3 of the triterpene ring (Figure 1.2) and have increased hemolytic activity (Liu et al., 2003), compared to those which have attachment at position C-6. Furthermore, the aglycone protopanaxadiol and Rg3 exhibit strong hemolytic activity even in the absence of free-radical initiators and the percentage of hemolytic activity increased as concentration increased (Liu et al., 2003). Hence, one of the biological effects of ginseng is reported to be a result of membrane modifying properties (Prokof'eva et al., 2002). Likewise, dammarane triterpenoids isolated from birch leaves sharing
similar structural characteristics to the aglycone protopanaxadiol from ginseng was found to be cytotoxic to Erlich ascite carcinoma cells (Prokof'eva et al., 2002). An increase in the membrane permeability of cells was reported, measured by the release of UV-absorbing substances from the cell and a decrease in thymidine ($^3$H) incorporation. A dose-dependent increase in membrane permeability was reported along with the increase in microviscosity (Prokof'eva et al., 2002).

Saponins from *Panax notoginseng* have been shown to alter lipid fluidity of platelet membranes in male Wistar rats; intra peritoneal administration (200 mg/kg) inhibited platelet aggregation and adhesiveness after permanent occlusion of the middle cerebral artery (Ma and Xiao, 1998). Ginsenoside Rg1 inhibited adrenaline and thrombin induced platelet aggregation in human platelets analyzed *in vitro* and reduced the elevation of cytosolic free calcium concentration (Kimura et al., 1988). Rg2 was reported to inhibit intracellular calcium mobilization from rabbit plasma (Teng et al., 1989).

Rg3 has been reported to reduce plasma membrane fluidity in bovine adrenal chromaffin cells (Tachikawa et al., 2001). Similarly, an increase in the concentration of cholesterol in cells resulted in the stiffening of the plasma membrane, and a reduction in the motion of the membrane (Burns and Duff, 2003). A reduction of membrane fluidity has also been reported in cells treated with 17β-estradiol and the anti-cancer drug tamoxifen. It is speculated that these compounds possibly associate with the hydrophobic portions inside the membrane (Clarke et al., 1990). Membrane protein function is dependent on structure and alterations of membrane composition will affect fluidity (Burns et al., 1979; Willmer, 1962). For example, 17α-hydroxyprogesterone decreased fluidity and increased membrane aggregation and permeability (Shivaji and Jagannadham, 1992). It is unclear if ginsenosides can interact with cells and
function as steroids, or are associated with steroid-like effects on the membrane by interaction with other components.

1.5.4 Effect of ginsenoside structure on bioactivity

The membrane effects of triterpene saponins, including ginseng, have been suggested to be a function of the attachment of sugar moieties to the triterpene ring structure. Cytotoxic effects of protopanaxadiol ginsenosides have been reported to decrease as sugar moieties are attached to position C-3; protopanaxadiol was reported to have lower LC50 compared to Rh2 and Rg3 in cultured cell experiments (Hasegawa et al., 1995; Popovich and Kitts, 2002). Mono-glucosides such as Rh2 and Rg3 and compound K (intestinal metabolite), were cytotoxic and exerted membrane altering properties in Erlich ascite carcinoma cells and in a liposome model (Popov, 2002). The effect on the membrane was a function of the cholesterol content. By increasing the cholesterol content, at neutral pH, the cytotoxic effect was reduced. Furthermore, pre-incubation of Rh2 and Rg3 with cholesterol free liposomes were reported to neutralize the cytotoxicity (Popov, 2002). Additions of functional groups (e.g. glucose) to the aglycone PD and PT of ginseng reduced apoptotic potential and reduced the potency to alter membrane integrity, measured by the release of lactate dehydrogenase from cells (Popovich and Kitts, 2002). Different types of saponins can have different effects on the membrane. Glycoalkaloids have the tendency to form complexes with plant sterols (Keukens et al., 1995), and sea-cucumber saponins have the strongest effect on cholesterol containing membranes (Popov, 2002). Structural characteristics of the saponins play a role in this effect. Ginsenosides such as Rb1, Re, Rg2, Rgl, Rd, Rf and Ro that have two attachments at positions C-3 or C-6 and C-20 do not affect the cell membrane or have cytotoxic effect. The attachment of the functional groups
possible prevents the penetration into the membrane by altering conformation (Popov, 2002) or by changing the polarity of the compound.

1.5.5 *In vivo* animal cancer models

A red ginseng extract was reported to inhibit the incidence of lung tumors in benzo(a)pyrene-induced newborn mice and red ginseng was found to be more effective compared to white fresh ginseng (Yun et al., 1995). In the aberrant crypt foci (ACF) assay, a model of colorectal cancer, red ginseng powder (0.5 g/kg, 2 mg/kg) was reported to be cytostatic as it inhibited the progression of established ACF induced by azoxymethane in Sprague-Dawley rat (F344) (Wargovich, 2001). Further reports indicate that dietary red ginseng powder fed to F344 rats during the initiation stage of 1,2-dimethylhydrazine-induced ACF had an inhibiting effect and this inhibition was greater for red ginseng than for white ginseng (Fukushima et al., 2001). Red ginseng was also found to inhibit the development of liver cancer induced by diethylnitrosamine in Wistar white rats. Red ginseng (5.6 g/kg/week) administered with water via a gastric tube reduced the rate of liver cancer (14.3%) in the later stage compared to an untreated control group (100%) (Wu et al., 2001). Ginseng has been reported to have a non-organ specific effect on cancer (Bespalov et al., 2001). Studies with white ginseng from plant culture, showed an inhibitory effect towards the development of tumors of the mammary gland, nervous system, kidney, uterine cervix and vagina after chemical induction of tumors, in a variety of experimental animals (Bespalov et al., 2001).

In mice that lack a thymus (Nude mice), oral administration of Rh2 by canula after inoculation of with human ovarian cancer cells resulted in a significant reduction in tumor volumes in all groups treated with Rh2 compared to ethanol-treated and cisplatin treated groups. Furthermore, tumor growth was also reported to be significantly reduced compared to a cisplatin
treated group. Cisplatin is a chemotherapy that is given as treatment for cancer and also referred to as cis-diaminedichloroplatinum (II) (CDDP). The survival rates of mice treated with either 15 or 120 µM Rh2 were also increased compared to ethanol and cisplatin treated groups (Tode et al., 1993). Tode et al., (1993) suggested that Rh2 may function by altering the cell surface of membranes. Red ginseng saponins, such as structurally related Rg3 inhibit lung metastasis in in vivo mice models. Lung metastasis was produced by metastatic tumor cells, B16-BL6 melanoma and colon 26-M3.1 carcinoma, in syngeneic mice. Red ginseng saponins were suggested to inhibit the adhesion and invasion of these metastatic cells and significantly decreased the number of blood vessels oriented toward the tumor mass (antiangiogenesis) (Mochizuki et al., 1995). Further studies on Nude mice inoculated with human ovarian cancer cells, showed that daily oral administration of Rh2 (0.4 mg/kg) produced greater tumor growth retardation than intraperitoneal administration of CDDP (2 mg/kg), a potent antitumor agent; however a dose-dependent relationship was not obtained (Nakata et al., 1998).

1.5.6 Human evidence

A case control study of ginseng intake and association with cancer risk from Korea showed that the odds ratio for ginseng consumers to develop cancer was 0.56 and that ginseng prepared as an extract or powder was more effective than fresh ginseng (Yun and Choi, 1990). Further case-control evidence showed that odds ratios were 0.56 in relation to ginseng intake and variable for different ginseng preparations such as fresh ginseng extract (0.57), white ginseng extract (0.33), and lowest odds ratio for red ginseng users (0.20) (Yun et al., 2001). Yun and Choi (1998) reported from a prospective cohort study of 4634 people over 40 years of age in Korea, that ginseng consumers had a relative risk of 0.40 (95% confidence interval) compared to non-consumers to develop cancer. The relative risk of consumers given fresh ginseng (0.31) and
for multiple and overlapping ginseng extract consumers were similar (0.34) and in a small subset of ginseng consumers no death was reported in 24 consumers of red ginseng (Yun and Choi, 1998). Furthermore, a dose-response relationship was reported and the risk ratios of gastric (0.33) and lung cancer (0.30) development were reduced even though these cancers have different mechanisms of carcinogenesis and etiology, which suggest ginseng has a non-organ specific preventative effect against cancer (Yun and Choi, 1998). Red ginseng was reported to increase disease free survival (68%) compared to non-red ginseng group (33%) and overall survival (76%) compared to control group (39%) in postoperative chemotherapy of stage III gastric cancer (Suh et al., 2002). This effect was suggested to have immune modulating properties.

1.6 EFFECT ON CULTURED CANCER CELLS

In recent years, the diverse effect of ginseng and individual ginsenosides has been studied using cultured cancer cell lines. The cell lines include human liver (Park et al., 2002), leukemia (Lee et al., 2000b; Popovich and Kitts, 2002), prostate (Liu et al., 2000), breast (Oh et al., 1999), and a variety of mouse and other cell types. The different types of ginsenosides do not inhibit cultured cancer cell proliferation to the same degree. Only the more hydrophobic ginsenosides, such as Rg3, Rh2, Rh1 and both of the aglycones of the protopanaxadiol and protopanaxadiol groups have been reported to have an impact on cell proliferation. More common ginsenosides, such as Rb1, Re, Rd, Re and Rg1, generally do not reduce the growth of cultured cancer cells (Park et al., 2002). Furthermore, ginseng extracts do not normally inhibit the growth of cultured cells because of the variable composition of individual ginsenosides, and the low concentration of the hydrophobic ginsenosides. Specifically, ginsenoside Rh2 has an effect on cell proliferation reported by a variety of different research groups (Fei et al., 2002; Oh et al., 1999;
Popovich and Kitts, 2002), however, the extent to which Rh2 affects the genomic regulation of the cell impacting protein expression or acts specially on the cellular membrane is unclear at present.

Ginsenosides Rh2, PD, and PT have been reported to induce apoptosis or programmed cell death detected either by DNA ladder pattern by electrophoresis (Fei et al., 2002) or by a build-up of sub-G1 cells measured by flow cytometry (Popovich and Kitts, 2002). The apoptotic cell death pathway is distinguished from the necrotic cell death as being an active death program guided by distinct cellular events. Further specific markers of apoptosis, such as caspase-3 or proteins involved in arresting or progression of the cell cycle have been measured after exposure to ginsenoside Rh2. Rh2 increased activation of caspase-3 in transfected SK-HEP-1 cells (Jin et al., 2000), and both caspase 3 and 8 in human melanoma cells (A375-S2) cells (Fei et al., 2002). Furthermore, Rh2 has been reported to decrease cyclin dependent kinase2 (cdk2) in three of four mouse cell lines (Ota et al., 1997), increased p27 kipl activity (Ota et al., 1997), increased p21 WAF1 and cyclin D but decreased cdk2 and cyclin E in breast cancer cells (Oh et al., 1999).

The genomic effects of ginsenosides measured by protein expression, such as the cell cycle regulators, have been associated with the composition of the culture media. The protein content or other serum component of media (BSA, or cholesterol) may influence the effect of ginsenosides in cell culture experiments (Ota et al., 1991) and reduce or prevent the effects of ginsenosides, especially Rh2 (Odashima et al., 1985), when compared to the aglycone. Serum-free media conditions resulted in a greater cellular concentration of PD compared to Rh2 at the same dose, and the cellular uptake of Rh2 and PD were reduced as concentration of serum increased (Ota et al., 1991). PD uptake in the cells was rapid and at least partially incorporated in the inner region of membrane (Ota et al., 1991). The growth inhibition of PD (1.3 μM) is
stronger than Rh2 (2.0 μM) in serum free media (Ota et al., 1991). PD is also stronger than Rh2 in 10% serum media but at much greater concentration (Popovich and Kitts, 2002). A reduction in the serum concentration of the media has an effect in cell systems and may enhance the biological activity of ginsenosides. A listing of the effects of specific ginsenosides on cultured cancer cells as a function of serum concentration is listed in Table 1.3 and Table 1.4, respectively.

1.6.1 Influence of ginsenosides and cholesterol on cellular membranes

Treatment of B16 mouse melanoma cells with Rh2 increased cell to cell and cell to substrate adhesiveness and Rh2 was found to be incorporated into the lipid fraction of cells (Ota et al., 1987). Cells also became more rigid, a trait that is similar to the reported effects of cholesterol enriched cells (Koter et al., 2003). Cholesterol enrichment of Erlich tumor cells resulted in reduced cytotoxicity of PD and Rh2 (Popov, 2002) and in a model membrane systems, an increased hemolysis and permeability was found when cholesterol content was reduced. Treatment of breast cancer cells (MDA-MB-231) with β-sitosterol, a plant sterol related structurally to ginsenosides was reported to be effective in reducing the total cholesterol content of cells, cholesterol synthesis and reduced cell density (Awad et al., 2003). β-sitosterol (16 μM) exposure to colon cancer cells (HT-29) resulted in a 26% decrease in membrane cholesterol.

Cholesterol is required for growth of all cells and is greater for tumor cells with quicker growth cycles, and restriction of cholesterol results in the inhibition of cell proliferation (Awad et al., 1996). Cholesterol starvation arrested cells at G2 boundary of the cell cycle and induced apoptosis in MOLT-4 and HL-60 cells and cholesterol supplementation reduced the effect (Martinez-Botas et al., 1999). A dual role for cholesterol has been suggested, a role in ensuring
the integrity of the membrane structure and a regulatory role. Furthermore, other sterols such as β-sitosterol, may also be used in place of cholesterol, as a cellular compensatory or survival mechanism when the cholesterol level is limiting (Buttke and Folks, 1992; Dahl et al., 1987). For example, removal of cholesterol from cell membranes, by treatment with methyl-β-cyclodextrin, resulted in Mitogen activated protein kinase (MAPK) activation, which is important in cell cycle progression (Chen and Resh, 2002). Statins used to treat hypercholesterolemia have also been reported to change properties of membrane fluidity reducing the amount of cholesterol deposited in membrane (Koter et al., 2003). In leukemia cells (THP-1), both aglycones PD and PT had an effect on membrane integrity assessed by the amount of lactate dehydrogenase (LDH) released from the cultured cells (Popovich and Kitts, 2002). In intestinal cells such as Caco-2 and Int-407, Rh2 and PD were found to have the greatest impact on LDH release from these cells compared to other ginsenosides (Popovich and Kitts, 2003).
Table 1.3. Cell culture studies using specific ginsenoside tested with media serum of 5% or less.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cells</th>
<th>LC50</th>
<th>Dose tested</th>
<th>Time</th>
<th>Major Finding</th>
<th>Serum %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh2</td>
<td>SK-HEP-1 and transfected bcl-2 cells</td>
<td>4 µg/mL (6.4 µM)</td>
<td>7.5 µg/mL, 1 d</td>
<td>Activation of caspase-3</td>
<td>5% CS, 12 h serum-free then Rh2 for experimental.</td>
<td>(Park et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Rh2</td>
<td>A375-S2</td>
<td>15 µmol/L 6 h</td>
<td>NS 1-12 h</td>
<td>Dose and time dependent inhibition. Sub-G1 buildup between 0-40 µmol/L but at 60 µmol/L declines. ↑ caspase 8 and 3.</td>
<td>5% FCS</td>
<td>(Fei et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>Rg3, Rg5, Rk1, Rs5, Rs4</td>
<td>SK-HEP-1</td>
<td>Rg 3-41µM, Rg 5-11, Rk 1-13, Rs5-37, Rs4-13, (Cisplatin 84 µM)</td>
<td>NS 1 d</td>
<td>Rg3, Rg5, Rk1, Rs5, Rs4 are cytotoxic, whereas Rb1, Rc, Rb2, Rd are not.</td>
<td>5% CS</td>
<td>(Park et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>Rh2</td>
<td>B16, Meth-A, A31-1-1, A-31-1-13</td>
<td>NS</td>
<td>15 µM 3-4 d</td>
<td>↓ cdk2 in 3 of 4 cell lines, Rh2 incorporated into cells.</td>
<td>2% FCS</td>
<td>(Ota et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Rh2</td>
<td>SK-HEP-1, HeLA, Chang liver, COS7, FT02B</td>
<td>SK-HEP-1, and HeLA 0.11 µM, Chang liver 0.12 µM, COS7-1 µM, FT02B-0.11 µM</td>
<td>SK-HEP-1 1 µM 1 d</td>
<td>↑ p27 kip1 no effect on cyclin E, cdk2, p21 WAF1.</td>
<td>5% CS, and serum-free for experimental test.</td>
<td>(Lee et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>Panaxadiol</td>
<td>SK-HEP-1</td>
<td>3.1 µM</td>
<td>10 µM 1-5 h</td>
<td>Depolarization of mitochondria membrane. ↑ caspase 9, 3, not 8, ↑ cytochrome c, ↑ cdk2.</td>
<td>5% CS</td>
<td>(Jin et al., 2003a)</td>
<td></td>
</tr>
<tr>
<td>Rh2</td>
<td>SK-HEP-1 (transfected)</td>
<td>NS</td>
<td>NS 1-4 h</td>
<td>INK1-up-regulated, transfected cells are more sensitive.</td>
<td>5% CS</td>
<td>(Ham et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Rs4</td>
<td>SK-HEP-1 (Hepatoma cells)</td>
<td>20 µM</td>
<td>25 µM 1 d</td>
<td>↑ p53 and p21 WAF1.</td>
<td>5% CS</td>
<td>(Kim et al., 1999b)</td>
<td></td>
</tr>
<tr>
<td>Rh2</td>
<td>Rat C6 glioma cells (transfected)</td>
<td>NS</td>
<td>7.5 µg/mL 6 h</td>
<td>Rh2 cell death related to ROS generation, ↑ caspase, no Bcl-XL.</td>
<td>10% CS for maintenance, serum free for experimental exposure.</td>
<td>(Kim et al., 1999a)</td>
<td></td>
</tr>
<tr>
<td>Rb1, M1</td>
<td>B16-B16 (intestinal metabolite)</td>
<td>5-40 µM (M1)</td>
<td>NS 1 d</td>
<td>Rb1 no effect, M1 dose and time dependant, inside cell after 15 min. ↑ p27 kip1 ↓ c-Myc and cyclin-D1.</td>
<td>10% for cell maintenance. 5% for cell experimental exposure.</td>
<td>(Wakabayashi et al., 1998)</td>
<td></td>
</tr>
</tbody>
</table>

(continued on next page)
Table 1.3. (continued).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cells</th>
<th>LC50</th>
<th>Dose tested</th>
<th>Time</th>
<th>Major Finding</th>
<th>Serum %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1, Rh2</td>
<td>B16 (mouse)</td>
<td>NS</td>
<td>Rh1-40 μM, Rh2-12.5 μM</td>
<td>2 d</td>
<td>Rh2 inhibit growth, stimulate melonogenesis, ↑ adhesiveness, ↑ agglutinability. Cell surface membrane and composition were changed, Rh2 incorporated into membranes, changed lipid organization, ↑ polarization — more rigid cell. Rh2 does not.</td>
<td>10% cell maintenance, 2% FCS for experimental exposure</td>
<td>(Ota et al., 1987)</td>
</tr>
<tr>
<td>PD, Rh2, Rg3, Comp K</td>
<td>L1210, Ehrlich ascite tumor cells</td>
<td>NS</td>
<td>18 h</td>
<td></td>
<td>Rg3, Rh2, K, ↑ liposome permeability without cholesterol, Rh2, Rg3 ↓ cytotoxicity as cholesterol level of cells ↑.</td>
<td>5% CS</td>
<td>(Popov, 2002)</td>
</tr>
</tbody>
</table>

CS-Calf serum, FCS-fetal calf serum, NS-not specified
Table 1.4. Cell culture studies using specific ginsenoside tested with a serum medium concentration of 10%.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cells</th>
<th>LC50</th>
<th>Dose Tested</th>
<th>Time</th>
<th>Major Finding</th>
<th>Serum %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rd2, Re, Rf, Rg1-3, Ro, LNCaP, L929</td>
<td></td>
<td>Rg3-650 μM (LNCaP)</td>
<td>0-500 μM</td>
<td>2 d</td>
<td>↓ PSA (prostate specific antigen) ↓ 5α reductase, ↑ bcl-2 and caspase-3.</td>
<td>10% FCS</td>
<td>(Liu et al., 2000)</td>
</tr>
<tr>
<td>Rh2</td>
<td>MCF-7</td>
<td>LC40-50 μM</td>
<td>50-100 μM</td>
<td>2 d</td>
<td>Dose-dependant inhibition, cytostatic not cytotoxic, ↑ p21WAF1 and cyclin D ↓ cdk2 and cyclin E. At 10%FCS no apoptosis found.</td>
<td>10% FCS. Changed to 0% for apoptosis detection.</td>
<td>(Oh et al., 1999)</td>
</tr>
<tr>
<td>Rh1, Rh2, Rh3, Rh4 PD, PT</td>
<td>HL-60</td>
<td>NS</td>
<td>25, 50 μM</td>
<td>2-4 d</td>
<td>Rh2 and Rh3 ↓ cells, induced differentiation. PD and PT reduced tumor invasion seem to be related to glucocorticoid receptor.</td>
<td>10% FCS</td>
<td>(Kim et al., 1998b)</td>
</tr>
<tr>
<td>PD, PT</td>
<td>HT 1080</td>
<td>22μM (PD) 50μM (PT)</td>
<td>PD-10 μM, PT-40 μM</td>
<td>16 h</td>
<td>No effect on viability needed methanesulfonate (alkylating agent). ↑ p53, p21, ↓ cdk2, cyclin E,D1.</td>
<td>10% FCS</td>
<td>(Park et al., 1999)</td>
</tr>
<tr>
<td>PD, PT</td>
<td>NIH 3T3</td>
<td>NS</td>
<td>100 μg/mL (209-217μM)</td>
<td>24 h</td>
<td>No effect on viability needed methanesulfonate (alkylating agent). ↑ p53, p21, ↓ cdk2, cyclin E,D1.</td>
<td>10% FCS</td>
<td>(Hwang et al., 2002)</td>
</tr>
<tr>
<td>Rb1, Rg3, Rh2, PD L1210, P388, A549, Me180</td>
<td></td>
<td>L1210-Rg3-47 μM, Rh2-22 μM, PD-18 μM. P388-Rg3-58 μM,Rh2-33 μM, PD-33 μM, A549-Rh2-31 μM, PD-28 μM, Me180-Rh2-28 μM, PT-28 μM</td>
<td>NS</td>
<td>NS</td>
<td>Rg3 is converted to Rh2 and PD by human fecal flora. Rh2 and PD cytotoxic.</td>
<td>NS</td>
<td>(Bae et al., 2002)</td>
</tr>
</tbody>
</table>

(continued on next page)
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cells</th>
<th>LC50</th>
<th>Dose Tested</th>
<th>Time</th>
<th>Major Finding</th>
<th>Serum %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IH-901, Rbl</td>
<td>HL-60, PC-14, Hep G2, MKN-45</td>
<td>HL-60-24.3 μM, PC-14-28 μM Hep G2-30 μM, MKN-45-56.6 μM</td>
<td>24.3 μM (HL-60)</td>
<td>4 d</td>
<td>Rbl no effect, ↑ caspase-3, no Bcl-2 change, ↑ cytochrome c.</td>
<td>10% FCS</td>
<td>(Lee et al., 2000b)</td>
</tr>
<tr>
<td>Rg3</td>
<td>KB, KBV20C, P388, P388/DOX (mouse)</td>
<td>NS</td>
<td>5-320 μM</td>
<td>NS</td>
<td>Rg3 restored sensitivity to cancer drugs in resistant cells (KBV20C), but not in drug sensitive (KB). Serum in medium affects sensitivity.</td>
<td>Cells (KB - 5% FCS)</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>Rh1</td>
<td>MCF-7</td>
<td>NS</td>
<td>50 μM</td>
<td>2 d</td>
<td>Weak phytoestrogen, no glucocorticoid activity.</td>
<td>10% CS</td>
<td>(Lee et al., 2003)</td>
</tr>
<tr>
<td>Rh1, Rh2</td>
<td>NIH 3T3 (mouse)</td>
<td>NS</td>
<td>Rh1 and Rh2 20, 50 μM</td>
<td>NS</td>
<td>↓ phospholipase C, ↓ intracellular diacylglycerol.</td>
<td>10% FCS</td>
<td>(Byun et al., 1997)</td>
</tr>
<tr>
<td>IH-901, Rbl</td>
<td>PC-14 PC/DDP (resistant), HL-60 MKN-45, HepG2</td>
<td>HL-60-24.3 μM, PC-14-25.9 μM MKN-45-56.6 μM HepG2-24.9 μM PC-14/DDP-20.3 μM</td>
<td>NS</td>
<td>4 d</td>
<td>Rbl has no cytotoxic effect. IH-901 three-fold more effective in CDDP (cisplatin) resistant cells.</td>
<td>10% FCS</td>
<td>(Lee et al., 1999)</td>
</tr>
<tr>
<td>PD, PT, Rh2, Rh1, Rg3</td>
<td>THP-1</td>
<td>PD 28 μM, PT 27 μM Rh2 24 μM Rh1 328 μM</td>
<td>27-328 μM</td>
<td>1-3 d</td>
<td>PD, PT, Rh2, Rh1 are cytotoxic. Rg3 is not cytotoxic, ↑ Sub-G1, ↑ LDH.</td>
<td>10% FCS</td>
<td>(Popovich and Kitts, 2002)</td>
</tr>
</tbody>
</table>

CS-Calf serum, FCS-fetal calf serum, NS-not specified.
OVERALL HYPOTHESES

Bioactive effects of ginsenosides can be characterized according to the hydrophobic or structural characteristics of the compound. North American ginseng plant material can be a source of bioactive ginsenosides.

OVERALL OBJECTIVES

To identify, evaluate and recover sources of bioactive ginsenosides.

PART 1. IDENTIFICATION OF BIOACTIVE CONSTITUENTS OF NORTH AMERICAN GINSENG (GINSENSOIDES).

NULL HYPOTHESIS \( (H_0) \): Bioactive effects of ginsenosides on cultured cancer cells are influenced according to individual structural and compositional differences.

OBJECTIVE 1: To identify ginsenosides that have a measurable effect on cell viability in three cultured cells lines.

OBJECTIVE 2: To investigate how active ginsenosides influence cultured cell proliferation.

PART 2. CHARACTERIZATION OF RH2 IN NORTH AMERICAN GINSENG PLANT

NULL HYPOTHESIS \( (H_0) \): Rh2 is present and can be recovered from North American ginseng plant material.

OBJECTIVE 1: To determine if bioactive, rare ginsenoside Rh2 is present in North American ginseng plant materials.

OBJECTIVE 2: To compare the application of thermal extraction and room temperature extraction for Rh2 generation.
NULL HYPOTHESIS (H₀)²: Rh2 is formed during thermal extraction of ginseng plant material.

OBJECTIVE: To sample the hot water reflux extraction of ginseng for the presence of ginsenoside Rh2.

NULL HYPOTHESIS (H₀)³: Rh2 is a thermal breakdown product of abundant ginsenosides Rb₁ and Rc.

OBJECTIVE: To determine if thermal treatment of ginsenoside Rb₁ and Rc results in the production of ginsenoside Rh2.

PART 3: DEVELOPMENT, ASSESSMENT OF BIOACTIVITY AND PROPOSED MECHANISM OF AN ENRICHED RH2 EXTRACT FROM THE LEAVES OF NORTH AMERICAN GINSENG

OBJECTIVE: To develop an enriched Rh2 extract from North American ginseng leaf material.

NULL HYPOTHESIS (H₀)⁴: The enriched Rh2 ginseng extract will affect cultured cell viability.

OBJECTIVE: To assess the enriched Rh2 ginseng extract effect on two cultured cancer cells (THP-1, Caco-2).

NULL HYPOTHESIS (H₀)⁵: Cell death is a result of a combination of apoptosis and decreased cell membrane function.

OBJECTIVE: To determine whether the active ginsenosides and the enriched Rh2 ginseng fraction impact cultured cell viability via alteration of cell membrane function or through apoptotic mechanism, or a combination of both.
PART 1. IDENTIFICATION OF BIOACTIVE CONSTITUENTS OF NORTH
AMERICAN GINSENG (GINSENOSIDES)

2 CHAPTER II

Preface

A version of Chapter II has been published in Archives of Biochemistry and Biophysics 2002.

2.1 ABSTRACT

Ginsenosides of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol classification including the aglycones, protopanaxadiol (PD), protopanaxatriol (PT) and ginsenosides Rh2, Rh1 were shown to possess reduce proliferation of human leukemia cells (THP-1). A similar efficacy was not apparent for ginsenoside Rg3. The concentration to inhibit the growth of 50% of cells (LC50) for PD, Rh2, PT and Rh1 were 13 μg/mL, 15 μg/mL, 19 μg/mL and 210 μg/mL, respectively. PD and PT induced DNA fragmentation at the LC50 after 72 h of treatment. Cell cycle analysis confirmed apoptosis with PD and PT treatment of THP-1 cells resulting in a build up of sub-G1 cells after 24, 48 and 72 h of treatment. Rh2, and dexamethasone treatments also increased apoptotic cells after 24 h, whereas Rh1 did not. After 48 and 72 h Rh2, Rh1 and dexamethasone similarly increased apoptosis, but these effects were significantly (P ≤ 0.05) lower than observed for both PD and PT treatments. Furthermore, treatments that produced the largest build up of apoptotic cells were also found to have the largest release of lactate dehydrogenase (LDH). It can be concluded from these studies that the additions of sugars to PD and PT aglycone structure reduces the potency to induce apoptosis, and alters membrane integrity. These cytotoxic effects to THP-1 cells were different from dexamethasone.
2.2 INTRODUCTION

Ginseng (Panax ginseng, C.A. Meyer) has a long history of traditional Chinese medicinal usage as a general tonic to promote health (Banthorpe, 1994). Ginseng is increasingly being employed as a herbal supplement and for more contemporary nutraceutical or functional food usages. Ginseng has been reported to possess antioxidant activity, affect both central nervous system and neuroendocrine functions, alter carbohydrate and lipid metabolism, and modulate immune function (Gillis, 1997; Kitts et al., 2000; Kitts and Hu, 2000). It is generally believed that the major bioactive components of ginseng are the triterpene saponins, also referred to as ginsenosides (Gillis, 1997). Over thirty different ginsenosides have been isolated and structures determined (Banthorpe, 1994; Kim et al., 1998b).

Ginsenosides share a similar basic structure, consisting of gonane steroid nucleus having 17 carbon atoms arranged in four rings. Differences in ginsenoside structure which include the type, position and number of sugar moieties attached by a glycosidic bond at positions C-3 and C-6 of the rings can characteristically influence biological response (Byun et al., 1997; Liu et al., 2000; Odashima et al., 1985). Based on these structural differences, two main categories of ginsenosides exist, the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol classifications. The 20(S)-protopanaxadiol classification contains the most abundant ginsenosides in ginseng, such as ginsenosides Rb1, Rb2, Rc, Rd, and rare compounds such Rg3, Rh2 and the aglycone PD. Pharmacokinetic studies conducted in rats have reported 23% absorption of ginsenoside (Rb1) after a period of 2.5 h (Takino et al., 1982). Specifically, ginsenosides Rg3 and Rh2 have been reported to possess bioactivity in cell culture experiments and can alter cancer cell proliferation, induce apoptosis and perturb normal cell cycle events (Kim et al., 1999b; Lee et al., 1996; Liu et al., 2000; Park et al., 1997). The 20(S)-protopanaxatriol classification contains ginsenosides Re,
Rg1, Rg2, Rh1 and the aglycone PT. It is noteworthy, that many ginsenosides, based on structural similarities to steroids, also interact with plasma membranes (Attele et al., 1999), possess some steroid-like activity (Lee et al., 1998b) and may be a functional ligand of the glucocorticoid receptor by competing with dexamethasone (Lee et al., 1997).

The purpose of this study was to test the hypothesis that the relative bioactivity of related ginsenosides derived from the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol families will decrease as the glycosidic attachment of sugar moieties located on position C-3 and C-6 of the gonane structure increases. A possible structure-function relationship for cell proliferation and apoptosis in cultured human (THP-1) cells was examined, that could be explained on the basis of the hydrophobic character of the ginsenoside. This cell line represents a model of human monocytes and macrophages.

2.3 MATERIALS AND METHODS

2.3.1 Test compounds and materials

Five ginsenoside standards were obtained from Canfo (Chendu, China) and purity confirmed by HPLC. The 20(S)-protopanaxadiol and 20(S)-protopanaxatriol families, which includes the 20(S)-protopanaxadiol aglycone (PD), ginsenoside-Rh2 (Rh2), ginsenoside-Rg3 (Rg3) and the 20(S)-protopanaxatriol aglycone (PT) and ginsenoside-Rh1 (Rh1) are shown in Figure 2.1. Purity assessments were greater than 98% as assessed by HPLC (Li et al., 1996). Dexamethasone (Dex), a synthetic glucocorticoid was obtained from Sigma (St. Louis, MO). The test compounds were initially dissolved in 70% ethanol and subsequently diluted with culture medium to the final concentration prior to use. Unless stated, all test materials were obtained from Sigma (St. Louis, MO).
2.3.2 Cell culture

Human acute monocytic leukemia suspension cell line (THP-1) was obtained from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with HEPES (10 mM), fetal bovine serum (10%) (Gibco, Grand Island, NY), 2-mercaptoethanol (0.05 mM), penicillin (100 U) and streptomycin (100 µg/mL) (Gibco, Grand Island, NY). Cultures were maintained at a cell concentration between $2 \times 10^5$ and $1 \times 10^6$ cells/mL, with culture medium added every three days. Cells were subcultured by total medium replacement using centrifugation every 5-6 days depending on cell number and incubated at 37 °C in a 5% CO₂ humidified incubator. Cell numbers were assessed by trypan blue (0.04%) exclusion dye using a hemocytometer. Viable cell numbers were assessed in quadruplicate.

2.3.3 Cell viability MTT assay dose response

A cell viability assay, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction by viable cells, was used in order to establish an LC50 (e.g. concentration to inhibit the growth of 50% of cells). Fifty microlitres of RPMI 1640 medium was added to each well of a 96 microwell plate. Test compounds (PD, Rh2, Rg3, PT, Rh1, Dex) were serially diluted by mixing 50 µL of test compound with 50 µL medium and in a two-fold dilution transferred to adjacent wells. THP-1 cells were seeded to each well to a final concentration of $5 \times 10^5$ cells/mL. Controls contained test model cells, culture medium but no test compounds. Cells were incubated for three days before MTT was added to a concentration of 0.5 mg/mL and incubated in the dark for 4 h as first described by Mosmann (1983). To solubilize the formazan crystal, 100 µL of SDS (10%) (Fisher, Springfield, NJ) in HCl (0.1 M)
was added to each well and incubated overnight. The optical density was read at 550 nm absorbance in a microplate reader (Biorad, Cambridge, MS).

2.3.4 Cell LDH activity

THP-1 cells were seeded at a concentration of $1 \times 10^6$ cells/mL in 24 well plates. The test compounds (PD, Rh2, PT, Rh1, and Dex) were added to THP-1 cells at LC50 determined previously from MTT assays; the concentrations used were 13, 15, 19, 210 and 13 $\mu$g/mL respectively. Untreated cells acted as control. Cells were incubated at 37 °C in a 5% CO$_2$ humidified incubator for 24, 48 and 72 h. Cell-free supernatant was obtained by centrifugation (400 x g) for 10 min. Two millilitres of Tris-EDTA-NADH buffer and 50 $\mu$L of cell-free supernatant were mixed and incubated in a 37 °C water bath for 10 min and 200 $\mu$L of pre-warmed (37 °C) pyruvate solution was added. The mixture was transferred to a 3 mL cuvet and the initial reaction velocity was recorded by continuous monitoring of the absorption at 340 nm at 37 °C (Shimadzu Corp, UV-160 Spectrophotometer, Kyto Japan). The concentration of the reaction components in the cuvette was Tris buffer 50 mmol/L (pH 7.4, 37 °C); EDTA, 5 mmol/L; pyruvate, 1.2 mmol/L; NADH, 150 $\mu$mol/L as reported (Moss and Henderson, 1994).

2.3.5 DNA fragmentation

DNA fragmentation was assessed by agarose gel electrophoresis. After 72 hours treatment of THP-1 cells ($1 \times 10^6$ cells/mL) with PD, Rh2, PT, Rh1, Dex (13, 15, 19, 210 and 13 $\mu$g/mL, respectively) or untreated cells as a control, cells were washed twice with PBS, lysed in Tris-HCL (10 mM), EDTA (10 mM), SDS (0.5%) and proteinase K (100 $\mu$g/mL) overnight at 55 °C. DNA was extracted twice by phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and once
with chloroform. DNA was precipitated with ammonium acetate (2.5 M, pH 5.2) and two-volumes ice-cold ethanol (100%) and washed in 70% ethanol as described in Ramachandra and Studzinski (1995). SDS and proteinase K treatment results in the inactivation of DNase (Hansen, 1974; Liao, 1974). Samples were dried under air and dissolved in TE buffer (10 mM Tris-HCL, 5 mM EDTA, pH 8.0). DNA was incubated with RNase A (100 µg/mL) for 30 min and separated in a 1.8% agarose gel for 2.5 h at 70 Volts. The gel was stained with ethidium bromide (0.5 µg/mL) and photographed under UV light. A DNA step-ladder marker was run in parallel with treated and untreated control samples.

2.3.6 Flow cytometry cell cycle analysis

Test compounds PD, Rh2, PT, Rh1 and Dex were added to THP-1 cells (1 x 10^6 cells/mL) at concentrations described above. Cells were incubated at 37 °C in a 5% CO2 humidified incubator for 24, 48, and 72 h with the untreated cells acting as a control. After treatment, cells in suspension were centrifuged for 10 min (400 x g). The supernatant was discarded and cells were further washed twice in PBS and re-centrifuged to remove the cell pellet. The pellet was vortexed vigorously and 1 mL of ice-cold 70% ethanol was added slowly and fixed overnight at 4 °C. Ethanol was removed by centrifugation (10 min, 300 x g) and gently vortexed followed by the addition of 1 mL of PBS containing propidium iodide (50 µg/mL) and RNase A (100 µg/mL). Samples were incubated at room temperature for 1 hour and analysed by FACscan flow cytometry (Becton-Dickinson, Mountain View, CA) as outlined in Noguch (1991). Flow cytometry data were analysed using WINMDI software package (La Jolla, California).
2.3.7 Statistical analysis

One-way ANOVA (SPSS release 9.0) was used to analyse the experimental data at 24, 48 and 72 h time periods. Significance was judged at \( P \leq 0.05 \) using Tukey post hoc multiple comparisons of observed means.

2.4 RESULTS

2.4.1 Dose response LC50 determination

Figure 2.2 shows the different concentration response effects of test compounds PD, Rh2, Rg3 (bottom panel) and PT, Rh1 and Dex (top panel) on the proliferation of THP-1 cells. The 20(S)-protopanaxadiol family of compounds were determined to have an LC50 of 13 \( \mu g/mL \) for the PD aglycone, followed by 15 \( \mu g/mL \) for Rh2, and Rg3 had no inhibitory effect on proliferation of the cells at concentrations greater than 250 \( \mu g/mL \). The 20(S)-protopanaxatriol classification of compounds were determined to have an LC50 of 19 \( \mu g/mL \) for the PT aglycone followed by Rh1 at 210 \( \mu g/mL \). Dexamethasone (Dex) had no effect on proliferation of the THP-1 cells at concentrations greater than 250 \( \mu g/mL \).

2.4.2 LDH activity

To assess the effect of PD, Rh2, PT, Rh1 and Dex on membrane integrity, lactate dehydrogenase (LDH) activity was measured in cultured THP-1 supernatant after exposure for 24, 48 and 72 h (Figure 2.3). After 24 h, LDH activity for PT treatment was significantly (\( P \leq 0.05 \)) higher (194 ± 3% of untreated cells) compared to other test samples. Rh2 was found to be 113 ± 8%, followed by PD (112 ± 21%), Rh1 (107 ± 3%) and Dex (100 ± 5%). After 48 h of treatment, PT was significantly (\( P \leq 0.05 \)) higher (206 ± 18%) than all test samples followed by
PD (143 ± 7%) which was in turn significantly (P ≤ 0.05) higher than Dex (111 ± 9%) and Rh2 (106 ± 3%) respectively, but not Rh1 (121 ± 9%). After 72 h, both PT (196 ± 7%) and PD (199 ± 31%) treatment were significantly (P ≤ 0.05) higher than Rh1 (131 ± 12%), Dex (126 ± 13%) and Rh2 (115 ± 8%) treatments.

2.4.3 DNA fragmentation

Figure 2.4 shows the impact of PD, Rh2, PT, Rh1, Dex treatments of THP-1 cells at the LC50 used to induce DNA fragmentation. PD (lane 1) and PT (lane 2) induced DNA fragmentation after exposure for 72 h. Rh1 (lane 4), Rh2 (lane 5), Dex (lane 6) and untreated cell (lane 3) showed no evidence of DNA fragmentation.

2.4.4 Cell cycle analysis

The effect of PD, Rh2, PT, Rh1 and Dex on THP-1 cell cycle events measured using flow cytometry showed that these test compounds produced different effects represented by DNA histograms (Figure 2.5). After 24 h, PT treatment produced a significantly (P ≤ 0.05) higher production of apoptotic cells (sub-G1) (42 ± 3%), compared to untreated cells (2 ± 0.1%) and other treatments at respective LC50s (Figure 2.6). PD (20 ± 2%), Rh2 (22 ± 5%) and Dex (15 ± 5%) treatments were also significantly (P ≤ 0.05) higher than untreated cells but Rh1 (4 ± 1) treatment had no effect. After 48 h, both PT (40 ± 2%) and PD (34 ± 3%) treatments were significantly (P ≤ 0.05) higher than untreated cells, followed by Rh2 (20 ± 1%), Dex (18 ± 2%) and Rh1 (10 ± 3%). After 72 h, PD (44 ± 8%) was found to have the highest build up of apoptotic cells followed by PT (30 ± 2%), Dex (22 ± 2%), Rh2 (18 ± 4%) and Rh1 (15 ± 1%), all of which were significantly (P ≤ 0.05) higher than untreated cells.
2.5 DISCUSSION

In this study it was shown that the concentrations of individual 20(S)-protopanaxadiol and 20(S)-protopanaxatriol ginsenosides that are required to inhibit proliferation of cultured human leukemia cells (THP-1) varies with specific ginsenoside. For the 20(S)-protopanaxadiol family of ginsenosides, PD was found to have the strongest effect relative to Rh2 whereas, Rg3 had no effect on cell proliferation. A similar finding was found for PT of the 20-(S) protopanaxatriol family of ginsenosides, relative to the effect on proliferation compared to Rh1. These results indicate that specific differences in ginsenoside chemical structure will influence the cytotoxic properties and proliferation of THP-1 cells. The THP-1 cell line was chosen as a model cell system with a range of similar properties to human monocytes and macrophages (Kim et al., 1999a; Klegeris et al., 1997; Lorton et al., 1996; Nakayama et al., 2000).

Ginsenosides are characterized according to the number and position of sugar moieties on the sterol chemical structure (Figure 2.1). Rh2 of the 20(S)-protopanaxadiol classification differs from the structure of the PD by the addition of one glucose moiety at position C-3 and Rg3 has the addition of two glucose moieties at the same position. Rh1 of the 20(S)-protopanaxatriol family of compounds differs from PT by the addition of one glucose moiety at C-6 (Figure 2.1). Rh2 has been shown to suppress proliferation in a number of human cancer cells including, breast, prostate, hepatic, intestinal and animal cell lines (Kim et al., 1999a; Lee et al., 1996; Oh et al., 1999; Park et al., 1997). Rh2 and other ginsenosides have also been reported to act on specific membrane proteins or penetrate the plasma membrane and initiate genomic effects in addition to behaving as steroid hormones, and potent signalling molecules (Attele et al., 1999). Much less is known about the 20(S)-protopanaxatriol family of ginsenosides. Rh1 has been reported to inhibit proliferation of NIH 3T3 mouse fibroblast cell line (Byun et al., 1997), but did
not influence growth of B16 melanoma cells (Odashima et al., 1985). Dexamethasone (Dex), a synthetic glucocorticoid was chosen in this study to compare the effects of ginsenosides to steroids. Our results indicate that PD, Rh2, PT and Rh1 had a substantially stronger effect at inhibiting proliferation of cancer cells than Dex.

In the present study, characteristic effects of specific ginsenosides on cell proliferation were achieved by examining the affinity of test compounds to induce apoptotic or programmed cell death. Apoptosis was effectively measured by cell cycle analysis using flow cytometry and by DNA fragmentation. In cell cycle analysis, the build up of sub-G1 cells is characteristic of apoptosis (Darzynkiewicz et al., 1995), and agarose gel electrophoresis fragmentation of chromosomal DNA into 180 bp fragments is an effective marker of late apoptosis (Wyllie, 1980). There are many reports showing the induction of apoptosis by ginsenosides Rh2. For example, Rh2 has been reported to activate caspase-3 protease, a major pro-enzyme involved in apoptosis. Rh2-induced apoptosis has also been described to trigger activation of cyclin A-associated-cyclin-dependent kinase 2 (cyclin A-Cdk2) by p21WAF1/CIP1 in SK-HEP-1 cells (hepatoma cells) (Jin et al., 2000). The p21 WAF1/CIP1 inhibitor is transcriptionally regulated by the p53 tumor suppresser, and is important in the G1/S phase DNA-damage checkpoint control machinery. In MCF-7 human breast cancer cells, Rh2 inhibits growth in an irreversible, concentration dependent manner, while also inducing a G1 arrest in the cell cycle, and an up-regulation of the expression of Cdk inhibitor p21WAF1/CIP1 that results in reduced protein levels of cyclin D (Oh et al., 1999).

No effect on DNA fragmentation of cancer cells was found following treatments of Rh2 at a LC50 of 15 μg/mL, Rh1 (210 μg/mL) or Dex (13 μg/mL). The aglycones of Rh2 (PD) and Rh1 (PT) did however, induce DNA fragmentation at a LC 50 of 13 μg/ml and 19 μg/mL,
respectively. Both PD and PT treatments were found to have varied effects on apoptosis measured by flow cytometry. Flow cytometry is a unique and effective technique that provides rapid, quantitative analyses on single living, or dead cells (Sharrow, 1996) and will identify cells in sub-populations. This procedure enabled us to determine that PT treatment produced the largest increase in the percentage of cells in the sub-G1 phase of the cell cycle after 24 h, whereas, PD treatment induced the largest build-up after 72 h. Cell-cycle analysis indicated a build-up of sub-G1 cells for Rh2 and Dex after 24 h, and Rh2, Rh1 and Dex after 48 and 72 h, respectively. The synthetic glucocorticoid Dex, which is known to induce apoptosis (Wyllie, 1980), was found to have a smaller increase of cells in sub-G1 phase of the cell cycle, compared to PD after 48 and 72 h and PT after 24 and 48 h, respectively. Detection and confirmation of apoptosis by DNA fragmentation was not conclusive for Rh2, Rh1 and Dex treatments, thus indicating varying levels of apoptotic cell accumulation. The use of DNA fragmentation, which can resolve late apoptotic events but may lack the sensitivity of cell cycle analysis, indicated that both PD and PT affected cell cycle and induce apoptosis to a greater extent than dexamethasone. This finding would suggest that PD and PT bioactivity occurs independent of steroid activity, a popular suggestion that exists in the literature (Lee et al., 1997; Lee et al., 1998b).

It is particularly noteworthy that PD significantly increased LDH activity when compared to Rh2 after 48 and 72 h of treatment. In contrast, PT treatment resulted in greater increase in THP-1 LDH activity compared to Rh1 for all treatment periods. LDH, a stable enzyme present in all cells and found only in the cytoplasm (Cominacini et al., 1997), is released when the cell membrane is damaged. LDH release is therefore a useful marker of membrane integrity. The fact that aglycones (PD and PT) affect cell membrane function in possibly different ways compared to corresponding ginsenoside compounds Rh2 and Rh1, strongly indicates that these
bioactivities are specifically related to an effect on apoptosis. This suggestion was confirmed herein with findings that showed treatments that produced the largest release of LDH activity, also exhibited the greatest build-up of apoptotic cells. Ginsenosides are known to pass directly into the nucleus or alternatively embed into the cell membrane (Odashima et al., 1985; Ota et al., 1987). Our findings therefore suggest the possibility of an increased permeability of THP-1 cell membranes by PD and PT treatment in a manner similar reported to that reported for phytosterols (Awad and Fink, 2000). For example, the increase in cell permeability may be explained, in part, by the different hydrophobic properties of individual test ginsenosides and thus preferential uptake in cell membrane in place of endogenous sterol.

To our knowledge, these findings are the first to show that the PD aglycone of Rh2 and Rg3 and the PT aglycone of Rh1 have a relatively stronger affinity to induce apoptosis than other structurally related ginsenosides. These differences cannot be compared to the known effects of dexamethasone. Moreover, differences between specific ginsenosides reported herein are suggested to be related to the structural conformation and presence of sugars at position C-3 and C-6 which contribute to unique differences and will influence the hydrophobic character of the compounds required to interact with cell membrane function. Future studies are needed to determine if the relative ginsenoside compositional differences specific to Asian (Panax ginseng, C.A. Meyer) and North American (Panax quinquefolius) ginseng sources, constitute sufficient advantage for selection of use in the development of novel nutraceutical products.
Figure 2.1. The structure of test compounds 20(S)-protopanaxadiol aglycone (PD), ginsenosides Rh2, Rg3 and 20(S)-protopanaxatriol aglycone (PT) and Rh1.
Figure 2.2. Dose response relationship between test compounds (PT, Rh1, Dex and PD, Rh2, Rg3), in THP-1 cells after three days measured in triplicate by MTT viability assay as outlined in the Material and Methods. Figure labels corresponding to the top panel (A) are represented by PT (●), Rh1 (■), and Dex (▲). The bottom panel (B) labels are represented by PD (◊), Rh2 (□), Rg3 (△). Values are expressed as a percentage of untreated cells (mean ± SD).
Figure 2.3. Lactate dehydrogenase (LDH) activity was measured in cell-free supernatant (THP-1 cells) after 24, 48 and 72 h of treatment at LC50 determined by MTT assay. LDH activity was determined in cell-free supernatant as outlined in the Material and Methods. Data are expressed as percentage of untreated samples (mean ± SD, n = 3). Bars within the same time period possessing different letters are significantly different (P ≤ 0.05).
Figure 2.4. DNA fragmentation of THP-1 cells after exposure to test compounds compared to untreated sample for 72 h. The DNA was extracted as described in Materials and Methods, and separated on a 1.8 % agarose gel for 2.5 h at 70 volts. DNA fragmentation was detected by ethidium bromide staining and was photographed under UV light. The lane marked (M) corresponds to DNA stepladder marker labelled with appropriate base pairs amount; lane 1 represents PD treated cells, lane 2 (PT), lane 3 (untreated), lane 4 (Rh1), lane 5 (Rh2), and lane 6 (Dex).
Figure 2.5. DNA cell cycle histograms of untreated cell compared to cells treated with PD, PT, Rh2, Rh1 and Dex at the respective LC50 concentration for 24, 48 and 72 h. Cells were fixed in ethanol and stained with PI as described in the Materials and Methods. DNA histograms shown are representative histograms of three separate experiments.
Figure 2.6. Percentage of apoptotic cells (sub-G1) following PD, PT, Rh2, Rh1 and Dex treatment measured by flow cytometry. Bars of the same time period (mean ± SD, n = 3) with different letters are significantly different compared to control (Con) cells (P ≤ 0.05).
2.6 SUMMARY AND FUTURE STUDIES

Four compounds PD, PT, Rh2 and Rh1 have been identified as having a characteristic effect on cultured leukemia cell viability. A similar efficacy was not apparent for ginsenoside Rg3 a structurally related protopanaxadiol ginsenoside. Cell cycle analysis showed that PD and PT treatment of THP-1 cells resulted in a build up of sub-G1 cells after 24, 48 and 72 h of treatment. Rh2 also increased apoptotic cells after 24 h, whereas Rh1 did not. Furthermore, treatments that produced the largest build up of apoptotic cells were also found to have the largest release of lactate dehydrogenase (LDH). It can be concluded that the presence of specific sugars to the PD and PT aglycone structure reduces the potency to induce apoptosis, and alternately alter membrane integrity. Therefore, ginsenosides have variable effects on cultured cells and the effects may relate to the hydrophobic or structural characteristics of the compounds.

It is still unclear if the aglycones PD, PT and ginsenosides Rh2 and Rh1 have similar effects in other cultured cancer cell lines. Of particular importance is the effect on cultured gastrointestinal cells. Gastrointestinal cells represent the first route of exposure to bioactive plant compounds and are an important model to study the interaction of compounds of different polarity and absorption.

Furthermore, aglycones PD, PT and ginsenosides Rh2 and Rh1 are considered rare compounds that are not found in all types of ginseng and ginseng products. Typically, ginseng products are standardized according to six more abundant ginsenosides and it is not clear if these compounds have characteristically different response on cultured cells compared to rare ginsenosides.
3 CHAPTER III

Preface

A version of Chapter III has been published in Canadian Journal of Physiology and Pharmacology 2004.

3.1 ABSTRACT

Ginsenosides derived from 20(S)-protopanaxatriol (PT) and 20(S)-protopanaxadiol (PD) groups had similar characteristic cytotoxic effects on the growth of two intestinal cell lines namely, Int-407 and Caco-2. Pure Rh2, a ginsenoside structurally related to PD inhibited intestinal cell growth at greater than twice the concentration of PD, while Rh1 a ginsenoside structurally related to aglycone PT, had no cytotoxic effect. Concentrations, which produce growth inhibition of 50% of cells (LC50) for compounds PD, PT and Rh2, were 23 μg/mL, 26 μg/mL and 53 μg/mL, respectively for Int-407 cells. In comparison, LC50 values for PD and PT were determined to be 24 μg/mL and for Rh2 was 55 μg/mL in Caco-2 cells. A standardized North American ginseng extract with a measured and known ginsenosides composition did not induce cytotoxicity in either of the intestinal cell lines. Cycle analysis showed characteristically different (P ≤ 0.05) effects of ginsenosides PD, Rh2 and PT in both cell lines. Rh2 treatment of Int-407 produced a significantly (P ≤ 0.05) higher production of sub-G1 (apoptotic) cells (35 ± 1%), compared to untreated cells (14 ± 0.3%) after 24 h. PD and Rh2 treatments were both significantly (P ≤ 0.05) higher than untreated cells after 48 and 72 h. Similar results were obtained for treatment of Caco-2 cells. Lactate dehydrogenase (LDH) activity in both cell lines was similar for PD and Rh2 and higher (P ≤ 0.05) than PT treatment at most time periods. These results show a specific structure-function relationship for bioactive ginsenosides in two contrasting intestinal cell types.
3.2 INTRODUCTION

Acceptance of traditional herbal and nutraceutical products is growing. Ginseng is one of the most utilized components of traditional Chinese medicine, and now occupies a large portion of the nutraceutical market (Brevoort, 1998). Standardization of ginseng formulations by the amount of triterpene saponins, also known as ginsenosides, is important for consumer confidence. Typically, there are six individual ginsenosides, which comprise the standard reference; they are Rb1, Rb2, Rg1, Rc, Rd and Re. However, these reference ginsenosides may not be exclusively related to ginseng bioactivity. Previous work from our laboratory has shown that certain ginsenosides classified according to the aglycone structure, exhibited a structure-function relationship and related affinity for cytotoxicity and apoptosis in leukemia cells (THP-1) (Popovich and Kitts, 2002). Ginseng and other herbal formulations are taken orally, and consequently the gastrointestinal cells are exposed to a greater proportion of compounds compared to other cell systems. Bacteria collected from human fecal material have been reported to hydrolyze ginsenosides Rb1 and Rb2 to specific metabolites (Hasegawa et al., 1997). For example, compound IH-901 is formed after Rb1 is inoculated with human fecal bacteria (Lee et al., 2000b). In a pilot study of two human volunteers, ginsenoside Rb1 was detected by LC-MS analysis in the urine and plasma, furthermore, hydrolysis products and the main intestinal bacterial metabolite was also detected (Tawab et al., 2003). The bacterial metabolite structures differ slightly from both protopanaxadiol and protopanaxatriol aglycones.

Gastrointestinal cell lines can be an important model for the bioactive standardization of ginsenosides. Bioactive compounds from ginseng could interact with cell membranes and initiate cellular responses, without being fully absorbed, and thus influence gastrointestinal function. Therefore, ginsenosides and related plant compounds may have an integral role in the health of
the gastrointestinal system from the standpoint of regenerating healthy cells and 
eliminating pre-cancerous and cancerous cells.

The purpose of this study was to investigate the relative bioactivity of a standardized 
North American ginseng extract, and individual pure ginsenosides to induce cytotoxicity and 
apoptosis in two distinct intestinal cell lines, Int-407 and Caco-2. Int-407 represents an 
embryonic cell line and Caco-2 represents a colorectal adenocarcinoma cell line and both 
represents models for evaluating the bioactivity of plant derived compounds and the effect on 
gastrointestinal cells.

3.3 MATERIALS AND METHODS

3.3.1 Test compounds and materials

A standardized North American ginseng root extract was obtained by solvent extraction 
according to the method of Hu and Kitts (2001). The composition of the standardized ginseng 
eextract was analyzed by HPLC (Li et al., 1996) and by TLC (Sanada et al., 1974) and total 
ginsenoside content was determined spectrophotometrically (Hiai et al., 1975a). Briefly, for the 
HPLC analysis a Hewlett-Packard (HP) series 1100 high-performance liquid chromatograph was 
used with a diode array detector (Agilent Technologies). The column used was an HP, Zorbax 
C18, 4.0 x 150 mm, 3.5 μm. The solvent systems consisted of acetonitrile (HPLC grade) and 
nano-pure water (Barnstead Dubuque, IA) with a flow rate of 1 mL/min. Furthermore, total 
ginsenosides content was determined by the reaction that was formed by heating vanillin and 
sulfuric acid with ginseng samples and absorption measured at 544 nm (Shimadzu Corp, UV-160 
Spectrophotometer, Kyto Japan). Three ginsenoside standards Rb1, Rc and Re were obtained 
from Sigma (St. Louis, MO). Four additional ginsenoside standards 20(S)-protopanaxadiol (PD),
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20(S)-protopanaxatriol (PT) and ginsenosides Rh2 and Rh1 were obtained from Canfo (Chengdu, China). Purity of ginsenosides standards were confirmed by HPLC (Li et al., 1996). The structures of the four ginsenoside standards are shown in Figure 3.1.

3.3.2 TLC

Powdered ginseng extract was solubilized in methanol and separated using chloroform-methanol-water (65:35:10, v/v/v) lower phase (Fischer Scientific, Fair Lawn NJ, Barnstead E-pure water purifier Dubuque, IA). Plates were visualized by spraying sulfuric acid (10% H₂SO₄, v/v) in ethanol, and heating at 125 °C for 4 min. The plate was scanned using Biorad GS-670 densitometer and Molecular Analyst software (Biorad, Cambridge, MA).

3.3.3 Cell culture

Intestine 407 (Int-407) and Caco-2 cell lines were obtained from ATCC (Manassas, VA). Cells were maintained separately in minimum essential medium supplemented with fetal bovine serum (10%) (Gibco, Grand Island, NY), penicillin (100 U) and streptomycin (100 µg/mL) (Gibco, Grand Island, NY). Cells were subcultured prior to confluence by removal of old medium, followed by addition of 3 ml PBS and 3 mL of trypsin (0.25%) and EDTA (0.03%) to detach the cells. A subcultured ratio of 1:2 was maintained and cells were incubated at 37 °C in a 5% CO₂ humidified incubator. Viable cells for cell counting was assessed by trypan blue (0.04%) exclusion dye using a hemocytometer. Viable cell numbers were assessed in quadruplicate.

3.3.4 Cell viability MTT assay dose response

A cell viability assay, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction by viable cells, was used in order to establish an LC50 (e.g.
concentration to inhibit 50% of cells) for ginseng samples. Int-407 and Caco-2 cells were seeded to a final concentration of 5 x 10^5 cells/mL in 96 microwell plates and allowed to attach overnight in a humidified incubator (37 °C, 5% CO2). Ginsenoside standards and ginseng root extract were initially dissolved in 70% ethanol and subsequently diluted with culture medium to the final concentration prior to use. Test compounds and ginseng extract were serially diluted and transferred to adjacent wells as described in Section 2.3.3.

3.3.5 Cell LDH activity

Int-407 and Caco-2 cells were seeded at a concentration of 1 x 10^6 cells/mL in 24 well plates in separate experiments. Test ginsenosides (PD, PT, Rh2) were added to wells to the LC50 concentration determined previously from MTT assays. The ginsenoside concentrations used for Int-407 cells were 23, 26 and 53 µg/mL respectively for PD, PT and Rh2. In a similar experiment using Caco-2 cells, 24 µg/mL for both PD and PT and 55 µg/mL for Rh2 were used. Untreated cells represented the control. Cells were incubated at 37 °C in a 5% CO2 humidified incubator for 24, 48 and 72 h. Cell-free supernatant was obtained by centrifugation (400 x g) for 10 min and LDH assay was conducted as previously reported in Section 2.3.4.

3.3.6 Flow cytometry cell cycle analysis

In separate experiments, Int-407 and Caco-2 cells were seeded to a final concentration of 1 x 10^6 cells/mL and treated with test compounds PD, PT and Rh2 at concentrations described above. Int-407 and Caco-2 cells were incubated at 37 °C in a 5% CO2 humidified incubator for 24, 48, and 72 h, with the untreated cells acting as a control. Attached cells were collected by addition of 100 µL of trypsin (0.25%) and EDTA (0.03%) and manually dispersed. These cells
were combined with floating cells and the suspension was centrifuged for 10 min (400 x g) as previously reported in Section 2.3.6.

### 3.3.7 Statistical analysis

Oneway ANOVA (SPSS release 9.0) was used to analyze the experimental data at 24, 48 and 72 h time periods. Significance was judged at P ≤ 0.05 using Tukey post hoc multiple comparisons of observed means.
3.4 RESULTS

3.4.1 Dose response and LC50 determination of ginsenosides

Figures 3.2A, 2B show the different concentrations of test ginsenosides PD, PT, Rh2 and Rh1 required to induce cytotoxicity in Int-407 and Caco-2 cells. PD, PT and Rh2 had LC50 values of 23 μg/mL, 26 μg/mL, 53 μg/mL respectively, in the Int-407 cell line. In the Caco-2 cell line, PD and PT both exhibited a LC50 of 24 μg/mL, followed by 55 μg/mL for Rh2. Ginsenoside Rh1 did not show the same cytotoxic in either Int-407 or Caco-2 cells.

3.4.2 HPLC, TLC and total ginsenoside analysis of standardized extract

A representative TLC separation and densitometric profile of ginsenosides contained in the North American root extract is shown in Figure 3.3. The ginsenoside content of the extract was confirmed by HPLC analysis and and was concluded to be Rb1 (3.67%), Re (1.92%), Rc (0.5%), Rd (0.4%), Rg1 (0.26%) and Rb2 (0.08%). The concentration of ginsenosides were determined by comparison to standard curves (Rb1, Re, Rc, Rd, Rg1, Rb2). Total ginsenoside content measured spectrophotometrically was found to be 7.9%, which is slightly greater than the six ginsenosides used for HPLC analysis (total 6.9%) and may be due to additional unidentified components seen in the TLC profile.

3.4.3 Dose response of standardized extract and main ginsenosides

The effects of the standardized ginseng root extract on Int-407 and Caco-2 cell lines are shown in Figure 3.4. The extract did not inhibit growth of either cell line up to a maximum concentration of 1.5 mg/mL. The effects of the three main ginsenosides found in the North American ginseng root extract, which were tested for cytotoxicity are shown in Figures 3.5A and
5B. Ginsenosides Rb1, Rc and Re did not affect the growth of Int-407 cells up to a concentration of 250 μg/mL and had no effect on Caco-2 cells.

3.4.4 LDH activity

Lactate dehydrogenase (LDH) activity was measured in cultured Int-407 supernatant after exposure for 24, 48 and 72 h (Figures 3.6A), to assess the effect of test ginsenoside standard compounds on cell membrane integrity. Ginsenosides PT, PD and Rh2 were used at respective LC50 concentrations determined above. The percentage of untreated Int-407 cell LDH activity following Rh2 treatment was significantly (P ≤ 0.05) higher (167 ± 20% of untreated cells) compared to ginsenosides PD (116 ± 2%) and PT (82 ± 9%) after 24 h. Rh2 induced significantly (P ≤ 0.05) higher (234 ± 11%) LDH activity than PD (147 ± 9%) and PT (96 ± 9%) after 48 h treatment. Both Rh2 (198 ± 9%) and PD (182 ± 24%) treatment were significantly (P ≤ 0.05) higher than PT (123 ± 4) after 72 h treatment. Similar experiments conducted in Caco-2 cell showed that LDH activity of untreated cells were not significantly altered by ginsenoside standards after 24 h of incubation (Figures 3.6B). However, after 48 h exposure, Rh2 (283 ± 13%) and PD (232 ± 20%) treatments produced significantly (P≤0.05) higher LDH activity than PT (169 ± 7%). Moreover, after 72 h Rh2 (353 ± 11%) and PD (301 ± 32%) produced significantly (P ≤ 0.05) higher percent change in LDH activity than PT (242 ± 5%).

3.4.5 Cell cycle analysis

A differential effect of PD, PT and Rh2, on Int-407 and Caco-2 cell cycle events occurred as evidenced by flow cytometry measurements. The ginsenoside concentrations used for Int-407 cells were 23, 26 and 53 μg/mL respectively for PD, PT and Rh2 and 24 μg/mL for both PD and PT and 55 μg/mL for Rh2 in Caco-2 cells. Rh2 treatment of Int-407 induced a significantly (P ≤
0.05) higher production of apoptotic cells (sub-G1) (35 ± 1%), compared to untreated cells (14 ± 0.3%) and other treatments at respective LC50s (Figures 3.7A) after 24 h of culture. PD (15 ± 1%) and PT (13 ± 1%) treatment failed to induce apoptosis in Int-407. After 48 h, both PD (27 ± 6%) and Rh2 (24 ± 3%) treatments increased Int-407 apoptosis at a significantly (P ≤ 0.05) higher extent than untreated cells. A similar result was also obtained after 72 h, where PD (31 ± 6%) and Rh2 (37 ± 5%) treatments were all significantly (P ≤ 0.05) higher than untreated cells and PT treated cells, respectively. Treatment of Caco-2 cells with the same ginsenosides also produced a significant (P ≤ 0.05) build-up of apoptotic (sub-G1) cells after 24 h for Rh2 (9 ± 1%), but not for PD (7 ± 3%) or PT (3 ± 0.3%) compared to untreated cells (4 ± 0.3%) at respective LC50s (Figures 3.7B). However, after 48 h of exposure, Rh2 treatment (20 ± 1%) significantly (P ≤ 0.05) enhanced the production of apoptotic cells compared to PD (7 ± 0.3%) and PT (6 ± 1%). These effects were also significantly (P ≤ 0.05) higher than untreated Caco-2 cells. Rh2 treatment after 72 h of treatment produced the highest (P ≤ 0.05) build-up of Caco-2 apoptotic cells (42 ± 1%), followed by PD treatments (16 ± 1%), both of which were significantly (P ≤ 0.05) higher than PT (10 ± 1%). Representative flow cytometry cell cycle histograms for Int-407 and Caco-2 are listed in Appendix 1, respectively.
3.5 DISCUSSION

In this study, it was shown that the concentration of ginsenosides tested individually, or collectively as contained in a standardized North American ginseng extract varied considerably in relative efficacy to induce cytotoxicity in two distinct intestinal cells lines. Neither the standardized North American ginseng root extract, nor the main ginsenosides found present in the root extract tested exhibited an affinity to trigger cell death in Int-407 nor Caco-2 cells. In contrast, ginsenosides, PD, PT and Rh2, not present in the North American ginseng root extract, was effective at inducing cytotoxicity. Our finding herein supports our previous results that concluded that specific differences in individual ginsenosides chemical structure are important for eliciting cytotoxic properties in a leukemia cell line (Popovich and Kitts, 2002). In specific terms, the aglycones PD and PT had the strongest cytotoxic effect on intestinal cells, followed by Rh2, whereas, Rh1 had no effect. Rh2 is structurally similar to PD, and Rh1 is related to PT by the addition of a glucose moiety, located at position C-3 and C-6, respectively (Figure 3.1).

In the Int-407 cell line, Rh2 and PD produced the greatest percentage of sub-G1 (apoptotic) cells relative to both PT and untreated cells. Rh2 treatment of Caco-2 cells also yielded the highest percentage of sub-G1 at all incubation time periods. The Int-407 cell line is a human embryonic nonmalignant cell line originating from fetal tissue of approximately two months gestation, whereas Caco-2 is a human colon adenocarcinoma cell line. Both intestinal cell lines show typical epithelial morphology and growth and are useful models for studying the gastrointestinal tract (Bestwick and Milne, 2000; Gork et al., 1999; Gronroos et al., 1995; Henle and Deinhardt, 1957). In both cell lines, Rh2 and PD showed the greatest effect on apoptosis, whereas PT had no measurable effect on apoptosis for both Int-407 and Caco-2 cells. Rh2 has been reported to have an impact on cell proliferation, apoptosis and cell cycle regulation (Jin et
Ginsenosides derived from ginseng that have PD and Rh2 present may play an integral part of the ability of gastrointestinal cells to regenerate and eliminate pre-cancerous cells, and thus maintain a healthy gastrointestinal tract. A prospective case-control study in Korea found that regular consumers of ginseng exhibited a reduced relative risk (0.33) to gastric cancer development compared to non-consumers (Yun and Choi, 1998). In this study, it was found that PT had an effect on cell proliferation, but did not increase the accumulation of sub-G1 cells in either intestinal cell line. Therefore, it is possible that PT acts on cultured intestinal cells through a different process. PT ginsenosides have been reported to have different effects on endothelium-dependent relaxation in the rat aorta (Kang et al., 1995), glucose uptake (Han et al., 1999), and cell cycle protein levels of cyclin E when compared to PD (Hwang et al., 2002). The PT aglycone has an additional hydroxyl group at position C-6 of the ring structure compared to PD aglycone (Figure 3.1), and this increase in polarity may reduce membrane contact when compared to the more hydrophobic PD aglycone. Ginsenosides, Rh2 and PD, have been detected in the lipid fraction of cultured melanoma cells (B16) after 6 h of exposure (Ota et al., 1991). Similarly, ginsenosides Rh2 have been shown to influence cell membrane function in Erlich ascite tumor cells (Popov, 2002). An interaction with intestinal membrane function as measured by LDH release, a useful marker for cell membrane damaged and integrity (Sung et al., 1995) is also reported herein. Our findings showed that, Rh2 and PD treatment of Int-407 cells resulted in a relatively larger release of LDH activity compared to PT treatments, which had no substantial effect. In Caco-2 cells, treatments with test compounds were similar to the results obtained for Int-407, with Rh2 showing maximum release at all time periods. Therefore, aglycone ginsenosides PD and PT can affect cell membrane
function differently, which may be related to structural affinities allowing incorporation into membranes and inducing differences in apoptotic response.

Standardization of ginseng products relative to ginsenoside content is required to establish confidence in potential claims of efficacy for standards of evidence. However, as shown, a standardized ginseng extract and the main ginsenosides used to standardize ginseng products (Rb1, Re and Re) had little bioactivity as measured in this study by cultured intestinal cell growth. Therefore standardization of ginseng extracts according to the accepted practice, using a selected small number of ginsenosides does little to imply potential bioactivity. New standardization practices are needed to include ginsenosides with measurable bioactive effects such as Rh2 and its aglycone PD.

Our results show for the first time that the protopanaxadiol ginsenosides Rh2 and PD were effective apoptosis inducers in both Caco-2 and Int-407 intestinal cells. This response was attributed to differences in individual ginsenoside glycosylation and thus the hydrophobic/hydrophilic balance.
Figure 3.1. The structure of test compounds 20(S)-protopanaxadiol (PD), 20(S)-protopanaxatriol (PT), ginsenosides Rh2, and Rh1.
Figures 3.2A, 2B. Dose response relationship between test compounds (PD, PT, Rh2, and Rh1), in Int-407 and Caco-2 cells after three days measured in triplicate by MTT viability assay as outlined in the Material and Methods. Figure labels corresponding to the Int 407 cells top panel (A) are represented by PD (△), PT (□), Rh2 (●), and Rh1 (×). Caco-2 cells bottom panel (B) labels are represented by PD (△), PT (□), Rh2 (●), and Rh1(×). Values are expressed as a percentage of untreated cells (mean ± SD).
Figure 3.3. Thin-layer chromatogram of ginsenosides found in North American ginseng extract. Ginsenosides were separated using a solvent system of chloroform-methanol-water (65:35:10, v/v/v) lower phase and visualized by spraying with sulfuric acid (10% H₂SO₄) in ethanol. The plate was scanned using Biorad GS-670 densitometer and profile analyzed by Molecular Analyst software (Biorad, Cambridge, MA).
Figure 3.4. Dose response relationship of a standardized North American ginseng extract measured in Int-407 and Caco-2 cells after three days measured in triplicate by MTT viability assay as outlined in the Material and Methods. Figure labels correspond to Int 407 cells (■), and Caco-2 cells (♦). Values are expressed as a percentage of untreated cells (mean ± SD, n = 3).
Figures 3.5A, 5B. Dose response relationship between the major ginsenosides compounds found in standardized North American ginseng extract (Rb1, Rc, Re), in Int-407 and Caco-2 cells after three days measured in triplicate by MTT viability assay as outlined in the Material and methods. Figure labels corresponding to the Int 407 cells top panel (A) are represented by Rb1 (▲), Rc(□), and Re (♦). Caco-2 cells bottom panel (B) labels are represented by Rb1 (▲), Rc(□), and Re (♦). Values are expressed as a percentage of untreated cells (mean ± SD, n = 3).
Figures 3.6A, 6B. Lactate dehydrogenase (LDH) activity was measured on separate occasions in both Int-407 (top panel A) and Caco-2 cells (bottom panel B) after 24, 48 and 72 h of treatment with PT, PD and Rh2, respectively at the LC50 determined by MTT assay. LDH activity was determined in cell-free supernatant as outlined in the Material and Methods. Data are expressed as percentage of untreated samples (mean ± SD, n = 3). Bars within the same time period possessing different letters are significantly different (P ≤ 0.05).
Figures 3.7A, 7B. Percentage of apoptotic cells (sub-G1) following PD (23 μg/mL), PT (26 μg/mL), and Rh2 (53 μg/mL) in Int-407 cells and PD (24 μg/mL), PT (24 μg/mL) and Rh2 (55 μg/mL) in Caco-2 treated cells measured by flow cytometry for 24, 48 and 72 h. Int-407 (top panel A) and Caco-2 (bottom panel B) cells were fixed on separate occasions in ethanol and stained with PI as described in the Material and Methods. Data are expressed as a percentage (mean ± SD, n = 3). Bars of the same time period with different letters are significantly different compared to control (con) cells (P ≤ 0.05).
3.6 SUMMARY AND FUTURE STUDIES

Ginsenoside Rh2, and aglycones PD and PT had similar characteristic cytotoxic effects measured on the growth of two intestinal cell lines (Int-407 and Caco-2). A standardized North American ginseng extract, with a known ginsenoside composition, showed no cytotoxicity in both intestinal cell lines. Furthermore, the main ginsenosides commonly used to standardize ginseng products also showed no effect on cultured cell viability and may not reflect bioactive potential of extracts. Cycle analysis showed characteristically different effects of ginsenosides PD, Rh2 and PT in both cell lines. Rh2 treatment of Int-407 produced a significantly higher ($P \leq 0.05$) production of sub-$G_1$ (apoptotic) cells, compared to untreated cells after 24 h. PD and Rh2 treatments were both significantly higher ($P \leq 0.05$) than untreated cells after 48 and 72 h. Similar results were obtained for treatment of Caco-2 cells. Lactate dehydrogenase (LDH) activity in both cell lines was similar for PD and Rh2 and higher than PT treatment at most time periods. These results show a specific structure-function relationship for bioactive ginsenosides in two contrasting intestinal cell types.

Therefore, three compounds (PD, PT and Rh2) have been identified as having cytotoxic properties in three distinct cultured cell models. Ginsenoside Rh2 is considered to be a rare compound found only in Asian red ginseng; red ginseng is subject to a thermal process added to the normal preservation (drying) regime that changes the colour and most likely the ginsenoside composition of ginseng and related products. However, it is uncertain if Rh2 is found in North American ginseng plant material.
PART 2. CHARACTERIZATION OF RH2 IN NORTH AMERICAN GINSENG PLANT

4 CHAPTER IV

Preface

A version of Chapter IV has been published in Phytochemistry 2004.

4.1 ABSTRACT

Rg3 and Rh2 ginsenosides are primarily found in Korean red ginseng root (*Panax ginseng* C.A. Meyer) and valued for bioactive properties. Both Rh2 and Rg3 were quantified from ginseng leaf and Rg3 from root extracts derived from North American ginseng (*Panax quinquefolius*) by HPLC and ion fragments detected using ESI-MS. Ginseng leaf contained 11.3 ± 0.5 mg/g Rh2 and 7.5 ± 0.9 mg/g Rg3 in concentrated extracts compared to 10.6 ± 0.4 mg/g Rg3 in ginseng root. No detectable Rh2 was found in root extract by HPLC, but ESI-MS analysis showed the presence of Rh2. Ginsenosides Rg3 and Rh2 were found following a hot water reflux extraction, but were not detected when extracted with 80% ethanol at room temperature. Therefore ginsenosides Rg3 and Rh2 are not naturally present in North American ginseng, but are a product of a thermal process. ESI-MS analysis showed that Rg3 and Rh2 formation, among other compounds, were found and were a function of heating time; Rg3 and Rh2 were a breakdown product of the more abundant ginsenosides Rb1 and Rc. Our finding that heat processed North American ginseng leaf is an excellent source of Rh2 ginsenoside is an important discovery considering that ginseng leaf material is obtainable throughout the entire plant cycle for recovery of valuable ginsenosides for pharmaceutical usages.
4.2 INTRODUCTION

Ginseng root is a valuable agricultural commodity grown for use in many traditional medicinal therapies. More contemporary usage of ginseng, especially in North America have included usages in formulations prepared for herbal supplements or functional food usages; certain ginsenosides may also hold pharmaceutical potential for drug development. Herbal product usage are based on specific health expectations that focus on immunological, anti-cancer, metabolic, neurological benefits (Kitts and Popovich, 2003) and anti-oxidant properties (Hu and Kitts, 2001). The primary active ingredients are a group of triterpene saponins also referred to as ginsenosides and specific ginsenosides such as Rg3 and Rh2 have been proposed as chemotherapeutic agents. Two main sources of ginseng root are typically used, Asian ginseng (Panax ginseng, C.A. Meyer) and North American ginseng (Panax quinquefolius). Much less is known about North American ginseng leaf compared to the root. Annual recovery of ginseng leaf could be a feasible alternative source of ginsenosides compared to the long growth cycle required for harvesting the root for nutraceutical products. Starratt et al. (2001) reported that the leaves are a source of ginsenosides but have not been capitalized or exploited at present. Processing conditions have been reported to affect the composition of ginsenosides. For example, Korean red ginseng is the same variety as Asian ginseng but subjected to steam preservation after harvesting (Kim et al., 2000).

Ginsenoside Rh2 is currently sought after for its bioactive properties; Rh2 can reduce the proliferation of a variety of cultured cancer cells and can influence apoptosis (Fei et al., 2002; Nakata et al., 1998; Park et al., 1997; Popovich and Kitts, 2002). Rg3 has also been shown to possess anti-tumor properties and have an effect on drug resistant cultured cancer cells (Keum et al., 2003; Kim et al., 2003).
The purpose of this study was to test the hypothesis that North American ginseng (*Panax quinquefolius*) leaf and root are potentially a source of rare ginsenosides Rg3 and Rh2, following a thermal process required to specifically generate these products.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Experiment 1. The effect of heat on ginseng extract preparation

Two extraction techniques were used to determine the effect of heat on ginsenoside composition of leaf. The first technique consisted of a hot water extraction of ginseng under reflux and the second technique consisted of room temperature extraction using 80% ethanol and constant stirring. These techniques will be referred herein as hot water reflux and 80% ethanol extraction respectively.

North American ginseng root and plant components were collected from two-year old ginseng (Panax Q farms, Vernon BC). Ginseng root and leaf were separated, washed, carefully dried and lyophilized 48 h after harvesting. Samples were subsequently vacuum packaged and stored at −18 °C until analysis. The hot water reflux extraction procedure consisted of two grams of freeze-dried leaf that were powdered and blended separately in 500 mL of distilled water for 5 min. Samples were refluxed for 1.5 h at 100 °C. The extracts were vacuum filtered through Whatman no. 4 filter paper. The hot water reflux extraction was applied to a polymeric adsorbent Amberlite XAD-4 (Sigma St. Louis MO) column (with an average pore diameter of 40Å, bed volume of 60 cm$^3$ and flow rate of 8 mL/min) and washed with 1000 mL of distilled water. The extract was eluted from the column using 500 mL of absolute ethanol, which was subsequently reduced under heat and a stream of nitrogen. The 80% ethanol extract preparation differed from the hot water reflux extraction by the absence of heat. Two grams of freeze-dried leaves were
powdered and blended separately in 500 mL of 80% ethanol for 5 min and extracted using a magnetic stirrer for 24 h at room temperature. The extract was vacuum filtered through Watman no. 4 filter paper, concentrated under a stream of nitrogen to a volume of 100 mL and combined with 400 mL distilled water and applied to an Amberlite XAD-4 column as described above. The ethanol was evaporated under a stream of nitrogen (12 h). The extract was lyophilized and stored at -18 °C until analyzed.

4.3.2 HPLC-ESI-MS

A Hewlett-Packard (HP) series 1100 high-performance liquid chromatograph was used with a diode array detector coupled to an HP Vectra computer running Chem Station for LC 3D (Agilent Technologies). The column used was an HP, Zorbax C18, 4.0 x 150 mm, 3.5 μm. An Agilent 1100 MSD mass spectrometer with electrospray ionization was used for ESI-MS analysis. The MS conditions used for analysis were negative ion mode, drying gas 10.0 L/min, temperature 300 °C, nebulizer pressure 60 psig, and scan range of 400-1400 μ. Solvent systems consisted of acetonitrile (HPLC grade) and nano-pure water (Barnstead Dubuque, IA) with a flow rate of 1 mL/min. The solvent gradient program used for HPLC quantification was H₂O (A) and acetonitrile (B) (91:9) at time 0 changing to A:B (88:12) at 5 min, A:B (75:25) at 15 min, A:B (65:35) at 35 min, A:B (30:70) at 60 min and finally (B) 100% by 70 min. Test samples and ginsenoside reference standards were prepared in HPLC grade methanol and injected in a 10 μL volume, monitored at a wavelength of 203 nm and measured in triplicate. The concentration of ginsenosides were determined by standard curves prepared by injecting different concentrations of ginsenoside standards (Rg1, Re, Rb1, Re, Rb2, Rd, Rg3, Rh2; concentration ranged from 2 – 2000 μg/mL).
4.3.3 Experiment 2. Thermal generation of Rg3 and Rh2 from abundant ginsenosides

Experiment 1 showed that ginsenoside Rh2 was formed by thermal generation using hot water reflux extraction. The second set of experiments was designed to measure the time required to produce Rg3 and Rh2 and to identify the ginsenosides that contribute to the formation of Rg3 and Rh2.

Two grams of freeze-dried leaf were powdered and blended separately in 500 mL of distilled water for 5 min and refluxed for 60 min at 100 °C. Samples of heated extract were removed at timed intervals of 10, 20, 30 and 60 min and filtered through Whatman no. 4 filter paper. The extracts were applied separately to a fresh polymeric adsorbent Amberlite XAD-4 (Sigma St. Louis MO) column (with an average pore diameter of 40Å, bed volume of 16 cm$^3$ and flow rate of 8 mL/min) and washed with 100 mL of distilled water. The extracts were eluted from the column separately using 100 mL of ethanol (100%) and subsequently reduced under heat and a stream of nitrogen and analyzed by ESI-MS.

4.3.4 Ginsenoside breakdown products

Ginsenoside standards Rb1 and Rc were initially dissolved separately in 100 μL of ethanol (70%) and subsequently refluxed with 20 mL of nano-pure water (Barnstead Dubuque, IA) for 1.5 h at 100 °C. The samples were concentrated under heat, analyzed by ESI-MS and compared to unheated reference standards.

4.4 RESULTS AND DISCUSSION

These experiments showed for the first time that using a simple hot water extraction enables the use of North American ginseng leaf as a valuable source of rare ginsenosides. Table
4.1 lists the ginsenoside fingerprint information identified by HPLC. Root samples extracted by hot water reflux contained greater amounts of ginsenosides Rg1, Re, Rb1, Rc and Rd compared to leaf samples. Ginsenoside Rg3 was detected in both leaf and root. However, ginsenoside Rh2, an otherwise rare ginsenoside was detected only in the leaf and not in the root, by HPLC, when employing hot water reflux extraction and preparation process. The HPLC limit of detection for ginsenoside Rh2 was determined to be 2 μg/mL and the MS detector was effective at 0.02 μg/mL. Ginsenosides exhibited quasi-molecular ion [M-H]⁻ and for some ginsenosides adduct ions [M+ Cl]⁻ and [M+ Aco]⁻. The main source of both ginsenosides Rg3 and Rh2 have been reported to be exclusively Korean red ginseng root (Kim et al., 2000; Park et al., 2002; Shin et al., 2000). Preparation of Korean red ginseng relies on steam processing to ensure preservation which may alter the ginsenoside composition. Steaming ginseng has been shown to produce ginsenosides that are not present in raw ginseng (Kim et al., 2000). The effect of heat produced from a hot water reflux extraction procedure, were compared to a room temperature extraction using 80% ethanol to determine if Rg3 and Rh2 were indeed formed as a result of the addition of thermal energy. Table 4.2 shows the major ion fragments detected by ESI-MS analysis of the water reflux extraction of ginseng root and leaf and the 80% ethanol extraction at room temperature. Ginseng root and leaf hot water reflux extract were found to have 23 and 19 different ion fragments, respectively, whereas, the 80% ethanol extract contained 14 fragments. Furthermore, the 80% ethanol extract contained a malonyl ginsenoside that was not present in the hot water reflux extract. Malonyl ginsenosides are heat-labile and readily demalonylate (Zhang et al., 2001). Ion fragments of ginsenosides Rg3 and Rh2, among others, were not recovered in the 80% ethanol extraction, but were found in the hot water extraction procedure. HPLC analysis of the leaf extracts (Table 4.1) showed the presence of both Rg3 (7.5
± 0.9 mg/g) and Rh2 (11.3 ± 0.5 mg/g) in the hot water reflux extract, but not in the 80% ethanol extraction. Conformational isomers of ginsenosides Rg3 and Rh2 (20(S) and 20(R)) were detected by comparing retention order and ion fragments produced during ESI-MS analysis. Isomers of Rg3, Rg2 and Rh1 ginsenosides have been also reported in "Shenmai" derived from red ginseng and ophiopogon (Ophiopogon Japonicus) (Bae et al., 2002; Haijiang et al., 2003).

Evidence is provided that Rg3 and Rh2 are formed during thermal processing of North American ginseng, and that these breakdown products are not naturally present in leaf or root materials. The second set of experiments were designed to identify the heating time required to form Rh2 after hot water reflux and to identify the compounds that may contribute to the formation of the rare ginsenoside Rh2. Table 4.3 shows the ESI-MS analysis of the hot water reflux of ginseng leaf that was sampled between 10 and 60 minute intervals. Fragments corresponding to malonyl ginsenosides disappeared after 30 min of heating. After 10 min of water reflux, ginsenosides Rg3 and Rh2 were not detected, however, both Rg3 and Rh2 began to appear after 20 min. The compounds needed for Rg3 and Rh2 formation were studied and Table 4.4 shows the ion fragments of protopanaxadiol ginsenosides Rc and Rbl produced after hot-water reflux. Both ginsenosides Rg3 and Rh2 were breakdown components from both ginsenosides Rc and Rbl after a hot water reflux. Breakdown products or artifacts generated from heat processing of saponins from Medicago sativa L have recently been reported by (Tava et al., 2003). They showed that after hot water reflux of plant material artifacts were produced which resulted in decreased polarity or higher retention time compared to original compounds when measured by TLC and HPLC. Furthermore, ginsenosides Rb1 and Rc were classified in the same category as Rg3 and Rh2, the 20(S)-protopanaxadiol group of ginsenosides. These compounds are organized according to the attachment of sugar moieties to position C-3 of the
triterpene ring structure (Figure 4.1). Conversions of ginsenosides Rb1, Rc and Rd have been reported and treatment with a specific β-glucosidase, resulted in the production of Rh2 (Bae et al., 2002; Zhang et al., 2001). Future studies are needed to generate quantitative data on the conversion of Rh2 and Rg3 from Rb1 and Rc precursors. A comprehensive time course analysis of Rh2 and Rg3 generation from Rb1 and Rc ginsenosides during optimized thermal process for conversion is required.

An additional source of the rare ginsenosides Rh2 and Rg3, which are valued for potential anti-cancer properties has been identified. In cultured cancer cell experiments, Rh2 has been shown to reduce proliferation. Popovich and Kitts (2002) previously found that the LC50 for Rh2 standard was 15 µg/mL in THP-1 human leukemia cells and 53-55 µg/mL in intestinal cell lines (Popovich and Kitts, 2003). Furthermore, Rh2 induced apoptosis in a variety of cell lines (Fei et al., 2002; Nakata et al., 1998; Park et al., 1997; Popovich and Kitts, 2002). Rg3 was effective in reducing proliferation of prostate cancer cells (Liu et al., 2000) and cisplatin resistance cells (Keum et al., 2003).

With the addition of heat in the extraction process for recovery of ginsenosides from ginseng leaf, the bioactive potentials of subsequent extracts are enhanced by generating Rh2 and Rg3. Further studies are required to quantitative the conversion of Rg3 and Rh2 from Rc and Rb1 during thermal processing of North American ginseng leaf materials and to assess the leaf extract ability to induce biological responses in human clinical studies which are critical for the development and use of this novel source of ginsenosides for potential pharmaceutical usage.
Figure 4.1. The structures of 20(S)-ginsenoside Rh2 and Rg3.
Table 4.1. Ginsenoside profile of North American ginseng root and leaf quantified by HPLC\textsuperscript{1}.

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<th>Rb1</th>
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<th>Rb2</th>
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<th>Rg3</th>
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</table>

\textsuperscript{1} Data are expressed as mean (± SD) measured in triplicate (mg/g dry weight). Asterisk (*) refers to hot water reflux extraction, \# refers to room temperature ethanol (80%) extraction, ND: not detected.
Table 4.2. ESI-MS ion fragments of North American ginseng root and leaf extraction.

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(*) identified from Haijiang et al. (2003), ‡ order of detection (refer to Appendix 2 for chromatograph), # molecular weight match, iso-isomer.
Table 4.3. ESI-MS ion fragments of North American ginseng leaf sampled during hot water reflux extraction.

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(*) identified from Haijiang et al. (2003), † order of detection, # molecular weight match, iso-isomer.
Table 4.4. ESI-MS ion fragments of breakdown products of ginsenosides Rc and Rb1 after hot water reflux extraction.

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(*) identified from Haijiang et al. (2003), † order of detection, # molecular weight match, iso-isomer.
4.5 SUMMARY AND FUTURE STUDIES

North American ginseng plant materials including the root and leaf were analyzed by HPLC for ginsenoside Rh2 and ions were confirmed by electrospray mass spectrometry (ESI-MS). In ginseng leaf, ginsenoside Rh2 and structurally related ginsenoside Rg3 were found by both HPLC and ELS-MS. However, in the root, only ESI-MS analysis showed the presence of ginsenoside Rh2. Therefore, ginseng leaf material is a better source of rare ginsenoside Rh2 and was identified as formed during thermal extraction. Furthermore, ginsenoside Rh2 and related Rg3 appeared after 20 min of hot water reflux and at subsequent intervals. Two possible sources of Rh2 generation have been identified and overall rare ginsenoside Rh2 was formed by the extraction process and is not naturally present in North American ginseng plant material. It is unclear if North American ginseng plant material can be an effective source material to concentrate or enrich ginsenoside Rh2 in the preparation of bioactive extracts.

Furthermore, bioactive compounds have been identified and effects on cultured cells have been characterized, however, the specific effects involving membrane permeability, apoptosis, and necrotic modes of cell death remain to be elucidated and whether an enriched Rh2 from North American plant material similarly influence a bioactive response.
PART 3: DEVELOPMENT, ASSESSMENT OF BIOACTIVITY AND PROPOSED MECHANISM OF AN ENRICHED RH2 EXTRACT FROM THE LEAVES OF GINSENG

5 CHAPTER V

Preface

A version of Chapter V has been accepted for publication in *Journal of Biochemical and Molecular Toxicology*.

5.1 ABSTRACT

Certain ginsenosides, also known as triterpene glycosides, have been recently reported to have a characteristic effect on cultured intestinal and leukemia cell growth. Ginsenoside aglycones 20(S)-protopanaxadiol (PD), 20(S)-protopanaxatriol (PT) and ginsenoside Rh2 have been identified as having a strong effect on reducing cell viability. Furthermore, ginsenoside Rh2 is thought to be a rare ginsenoside not found in all ginseng products. Rather, Rh2 has been recently reported to be a breakdown product of thermal processing of North American ginseng. In this study, pure ginsenosides PD, PT, Rh2 standards and an enriched Rh2 fraction derived from ginseng leaf were tested in cultured Caco-2 cells for relative cytotoxic potency. PD and Rh2 LC50 were similar after 24 to 72 h, whereas a drop in PT LC50 occurred later at 48 and 72 h. Furthermore, PD and Rh2 affected membrane integrity as indicated by LDH secretion earlier than PT and the enriched Rh2 fraction (P ≤ 0.05). Ginsenoside Rh2 showed the greatest (P ≤ 0.05) build up of necrotic cells (18.3 ± 0.1%) at the respective LC50 after 24 h and PD (21.3 ± 0.3%) showed the largest effect after 44 h of exposure. The effect on apoptotic cells at 44 h of treatment were significantly different (P ≤ 0.05) for Rh2 (21 ± 0.4%), PD (14.6 ± 0.1%), enriched Rh2 leaf fraction (9.9 ± 0.6%) and PT (2.3 ± 0.1%) treatments. Caco-2 caspase-3 activity was different between ginsenoside exposure; Rh2 (10.6 ± 0.3 nM pNA) had the greatest (P ≤ 0.05) activity followed by the enriched Rh2 leaf fraction (8.3 ± 0.2 nM pNA), PT (7.3 ± 0.3 nM pNA). The PD (4.8 ± 0.04 nM pNA) treatment was similar to untreated cells (4.3 ± 0.05 nM pNA) in caspase-3 activity. These results show variable bioactive response in cultured intestinal cell to specific ginsenosides and an enriched Rh2 North American ginseng extract which may be explained on basis of hydrophobic/hydrophilic balance.
5.2 INTRODUCTION

The main bioactive compounds found in ginseng are generally believed to be ginsenosides and these compounds have been associated with a wide variety of physiological and pharmacological effects. Certain rare, non-polar ginsenosides have been reported to impact the growth of cultured cancer cells. Specifically, ginsenosides that are not normally found in all ginseng varieties or products, have been reported to have an effect on cultured cancer cells by reducing cell proliferation (Popov, 2002), blocking cell cycle (Lee et al., 1996), inducing apoptosis and altering membrane permeability and integrity (Popovich and Kitts, 2002). It was previously reported that three ginsenoside compounds, namely aglycones protopanaxadiol (PD), protopanaxatriol (PT) and ginsenoside Rh2 have a measurable effect on cultured cancer cells (Popovich and Kitts, 2002; Popovich and Kitts, 2004b). Ginsenoside Rh2 is thought to be a rare ginsenoside that is found only in red ginseng; red ginseng differs from white ginseng as a result of applying a thermal process procedure to extend shelf-life (Kim et al., 2000). Rh2 has also recently been found to be a breakdown product formed by the addition of thermal energy to the extraction of ginsenosides, and researchers have reported that North American ginseng leaf is a potential source material for Rh2 formation (Popovich and Kitts, 2004a). The main effect of cytotoxic ginsenosides, such as Rh2, on cultured cancer cells is thought to be the result of non-specific changes to the cell membrane that eventually leads to cell death (Popov, 2002). However, characterization of the type of cell death via either apoptotic or necrotic pathways has not been definitively established.

The purpose of this study was to characterize the efficacy of individual pure ginsenosides, and an enriched Rh2 extract produced from North American ginseng leaf materials, to induce cytotoxicity, necrosis and apoptosis in Caco-2 cells. Caco-2 is a human colorectal adenocarcinoma cell line and
represents a model for evaluating the bioactivity of plant derived compounds and the
effect on complex gastrointestinal cells.

5.3 MATERIALS AND METHODS

5.3.1 Cell culture

Caco-2, a colorectal adenocarcinoma cell line, was obtained from ATCC (Manassas, VA).
Cells were maintained in minimum essential medium supplemented with fetal bovine serum (10%) as
described in Section 3.3.3.

5.3.2 Test compounds and materials

North American ginseng (*Panax quinquefolius*) leaf was collected from two-year old ginseng
(Panax Q farms, Vernon BC) and washed, dried and lyophilized 48 h after harvesting and processed
as described in Section 4.3.1. The concentrated leaf extract samples were applied to a silica gel
(Sigma St. Louis MO) column with an average pore diameter of 60Å and eluted with
chloroform/methanol/water (65/35/10 v/v, lower phase) (Fisher, Springfield, NJ). The column had a
bed volume of 40 cm$^3$ and a flow rate of 0.2 mL/min to fractionate the extract. A total of 85 fractions
of 0.65 mL were collected with every 5th fraction being examined for ginsenoside content using TLC.
Fractions that contained ginsenoside Rh2 were combined and dried under a stream of nitrogen, and
referred herein as the enriched Rh2 leaf fraction (LFRh2) and is represented in Figures 5.5A. Three
ginsenoside standards 20(S)-protopanaxadiol (PD), 20(S)-protopanaxatriol (PT) and Rh2 were
obtained from Canfo (Chengdu, China) and purity was assessed by HPLC (98.4%, 98.1% and 98.2%
for PD, PT and Rh2, respectively). The test compounds were initially dissolved in 70% ethanol and
subsequently diluted with culture medium to a final working concentration prior to use.
5.3.3 **HPLC-ESI-MS**

A Hewlett-Packard (HP) series 1100 high-performance liquid chromatograph with a diode array detector and mass spectrometer was used and described previously in Section 4.3.2.

5.3.4 **Cell viability MTT assay dose response**

A cell viability assay, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction by viable cells, was used to establish LC50 values and previously described in Section 3.3.4.

5.3.5 **Cell LDH activity**

Caco-2 cells were seeded at a concentration of 1 x 10^6 cells/mL. The test compounds (PD, PT, Rh2, and LFRh2) were added to wells containing Caco-2 cells at concentrations of 25, 50, 40, 100 μg/mL respectively. Untreated cells at each time period were designated as control. Cells were incubated at 37 °C in a 5% CO₂ humidified incubator for 2, 4, 6, 12, 24, and 48 h and analyzed as previously reported in Section 3.3.5.

5.3.6 **Annexin-V and PI staining**

Caco-2 cells were seeded at a concentration of 1 x 10^6 cells/mL in six-well plates. Ginsenosides, PD, PT, Rh2 and LFRh2 were added to cells at concentrations of 25, 50, 40 and 100 μg/mL respectively for 2, 4, 24, and 44 h. Control cells consisted of untreated cells and culture medium. Cells were pelleted by centrifugation at 500 x g for 5 min and washed twice with cold PBS. Annexin-V-FITC apoptosis detection kit (BD Biosciences, Mississauga, ON) was used to detect apoptotic and necrotic cells using flow cytometry (Becton Dickinson, Franklin lakes, NJ). Annexin-V-FITC and propidium iodide (PI) were added separately according to the manufacturer’s
instructions. Briefly, cells were gently vortexed and incubated at room temperature for 15 min in the dark. Binding buffer (400 µL) was added to each tube and analyzed within the hour. Flow cytometry was conducted using four different control samples containing no stain, Annexin-V-FITC, PI and both stains. A WINMDI (La Jolla, California) software package was used for the flow cytometry analysis.

5.3.7 Caspase-3 activity

Caco-2 cells were seeded separately at 2 x 10^6 cells/mL and ginsenosides, PD, PT, Rh2, and LFRh2 were applied to cells at the concentration described above for 4 h. The exposure time was determined from Annexin-V and PI data before any large accumulation of apoptotic cells were observed (Table 5.1). Control cells consisted of untreated cells and medium. Cells were washed in PBS and detached using trypsin-EDTA as described above, combined with saved supernatant and pelleted by centrifugation 500 x g for 10 min. Supernatant was removed and a cell pellet was suspended in 50 µL of cold lysis buffer (BD Apop Alert Caspase colorimetric assay kit, Mississauga, ON). Furthermore, 50 µL of reaction buffer/DTT mix and 5 µL of 1 mM caspase-3 substrate (DEVD-pNA) were added to each sample and incubated at 37 °C for one hour. Control samples included a sample without the substrate. Samples were read in a microplate (Thermo Lab Systems) reader at 405 nm. A pNA calibration curve was constructed at concentrations of 0, 2.5, 5, and 10 nmole of pNA.

5.3.8 Total cell lysate fraction

Caco-2 cells were seeded separately at a concentration of 1 x 10^6 cells/mL in 24 well plates. Caco-2 cells were allowed to attach to the plate overnight and the enriched ginseng leaf Rh2 fraction was added to cells at concentrations of 100 µg/mL for 48 h. Cell medium was removed and cells
were first washed twice with PBS and detached as described for the LDH assay. Cells were pelleted, washed and stored until extraction. Cell pellets were combined into 1 mL of PBS and homogenized in a Wheaton cell homogenizer and the lipid component of the cells (cell lysate) were extracted with chloroform and methanol (1:2 v/v) and centrifuged at 2000 x g for 10 min. The supernatant layer was collected and further extracted with chloroform and methanol (1:1) and centrifuged. The bottom layer was collected and dried under a stream of N₂. Dried cell lysate was analyzed by TLC on Fischerbrand silica gel G glass backed plates as described above in Section 5.3.2.

5.3.9 Statistical analysis

One-way ANOVA (SPSS release 10.0) was used to analyze the experimental data. Significance was judged at P ≤ 0.05 using Tukey post hoc multiple comparisons of observed means. A probit regression analysis was performed to determine LC50 values by using Sigmaplot (release 6.1) graphical software.

5.4 RESULTS

HPLC quantification of ginsenoside Rh2 in the enriched Rh2 North American ginseng leaf fraction revealed a concentration of 20(S)-Rh2 of $367.6 \pm 29$ mg/g and $208 \pm 16$ mg/g of 20(R)-Rh2. Therefore, a total of 576 mg/g of both Rh2 conformational isomers were found in the enriched Rh2 leaf fraction. Other quantified ginsenosides in the leaf fraction were Rgl (149 ± 9 mg/g), Rg3 (S) (38 ± 3 mg/g), and Rg3 (R) (33 ± 5 mg/g). ESI-MS analysis confirmed the presence of Rh2 and Rg3 and other ion fragments. Figure 5.1 shows the effect of exposure time on cell viability measured by the MTT assay. PD had the strongest effect on the proliferation of cultured Caco-2 cells at all time periods tested. The LC50 for PD was similar at 24 and 72 h, whereas, PT needed a substantially greater (P ≤ 0.05) concentration at 24 h than at 72 h to induce a similar effect (Figure 5.2). The
enriched Rh2 leaf fraction reduced proliferation and was three fold greater than the effect of pure standard Rh2 at 24 and 48 h and two fold greater after 72 h.

The effect of different ginsenosides on LDH release is shown in Figure 5.3. Secretion of LDH into culture medium generally increased after 6 h of exposure to all compounds. At 48 h, Rh2 showed the greatest effect on LDH release, followed by PT, PD and the enriched Rh2 leaf fraction.

Apoptotic and necrotic cell accumulations between 2 and 44 h are given in Table 5.1. Maximum apoptotic cell accumulation was found for all compounds after 44 h exposure. The Rh2-induced an effect (21 ± 0.4%) was statistically (P < 0.05) greater than the effect of other test ginsenosides on apoptotic cells. This was followed by PD (14.6 ± 0.1%), and the enriched leaf Rh2 fraction (9.9 ± 0.6%), respectively. Ginsenoside Rh2 produced the greatest percentage of necrotic cells after 24 h, whereas, PD produced the greatest percentage of necrotic cells after 44 h of exposure. Caspase-3 activity was measured after exposure to test compounds for 4 h (Figure 5.4). The activity was greatest (P < 0.05) for Rh2 treated cells (10.6 ± 0.3 nM pNA); followed by the enriched leaf Rh2 fraction (8.3 ± 0.2 nM pNA), PT (7.3 ± 0.3 nM pNA), respectively. PD ginsenoside (4.8 ± 0.04 nM pNA) was similar to the untreated controls (4.3 ± 0.1 nM pNA). Figures 5.5A and 5B show the TLC separation of the enriched Rh2 fraction and evidence of incorporation of the enriched Rh2 leaf fraction into the Caco-2 total cell lysate.

5.5 DISCUSSION

The effect of PD, PT, Rh2 and the enriched ginseng leaf Rh2 fraction on cell growth was shown to be variable and dependent on both exposure time and ginsenoside concentration in culture. Pure ginsenoside compounds PD, and Rh2 induced cytotoxicity differently than PT. For example, PD LC50 was similar at 24 and 72 h, whereas, PT LC50 had significantly greater (P ≤ 0.05) differences between 24 and 72 h, respectively. The enriched Rh2 leaf fraction was confirmed to
contain greater than 50% Rh2 (including both isomers) and produced a characteristic cytotoxic effect on Caco-2 cells that was slightly stronger than PT at 24 h. A similar effect to that observed for Rh2 at 72 h was noted for Rh2 containing leaf extract and required almost twice the concentration. Membrane changes in cultured intestinal Caco-2, Int-407 cells (Popovich and Kitts, 2004b) and leukemia cells (THP-1) (Popovich and Kitts, 2002) following exposure to cytotoxic ginsenosides through the expression of cytoplasmic LDH release have been previously reported. Membrane LDH leakage is a useful marker of membrane permeability and integrity (Sung et al., 1995). In this study, all tested ginsenoside compounds were shown to affect Caco-2 cell membrane permeability, but the time and the magnitude of LDH release was dependent on the specific ginsenoside. Generally, Rh2 and PD produced changes in membrane integrity earlier than the PT standard and at lower concentrations. These differences are likely a result of differences in chemical composition which influences polarity of the ginsenoside. Both PD and PT ginsenosides are classified according to the attachment of sugar moieties to either aglycone backbone at positions C-3 or C-6 respectively (Popovich and Kitts, 2004b). It is likely that PD ginsenoside sources which include Rh2 have a greater and more efficient impact on cultured intestinal cell membranes than PT ginsenosides. This was also evident from the necrotic cells accumulation data determined by annexin-V and PI flow cytometry. Rh2 had the largest buildup of necrotic cells after 24 h, while PD showed the largest effect after 44 h of culture. Rh2 is structurally different from PD by the attachment of a glucose moiety at position C-3, and thus is bulkier and thus appears to induce greater effects on membrane permeability. Rh2 standard treated of B16 cells have been reported to have altered membrane morphology (Ota et al., 1987) which agrees with observations made from other saponins that have also been reported to alter membrane function (Glauert et al., 1962). Furthermore, Rh2 and PD ginsenosides produced greater apoptotic accumulation compared to PT. This finding
confirms our earlier report, of a build up of sub-G1 (apoptotic) cell from Caco-2 and Int-407 cells, respectively after Rh2 and PD exposure (Popovich and Kitts, 2004b). It is of particular interest that caspase-3 activity, an apoptosis response trigger, responded differently between the two ginsenoside compounds. Rh2, for example, showed a maximal effect after 4 h exposure, whereas, the enriched Rh2 fraction and PT produced more prolonged effects. PD standard was similar to control cell caspase-3 activity. PD, the most non-polar ginsenoside tested induced cytotoxicity of Caco-2 cells at a lower concentration than both Rh2 and PT. These results indicate that PD had an effect on caspase-3 activity earlier than the measured 4 h of exposure or that PD inhibited caspase-3 activity in a similar manner reported for triterpenes from Ginkgo biloba (Luo et al., 2002). Rh2 and panaxadiol have been reported to induce caspase-3 activity in hepatoma (SK-HEP-1) cells (Jin et al., 2003b); however, unlike the present study, the cells were cultured in serum free medium. The cholesterol content of the culture medium which can influence the content of the cell membrane is a potentially critical factor influencing the cytotoxic ginsenoside effect, since increasing the concentration of cholesterol of Erlich tumor cells has been shown to decrease the cytotoxicity of both PD and Rh2 (Popov, 2002). Furthermore, a reduction in membrane cholesterol will lead to apoptosis (Martinez-Botas et al., 1999). In this study, it is speculated that the affinity of ginsenoside exchange for cholesterol in the membrane of treated cells may explain the relative differences in cytotoxicity observed herein between ginsenosides and our Rh2 enriched leaf extract. This idea is based on our finding that Rh2 isomers were recovered in Caco-2 total cell lysate which includes cellular membranes.

In this study an enriched Rh2 extract from North American ginseng leaf was shown to be a valuable source of rare ginsenoside Rh2. Moreover the enriched extract contained greater than 50% Rh2 and influenced cytotoxic and membrane altering properties. Again, Rh2 (isomers) from the
enriched Rh2 leaf fraction were found to be present in the Caco-2 cell lysate. Ota et al. (1987) reported that Rh2, a protopanaxadiol ginsenoside, could be incorporated into the lipid fraction of B16 melanoma cells, whereas, Rh1 a protopanaxatriol ginsenoside was not (Ota et al., 1987). Therefore, pure ginsenoside standards and an enriched Rh2 leaf extract appears to express a cytotoxic effect on intestinal cells that involves altering membrane permeability before initiating both apoptotic and necrotic cell characteristics. The relative potency of cytotoxicity is likely due to the hydrophobic/hydrophilic balance of the individual ginsenoside groups and may impact the overall health of the gastrointestinal tract by influencing cell turnover.
Table 5.1. Apoptotic and necrotic Caco-2 cell accumulation after exposure to ginsenoside standards (PD, PT, Rh2) and the enriched Rh2 leaf fraction (LFRh2)\(^1\).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Time (h)</th>
<th>PD</th>
<th>PT</th>
<th>Rh2</th>
<th>LFRh2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic</td>
<td>2</td>
<td>1.6 ± 0.1(^a)</td>
<td>2.8 ± 0.1(^b)</td>
<td>1.6 ± 0.0(^a)</td>
<td>1.7 ± 0.1(^a)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.5 ± 0.1(^a)</td>
<td>1.9 ± 0.1(^c)</td>
<td>1.6 ± 0.1(^a)</td>
<td>2.0 ± 0.1(^c)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.5 ± 0.1(^b)</td>
<td>2.9 ± 0.1(^c)</td>
<td>5.3 ± 0.2(^d)</td>
<td>1.6 ± 0.1(^a)</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>14.6 ± 0.1(^c)</td>
<td>2.3 ± 0.1(^a)</td>
<td>21.0 ± 0.4(^d)</td>
<td>9.9 ± 0.6(^b)</td>
</tr>
<tr>
<td>Necrotic</td>
<td>2</td>
<td>8.3 ± 0.1(^b)</td>
<td>9.0 ± 0.1(^b)</td>
<td>18.3 ± 0.1(^c)</td>
<td>5.7 ± 0.8(^a)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.8 ± 0.3(^b)</td>
<td>15.0 ± 0.1(^b)</td>
<td>6.4 ± 0.9(^a)</td>
<td>7.7 ± 1.6(^a)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>11.2 ± 0.1(^a)</td>
<td>11.3 ± 0.2(^a)</td>
<td>36.2 ± 3.4(^b)</td>
<td>9.0 ± 0.3(^a)</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>21.3 ± 0.3(^d)</td>
<td>9.9 ± 0.1(^b)</td>
<td>18.0 ± 0.1(^c)</td>
<td>8.2 ± 0.3(^a)</td>
</tr>
</tbody>
</table>

\(^1\) Data are expressed as percentage, mean ± SD, n = 3. Treatment at the same time period (h) with different superscript letters are significantly different (P ≤ 0.05).
Figure 5.1. The effect of three ginsenoside standards (PD, PT, Rh2) and a leaf enriched Rh2 fraction (LFRh2) on Caco-2 cell viability measured by MTT assay as described in the Material and Methods. Each panel represents mean ± SD (n = 3). A = 24 h, B = 48 h, C = 72 h.
Figure 5.2. LC50s determined from regression analysis of the MTT assays data shown in Figure 5.1. Data are expressed as mean ± SD (n = 3). Bars of the same treatment with different letters are significantly different from each other (P ≤ 0.05).
Figure 5.3. Temporal pattern of LDH release from cultured Caco-2 cells after exposure to ginsenoside standards (PD, PT, Rh2) and the enriched Rh2 leaf fraction (LFRh2). LDH release was measured and expressed as a percentage of control cells at each time period. Ginsenosides, PD, PT, Rh2 and ginsenoside leaf enriched Rh2 fraction (LFRh2) were applied to cells at concentrations of 25, 50, 40 and 100 μg/mL as described in the Materials and Methods.
Figure 5.4. Caspase-3 activity measured from Caco-2 cells (2 x 10^6 cells/mL) treated with ginsenoside standards (PD, PT, Rh2) and the enriched Rh2 leaf fraction (LFRh2) for 4 h. Control (CON) cells consisted of untreated cells and medium. Data are expressed as mean ± SD (n = 3). Bars with different letters are significantly different compared to control cells (P ≤ 0.05).
Figures 5.5A, 5B. Evidence of incorporation of Rh2 isomers in Caco-2 cells. Panel (A) represents the ginsenoside composition of the enriched Rh2 leaf fraction (LFRh2) and four ginsenoside standards (STD). Panel (B) represents the lipid fraction of Caco-2 (C-2) cells after exposure to the enriched Rh2 leaf fraction and untreated control (CON) cells. Caco-2 cells were washed twice in PBS after exposure to the enriched Rh2 leaf fraction and detached as described in the Materials and Methods. Cell pellets were combined into 1 mL of PBS and homogenized in a Wheaton cell homogenizer and total cell lysate were extracted with chloroform and methanol. Dried cell lysate was analyzed by TLC on Fischerbrand silica gel G glass backed plates as described in the Material and Methods.
6 CHAPTER VI

Preface

A version of Chapter VI has been submitted for publication.

Popovich, D.G.; Kitts, D. D. A proposed mechanism for ginsenosides-induced cytotoxicity in leukemia cells (THP-1).
6.1 ABSTRACT

Ginsenosides protopanaxadiol (PD), protopanaxatriol (PT) and Rh2 were shown to produce characteristic inhibition of growth in cultured THP-1 leukemia cells. Furthermore, a ginseng extract derived from heat processed North American ginseng (*Panax quinquefolius*) leaf, and enriched for ginsenoside Rh2 also reduced proliferation of cultured THP-1 cells. Cytotoxicity for all test ginsenosides was dependent on concentration and exposure time. PD had the greatest cytotoxic effect, followed by Rh2, PT and the enriched Rh2 ginseng leaf fraction (LFRh2) respectively (P ≤ 0.05). Annexin-V-FITC and PI stained cells, measured by flow cytometry, showed a significant (P ≤ 0.05) increase in apoptotic cell numbers from 5% at 1 h exposure to 10% at 41 h of exposure to PD (LC50 @ 72 h = 13 µg/mL), PT (LC50 @ 72 h = 19 µg/mL), and Rh2 (LC50 @ 72 h = 15 µg/mL). The enriched Rh2 ginseng leaf fraction (LC50 @ 72 h = 52 µg/mL) also significantly (P ≤ 0.05) increased apoptosis (18 ± 0.4%) after 23 h. There was no further increase in apoptosis when respective ginsenoside concentrations were increased; however, the percentage of necrotic cells did significantly (P ≤ 0.05) increase for PD, LFRh2, Rh2 and PT, respectively. PD produced the greatest LDH release (P ≤ 0.05) after exposure of cells for 24 h, compared to other test ginsenosides. THP-1 caspase-3 activity was different (P ≤ 0.05) between ginsenoside exposure; Rh2 (7.6 ± 1.1 nM pNA) had the greatest (P ≤ 0.05) activity followed by the LFRh2 (5.9 ± 1.0 nM pNA), PT (5.0 ± 0.8 nM pNA), whereas PD was similar to control cells. It can be concluded from these studies that ginsenoside PD, PT, Rh2 and the LFRh2 characteristically reduced cell proliferation, due to alteration of the THP-1 leukemia cell membrane properties.
6.2 INTRODUCTION

Ginseng consumption has been reported to attenuate blood pressure (Han et al., 1998), stimulate nitrite oxide production (Gillis, 1997; Han and Kim, 1996), reduce fasting glucose (Vuksan et al., 2000), have estrogenic properties (Duda et al., 1999), enhance the immune system (Scaglione et al., 1996), exhibit antioxidant activity (Hu and Kitts, 2001; Kitts et al., 2000), inhibit proliferation of cancer cells (Popovich and Kitts, 2002) and improve overall survival of cancer patients (Suh et al., 2002). Historically, practitioners of alternative or traditional medicine have used ginseng to treat a diverse group of ailments (Kitts and Popovich, 2003). Despite the apparent panacean effects of ginseng, a common mechanism describing the diverse effects of ginseng has yet to be elucidated. It is apparent that the non-specificity of the general responses attributed to ginseng may be related to modulations of cell membrane function, permeability and possibly integrity that lead to apoptosis or necrosis (Foulkes, 1998).

Two main varieties of ginseng, which are categorized by the growing region, include North American ginseng (Panax quinquefolius) and Asian ginseng (Panax ginseng C.A. Meyer); the latter of which is further subdivided into white and red ginseng. Ginseng root has a long history of use, nearly 5000 years (Yun, 2001), and a market for the intact root has been already been clearly established. However, ginseng leaf is currently underutilized, but represents a potentially valuable source of ginsenosides (Starratt et al., 2001), also known as steroidal saponins. A method based on the addition of thermal energy to generate rare ginsenosides such as Rg3 and Rh2 in leaf preparations was previously developed (Popovich and Kitts, 2004a). This process has considerable potential importance to the ginseng producer and related pharmaceutical industry because ginsenoside Rh2 has been reported to inhibit proliferation of a variety of cultured cancer cells, such as hepatoma (Lee et al., 1996), leukemia (Popovich and Kitts, 2002), and colon cells (Popovich and Kitts, 2003). Structurally
related ginsenoside, Rg3, has also been reported to improved sensitivity of drug resistant cells (Kim et al., 2003), reduce proliferation of hepatoma cells (Park et al., 2002) and decrease prostate specific antigen (PSA) in LNCaP prostate cancer cells (Liu et al., 2000). Rh2 and Rg3 are generally regarded as rare, breakdown products of ginsenoside derived from heat processing of ginseng for preservation or extraction purpose from parent compounds such as precursors Rb1, Rc and Rd (Kim et al., 2000; Popovich and Kitts, 2004a; Shibata, 2001). Furthermore, these examples of rare ginsenosides, derived from red ginseng, have been reported to affect the regulation of the cell cycle signal transducers, such as p27 kipl (Lee et al., 1996), p21 WAF1, cyclin D (Oh et al., 1999) and cyclin-dependent kinase (cdk2) (Ota et al., 1997). These examples of cell cycle regulators influence apoptosis.

The hypothesis that ginsenosides affect cancer cell viability mainly through membrane related effects and that subsequent changes to the cell membrane are secondary to this non-specific action is the basis of this study. The objective of the current study therefore, was to demonstrate reduced cell proliferation by adversely affecting THP-1 leukemia cell membrane function with ginsenoside aglycones PD, PT, Rh2, and an enriched Rh2 fraction derived from North American ginseng leaf. The THP-1 cell line represents an immune system model with characteristics common to human monocytes and macrophage.

6.3 MATERIALS AND METHODS

6.3.1 Cell culture

A human acute monocytic leukemia suspension cell line (THP-1) was obtained from ATCC (Manassas, VA) and maintained as previously described in Section 2.3.2.
6.3.2 Test compounds and materials

North American ginseng leaf was collected from two-year old ginseng (Panax Q farms, Vernon BC) as described in Sections 4.3.1 and 5.3.2.

6.3.3 HPLC-ESI-MS

A Hewlett-Packard (HP) series 1100 high-performance liquid chromatograph was used with a diode array detector coupled was used to assess the test compound and was previously described in Section 4.3.2.

6.3.4 Cell viability MTT assay dose response

A cell viability assay, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction of viable cells, was used to establish an LC50 (e.g. concentration to inhibit 50% cell viability) as described in Section 2.3.3. Serial dilutions of test compounds (PD, PT, Rh2, Figure 6.1 and LFRh2) were made in culture medium and transferred directly to adjacent wells and incubated for 6, 12, 24, and 48 h.

6.3.5 Cell LDH activity

THP-1 cells were seeded at a concentration of 1 x 10^6 cells/mL in a 24 well plate. The test compounds (PD, PT, Rh2, and LFRh2) were added to stimulate THP-1 containing wells at 21, 40, 30, 105 μg/mL, respectively, concentrations determined from LC50 @ 48 h cell viability described above. Untreated cells at each time period acted as control. Cells were incubated at 37 °C in a 5% CO2 humidified incubator for 2, 4, 6, 12, 24, 48 and 72 h. Cell-free supernatant was obtained by centrifugation (400 x g) for 10 min and the LDH assay was conducted as previously reported in Section 2.3.4.
6.3.6 Annexin-V and PI staining

THP-1 cells were seeded at a concentration of $1 \times 10^6$ cells/mL in six well plates. Pure ginsenosides, PD, PT, Rh2 and LFRh2 were applied to cells at concentrations of LC50 @ 72 h (13, 19, 15 and 52 µg/mL respectively) for 1, 3, 5, 11, 19, 23 and 41 h. A separate experiment was setup at twice the LC50 @ 72 h (26, 38, 30 and 104 µg/mL, respectively) for 19 h. Control cells consisted of untreated cells and culture medium. Cells were pelleted by centrifugation at 500 x g for 5 min and washed twice with cold PBS. An annexin-V-FITC apoptosis detection kit (BD Biosciences, Mississauga, ON) was used as previously described in Section 5.3.6.

6.3.7 Cell cycle analysis

The enriched Rh2 ginseng leaf fraction was added to THP-1 cells ($1 \times 10^6$ cells/mL) at the LC50 @ 72 h concentration of 52 µg/mL. Cells were incubated at 37 °C in a 5% CO₂ humidified incubator for 24, 48, and 72 h with the untreated cells acting as a control. After treatment, cells centrifuged for 10 min (400 x g) and were conducted as previously reported in Section 2.3.6.

6.3.8 Caspase-3 activity

THP-1 cells were seeded separately at $2 \times 10^6$ cells/mL and ginsenosides, PD, PT, Rh2, and the LFRh2 were applied to cells at LC50 @ 72 h concentrations. Control cells consisted of untreated cells and medium. Cells were pelleted by centrifugation 500 x g for 10 min and analyzed as previously described in Section 5.3.7.
6.3.9 Statistical analysis

All data are expressed as mean ± SD (n = 3) and a one-way ANOVA was used to analyze the experimental data. Significance was judged at P < 0.05 using Tukey post hoc multiple comparisons of observed means.

6.4 RESULTS

HPLC of Rh2 quantification in the enriched Rh2 ginseng leaf fraction (LFRh2) revealed two isomers at 367.6 ± 29 mg/g (20(S)-Rh2) and 208 ± 16 mg/g (20(R)-Rh2), or a total of 576 mg/g for both Rh2 conformational isomers. Other quantified ginsenosides present in the leaf extract were Rg1 (149 ± 9 mg/g), Rg3 (S) (38 ± 3 mg/g), and Rg3 (R) (33 ± 5 mg/g). The ion chromatogram from ESI-MS analysis yielded a total of eight major peaks (labeled 1-8), of which six peaks were identified as ginsenosides (Figure 6.2). Chromatogram peaks numbered 1, 3, 4, 5, 7, 8 corresponded to ginsenosides Rg1, Rg2, 20(S)-Rg3, 20(R)-Rg3, 20(S)-Rh2, 20(R)-Rh2, respectively. Peak 2 was identified by a molecular weight match, as Rg6 and peak 6 is currently unknown. The major ESI-MS ion fragments are listed in Table 6.1.

Figure 6.3 shows the effect of exposure of THP-1 cell to ginsenoside on cell viability at 6, 12, 24 and 48 h respectively. PD, the aglycone of ginsenoside Rh2, had the strongest and most potent inhibitory effect on the proliferation of cultured THP-1 leukemia cells as measured by the MTT assay, at all time periods examined (P ≤ 0.05). Relatively less inhibition of cell proliferation was observed for pure ginsenosides Rh2, PT, and LFRh2, respectively. PD showed a significant (P ≤ 0.05) stepwise reduction in the LC50 determined from regression analysis of cell viability (Figure 6.4). The results obtained after 72 h culture of THP-1 cell with ginsenosides PD, PT and Rh2 have been previously reported (Popovich and Kitts, 2002) and are included into Figure 6.4 for data comparison.
The effect of exposure time of ginsenoside compounds (LC50 @ 48 h) to assess LDH release from cultured THP-1 cells is shown in Figure 6.5. PD (21 µg/mL), PT (40 µg/mL), Rh2 (30 µg/mL) showed a maximum release of LDH after 48 h, compared to untreated control cells at the same time period. LFRh2 (105 µg/mL) showed the greatest LDH release after 72 h. Test compounds were further compared at the same concentrations, between 5-80 µg/mL, to establish relative effectiveness to release LDH into culture medium (Figure 6.6). Ginsenoside samples produced a similar effect at concentration of both 5 and 10 µg/mL; however, after exposure to 20 µg/mL or greater, PD had the greatest (P < 0.05) effect on LDH release, followed by Rh2, PT, and LFRh2.

The percentage of apoptotic or annexin-V-FITC positive cells measured by flow cytometry is presented in Table 6.2 and representative flow cytometry dot plots are listed in Appendix 3. Apoptosis was induced by LC50 @ 72 h exposure of cells to PD, PT, and Rh2 at 13, 19 and 15 µg/mL respectively. LFRh2 (LC50 @ 72 h = 52 µg/mL) gave the greatest (P < 0.05) apoptotic effect after 23 h. The percentage of necrotic cells, measured by both annexin-V-FITC and PI positive cells, were greatest for the LFRh2 (P < 0.05) after 19 h exposure (Table 6.2). However, when the concentrations of PD PT, Rh2 and LFRh2 were increased (e.g. LC50 @ 72 h = 26, 38, 30 and 104 µg/mL, respectively), only necrotic cells showed increases whereas, the percentage of apoptotic cell did not (Figure 6.7).

The effect of the LFRh2 (LC50 @ 72 h = 52 µg/mL) on THP-1 cell cycle events, measured using flow cytometry, revealed a significantly (P ≤ 0.05) higher production of apoptotic cells (sub-G1) (19 ± 1%) after 24 h, compared to untreated cells (9 ± 1%). Further exposure of cells LFRh2 resulted in a 26 ± 1% after 48 h and 38 ± 1% after 72 h (Figure 6.8). Caspase-3 activity was measured after exposure to PD, PT, Rh2 and LFRh2 for 4 h (Figure 6.9). The activity was greatest (P ≤ 0.05) for
pure Rh2 standard treated cells (7.6 ± 1.1 nM pNA); followed by the LFRh2 (5.9 ± 1.0 nM pNA) and PT standard (5.0 ± 0.8 nM pNA), respectively. The pure PD ginsenoside standard (3.0 ± 0.03 nM pNA) was similar to the untreated controls (4.0 ± 0.7).

6.5 DISCUSSION

In this study three ginsenoside standards PD, PT, and Rh2 and, for the first time, an enriched Rh2 ginseng leaf fraction (LFRh2) were shown to exhibit different relative activities to induce cytotoxicity in a human leukemia cell line (THP-1). The effect was shown to be dependent on both the exposure time to ginsenosides and the total ginsenoside concentration.

Ginsenosides PD, Rh2 and PT increased the percentage of apoptotic cells, as measured by annexin-V-FITC and PI staining and flow cytometry. This finding confirms our earlier report of a build up of sub-G1 (apoptotic) cell from THP-1 cells, respectively after PD and PT exposure (Popovich and Kitts, 2002). For example, ginsenosides PD, Rh2 and PT effectively doubled the amount of apoptotic cells from 5% to 10% over the entire test period. An increase in apoptotic cells was greater, however, for the enriched Rh2 ginseng leaf fraction compared to other ginsenosides. The enriched Rh2 ginseng leaf fraction was confirmed to contain greater than 50% Rh2 (including both isomers) and produced a buildup of sub-G1 cells measured after 24 h of exposure, which served as a confirmation of apoptosis. It is noteworthy that doubling the concentration of ginsenosides did not increase apoptotic cells but did have a positive effect on necrotic or annexin-V and PI permeable cells. Cells that are permeable to PI and elicit annexin-V binding, showed necrotic cell characteristics and consequently have enhanced membrane permeability. Our results clearly indicate that ginsenosides alter membrane properties before apoptosis is initiated. It is of particular interest that caspase-3 activity, an apoptosis response trigger, responded differently between the PD and other test compounds. The Rh2 standard, the enriched Rh2 ginseng leaf fraction and the PT standard increased
caspase-3 activity, while the PD standard was similar to control cell for caspase-3 activity.

These results indicate that PD may inhibit caspase-3 activity in a similar manner reported for triterpenes from Ginkgo biloba (Luo et al., 2002). We suggest that a possible overlap exists between apoptotic and necrotic cell death making a clear definition of apoptotic cell death difficult. These findings are supported by the phenomenon of necrosis-like programmed cell death, reviewed by Leist and Jaattela (2001), which exhibit annexin-V binding while being caspase-3 independent. The fact that the PD standard showed the greatest and earliest cytotoxic effect compared to other test ginsenosides, indicates that PD may influence permeability of cells before an apoptotic response by the cells is initiated.

LDH release, a useful marker of membrane integrity and permeability (Sung et al., 1995), was also found in THP-1 cells to be a function of ginseng exposure time and concentration. For example, the longer exposure of THP-1 cells to test ginsenosides resulted in a characteristic greater increase in LDH. Moreover, increasing the concentration of the test ginsenoside compounds above a concentration of 20 µg/mL for PD and 40 µg/mL for PT and Rh2, respectively, also increased LDH release. A similar effect was also noted for the enriched Rh2 ginseng leaf fraction. When comparing the LDH results along with the annexin V-PI staining data, it can be concluded that these test compounds alter THP-1 cell membrane permeability. It is well known that saponins influence the permeability of biological membranes (Glauert et al., 1962), by forming complexes within the membrane that rearrange membrane lipids. The cholesterol content of the membrane for example is an important determinant for the actions of some saponins (Glauert et al., 1962). Popov (2002) effectively showed that increasing the cholesterol content of Erlich tumor cells decreased the cytotoxic effect of both PD and Rh2. Furthermore, a reduction in membrane cholesterol can led to apoptosis (Martinez-Botas et al., 1999). Awad et al. (2003) reported that exposure of cultured breast
cancer cells to β-sitosterol, which has some similarity in structure to certain ginsenosides, also reduced the effective total cholesterol content of cell membranes and increased the percentage of β-sitosterol in the cell membrane, while reducing cell density. Similarly, glycoalkaloids have been reported to act on the cultured cell membrane by causing membrane components to associate or group together and form a membrane bud while disrupting the integrity of the epithelial barrier (Patel et al., 2002).

The concentration of the enriched Rh2 ginseng leaf fraction required to induce cytotoxic effects was notably greater than, but comparable to pure Rh2 standard. The enriched Rh2 ginseng leaf fraction contained Rh2 isomers (56%), but other ginsenosides such as Rg3 and Rg1 were also present. Ginsenoside Rg1 is generally regarded to have no cytotoxicity (Park et al., 2002), where as ginsenosides Rg3 has a potential role in enhancing the observed effect of the enriched Rh2 ginseng leaf fraction, especially by influencing apoptosis compared to Rh2. Rg3 reduces the effectiveness of P-gp membrane efflux pumps and can reduce expression of this transporter (Kim et al., 2003), which has a role in resistance to chemotherapy (Pastan and Gottesman, 1991). The membrane pump is used to remove potentially toxic plant components and therefore a reduction in the P-gp membrane efflux pump would effectively increase the concentration of test compounds such as Rh2 in the cell that is required to induce an apoptotic effect (Pastan and Gottesman, 1991).

Ginsenosides PD and Rh2 were found to have the greatest cytotoxic effect on THP-1 cell viability and, it is suggested herein that this result is due to structural similarities to cholesterol. Ginsenosides, such as PD and Rh2, have a general non-specific effect on the cell membrane apart from reported genomic (apoptotic) effects. Analogous to the case with phytosterols, certain ginsenosides may also be able to replace endogenous sterol in the cell membrane, which would alter
membrane properties. Therefore, a general membrane effect on a wide variety of cells would effectively explain the non-specific associated diverse biological effects of ginseng and ginsenosides.

20(S)-protopanaxadiol  
20(S)-protopanaxatriol

Rh2

Figure 6.1. The structure of ginsenosides tested in this study.
Figure 6.2. ESI-MS total ion chromatograph of the enriched Rh2 ginseng leaf fraction, peaks numbered 1-8 correspond to ginsenosides listed in Table 6.1.

Table 6.1. Main fragmentation ions produced from the enriched Rh2 ginseng leaf fraction presented in Figure 6.2.

<table>
<thead>
<tr>
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<td>[M+Aco]-</td>
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<tr>
<td>1 Rg1</td>
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<td>799.4</td>
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<td>2 Rg6*</td>
<td>766</td>
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<tr>
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<tr>
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<td>621.4</td>
<td>657.3</td>
</tr>
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<td>8 Rh2-20(R)</td>
<td>622</td>
<td>621.4</td>
<td>657.3</td>
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</table>

*Molecular weight match
Figure 6.3. The effect of three ginsenoside standards and an enriched Rh2 ginseng leaf fraction (LFRh2) on THP-1 cell viability measured by MTT assay at different exposure time as described in the Material and Methods. A = 6 h, B = 12 h, C = 24 h, D = 48 h, data are expressed as mean ± SD (n = 3). PD (●), PT (■), Rh2 (▲), LFRh2(×).
Figure 6.4. LC50 of test ginsenosides (PD, PT, Rh2) and an enriched Rh2 ginseng leaf fraction (LFRh2) on THP-1 cells, determined from MTT regression analysis assays at different time periods; histogram bars correspond to the following: 6 h (open bar), 12 h (grey bar), 24 h (black bar), 48 h (dotted bar), 72 h (horizontal hatch). Data are expressed as mean ± SD (n = 3) for each, bars of the same ginsenoside treatment with different letters are significantly different from each other (P ≤ 0.5).
Figure 6.5. LDH release from cultured THP-1 cells after exposure to PD, PT, Rh2 and enriched Rh2 ginseng leaf fraction (LFRh2) at respective LC50 @ 48 h concentrations. LDH release was measured and expressed as a percentage of control cells at each time period. Data are expressed as mean ± SD (n = 3) for each, bars of the same ginsenoside treatment with different letters are significantly different from control activity (P ≤ 0.5), ns = not significant. Bars correspond to PD (open bar), PT (grey bar), Rh2 (black bar) and LFRh2 (horizontal hatch).
Figure 6.6. LDH released from cultured THP-1 cells after 24 h exposure to PD, PT, Rh2 and enriched Rh2 ginseng leaf fraction (LFRh2) at different concentrations. Data are expressed as percentage of control, mean ± SD (n = 3), bars at specific concentration with different letters being significantly different from each other (P ≤ 0.5). Bars correspond to PD (open bar), PT (grey bar), Rh2 (black bar) and LFRh2 (horizontal hatch). (Note LFRh2 @ 160 µg/mL = 301 ± 11 %).
Table 6.2. Apoptotic and necrotic cells identified by annexin-V-FITC and PI positive staining after exposure to PD, PT, Rh2 and enriched Rh2 ginseng leaf fraction and analyzed by flow cytometry.

<table>
<thead>
<tr>
<th>Hours</th>
<th>PD</th>
<th>PT</th>
<th>Rh2</th>
<th>LFRh2</th>
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<tbody>
<tr>
<td></td>
<td>Apoptotic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>5.3 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>5.9 ± 0.0</td>
</tr>
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<td>4.5 ± 0.0</td>
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</tr>
<tr>
<td>11</td>
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<td>11.1 ± 0.3</td>
<td>8.3 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>19</td>
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<td>7.8 ± 0.2</td>
<td>8.9 ± 0.2</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>23</td>
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<td>10.2 ± 0.4</td>
<td>8.5 ± 0.2</td>
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</tr>
<tr>
<td>41</td>
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<td>10.6 ± 0.3</td>
<td>9.9 ± 0.3</td>
<td>18.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Necrotic</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>6.0 ± 0.2</td>
<td>21.5 ± 0.3</td>
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</table>

Data are expressed as percentage, mean ± SD (n = 3). Treatment at the same time period (h) with different superscript letters are significantly different (P ≤ 0.05).
Figure 6.7. Representative flow cytometry dot plots of annexin-V-FITC/PI flow cytometry of THP-1 cells at treatment (PD, PT, Rh2 and LFRh2) at concentrations of LC50 @ 72 h and twice the LC50. The upper right box of each panel (dot plot) shows necrotic cells (annexin-V\(^+\)/PI\(^+\)) and the lower right outline shows apoptotic cells (annexin-V\(^+\)/PI\(^-\)). The histogram shows the percentage of necrotic cells after 19 hours of exposure to the LC50 @ 72 h (open bar) and 2 x LC50 @ 72 h (black bar). The inset table shows apoptotic cell accumulation. Data are expressed as mean ± SD (n = 3); histogram bars with different letters are significantly different from other (P ≤ 0.5), LFRh2 = enriched Rh2 ginseng leaf fraction, CON = untreated control cells.
Figure 6.8. Cell cycle analysis of THP-1 cells exposed to the enriched Rh2 ginseng leaf fraction (52 μg/mL).
Figure 6.9. Caspase-3 activity of PD, PT, Rh2 and the enriched Rh2 ginseng leaf fraction (LFRh2) of THP-1 treated cells at 4 h exposure. Data are expressed as mean ± SD (n = 3); bars with different letters are significantly different from control (CON) (P ≤ 0.5).
CHAPTER VII

7.1 OVERALL DISCUSSION

7.1.1 Part 1. Identification of bioactive constituents

Ginsenoside PD of the 20-(S) protopanaxadiol family of compounds was found to have a stronger effect on cell viability relative to Rh2 in leukemia cells (THP-1), whereas Rg3 a structurally related compound had no effect on cell viability. A similar finding was found for PT of the 20-(S) protopanaxatriol series, relative to the effect of Rhl on viability. Differences between specific ginsenosides reported herein are suggested to be related to the structural conformation and presence of sugars at position C-3 and C-6 which influence the hydrophobic character of the compounds required to interact with cell membrane function. This effect is similar to other reported effects of saponins on membranes (Glauert et al., 1962). In intestinal cells, (Int-407 and Caco-2) individual ginsenosides, or collectively as contained in a standardized North American ginseng extract, varied considerably in relative efficacy to affect cell viability. A standardized North American ginseng root extract, and the main ginsenosides found present in the root extract did not affect cell viability in Int-407 nor Caco-2 cells. In contrast, ginsenosides, PD, PT and Rh2, not present in the North American ginseng root extract, were generally effective at inducing cytotoxicity in THP-1 cells. Furthermore, PD, PT and Rh2 showed a propensity to influence apoptosis mostly through alterations of cellular membrane permeability and integrity. It was found, for the first time, that ginsenosides that produced the most apoptotic cells also showed the greatest cellular leakage of LDH, a useful marker of membrane integrity.
7.1.2 Part 2. North American ginseng plant material

A valuable source of rare ginsenosides was identified from North American ginseng leaf by applying thermal energy during the extraction process. Ginsenoside Rh2, an otherwise rare ginsenoside was detected only in the leaf and not in ginseng root samples after application of thermal energy. The main source of Rh2 has been reported to be exclusively Korean red ginseng root (Kim et al., 2000; Park et al., 2002; Shin et al., 2000) which relies on steam processing to ensure preservation and may alter the ginsenoside composition (Kim et al., 2000). Evidence was shown that Rh2 and structurally related Rg3 are indeed formed as a result of the addition of thermal energy and could not be detected in a cold extraction process. Evidence is provided for the first time that Rg3 and Rh2 are formed during thermal processing of North American ginseng, and that these breakdown products are not naturally present. Furthermore, it was found that heating time was important for formation of rare ginsenosides; after 10 min of water reflux, ginsenosides Rg3 and Rh2 were not detected, however, both Rg3 and Rh2 appeared after 20 min. Ginsenosides Rg3 and Rh2 were shown to be degradation products of larger protopanaxadiol ginsenoside compounds sources such as Rc and Rb1. An additional source of the rare ginsenosides Rh2 and Rg3, which are valued for potential anti-cancer properties, was also identified. Popovich and Kitts (2002) previously found that ginsenoside Rh2 was effective at reducing cell viability in THP-1 human leukemia cells and in intestinal cell lines (Popovich and Kitts, 2003). Other researchers have found that Rh2 induced apoptosis in a variety of cell lines (Fei et al., 2002; Nakata et al., 1998; Park et al., 1997; Popovich and Kitts, 2002) and Rg3 was effective in reducing proliferation of prostate cancer cells (Liu et al., 2000) and cisplatin resistance cells (Keum et al., 2003).
7.1.3 Part 3. Development, assessment and proposed mechanism of an enriched Rh2 extract

An enriched Rh2 fraction derived from North American ginseng was demonstrated, for the first time, and the effect on cell viability was shown to be variable and dependent on both ginsenoside exposure time and concentration in a similar manner observed for ginsenoside PD, PT and Rh2. In these studies, all ginsenosides tested and the enriched Rh2 leaf fraction, affected Caco-2 and THP-1 cell membrane permeability, as measured by LDH release. The time and the magnitude of LDH release was shown to be dependent on the specific ginsenosides. Rh2 treated cells have been reported to alter membrane morphology (Ota et al., 1987) which agrees with observations made from other saponins that have also been reported to alter membrane function (Glauert et al., 1962). In Caco-2 cells, Rh2 and PD ginsenoside standards produced greater apoptotic accumulation compared to PT standard. This finding confirms our earlier report, of a build up of sub-G1 (apoptotic) cell from Caco-2 and Int-407 cells, respectively, after Rh2 and PD exposure (Popovich and Kitts, 2004b). It is of particular interest that caspase-3 activity, an apoptosis response trigger, responded differently between the two ginsenoside compounds and was similar for Caco-2 and THP-1 cells. In general, the relative potency of ginsenosides is likely due to the hydrophobic/hydrophilic balance of the individual ginsenosides that involves alterations in membrane permeability before initiating apoptotic and necrotic cell characteristics.

7.2 OVERALL CONCLUSION AND FUTURE STUDIES

P-glycoprotein (P-gp) is a membrane glycoprotein encoded by the \textit{MDR1} gene that is associated with resistant to anticancer drugs (Hasegawa et al., 1995). P-gp is an energy dependent drug efflux pump, which is involved in both intrinsic and acquired drug resistance. Expression of this transporter plays a role in resistance to chemotherapy (Pastan and Gottesman, 1991), effectively
pumping chemotherapy drugs from the cell thereby lowering the effective concentration.

Furthermore, resistance is often encountered from drugs that are amphipathic and have been isolated from plants and microorganisms (Pastan and Gottesman, 1991). Future studies with bioactive ginsenosides and Rh2 enriched extract should be evaluated on the potential effect to stimulate the P-gp efflux pump. P-gp transporter has been purposed to be an evolutionary adaptation to remove toxic compounds from the bile or intestine (Pastan and Gottesman, 1991). Higher levels of MDR1 expression is found in colon, kidney, liver, adrenal, pancreas and in leukemia cells. Expression of MDR1 after chemotherapy was found to be increased in leukemia, breast cancer, lymphomas among others cells (Pastan and Gottesman, 1991). Identifying agents that will slow or stop the efflux pump is a valuable addition to chemotherapy and certain ginsenosides have been tested in combination with resistant drugs. Daunomycin (DAU) and vinblastine (VBL) resistant leukemia cells (P388 ADM) were treated with 20(S)-Rh2, 20(S)-PT and compound K, an intestinal biotransformed metabolite of Rb1, to effectively reverse both DAU and VBL drug resistant cells. Ginsenosides have been suggested to inhibit the efflux pump according to structural characteristics related to the attachment of bulky side changes at position C-3 of the triterpene ring (Molnar et al., 2000). Bulky side chains of certain ginsenosides (Rb1, Re, Rd, Re) may not have adequate conformation to exert an effect on the glycoprotein or membrane structures. Furthermore, ginsenosides Rg3 was effective at increasing the sensitivity of vincristine (VCR) resistant cells (KBV20C); Rg3 competitively inhibited binding to P-gp (Kim et al., 2003) and increased survival time of mice transplanted with multidrug resistant P388 leukemia tumor compared to adriamycin treatment (Kim et al., 2003). With these findings taken into consideration, future studies should be planned to establish whether bioactive ginsenosides (PD, PT, Rh2) directly influence the P-gp membrane pump, or generally alter the conformation or composition of the cellular membrane.
Although membrane properties are reported to be altered by exposure to ginsenosides, it remains unclear as to the extent to which membrane modification is responsible for all of the observed effects of ginsenosides. However, it is likely that ginsenosides share similar dual roles that are both non-specific and genomic and common for cholesterol and other steroids, provided certain conditions are met. Reports stating ginsenosides can alter protein expression or modify gene expression have generally been reported in low, or cultures containing serum free media. Serum free media would stress the cell system potentially magnifying or forcing a genomic effect. Low serum formulations would have low or no cholesterol available for cells to maintain optimal growth conditions. Two possible outcomes may arise. Tumor cells require cholesterol for growth; other sterols or sterol-like compounds may be used in place of cholesterol, but may affect function. Exposure to β-sitosterol or other similar compounds can result in changes to the membrane composition and incorporation. Likewise, ginsenosides may also influence membrane properties, whereby cells incorporate PD or Rh2 or structurally related compounds into membranes in the place of cholesterol, or as a result of low cholesterol availability and an increased cellular demand for steroidal ring structure. These compounds would have an impact of membrane fluidity or permeability of the cells either by aggregation together in micelles, thus behaving as a detergent, or by opening small holes in the membrane. Membrane depolarization and an increase in ion permeability may also follow. Furthermore, dysfunctional membrane protein or receptors may result in a change in membrane composition which alters function. It is concluded that these effects are probably observed before a substantial genomic effect of ginsenoside supplementation can occur. However, more comprehensive studies are needed to determine the components of the serum in the cell culture media, such as cholesterol or binding proteins that can interact with ginsenosides and influence cytotoxicity in cell culture experiments.
It is certain that some ginsenosides can have an effect on biological systems and evidence for a membrane altering mechanism is mounting. A general, non-specific effect of ginsenosides on cells membrane composition or function would effectively explain the milieu of effects historically and scientifically reported for ginseng and ginsenosides components.
REFERENCES


Popovich, D. G.; Kitts, D. D. Ginsenosides can inhibit proliferation and induce apoptosis in cultured leukemia and intestinal cells but effects vary according to the structure of the compounds. *Faseb J.* 2003, 17, A762-A762.


Appendix A1.1 DNA cell cycle histograms of Control (untreated), PD, PT, Rh2, treated Int-407 cells for 24, 48 and 72 h. Cells were fixed in ethanol and stained with PI as described in the Materials and Methods (Section 3.3.6). DNA histograms shown are representative histograms of three separate experiments.
Appendix A1.2. DNA cell cycle histograms of control (untreated), PD, PT, Rh2, treated Caco-2 cells for 24, 48 and 72 h. Cells were fixed in ethanol and stained with PI as described in the Materials and Methods (Section 3.3.6). DNA histograms shown are representative histograms of three separate experiments.
APPENDIX 2

HPLC -UV and ESI-MS total ion representative chromatogram of North American ginseng root and leaf extractions.

Appendix A2.1. Representative chromatograph of HPLC-ESI-MS analysis of North American ginseng root (hot water extraction). Top panel represents HPLC chromatograph where the bottom panel represents total ion chromatograph. Peaks labeled with asterisks (*) were identified from Haijiang et al. (2003), (#) corresponds to a molecular weight match, iso refers to isomer.
Appendix A2.2. Representative chromatograph of HPLC-ESI-MS analysis of North American ginseng leaf (hot water extraction). Top panel represents HPLC chromatograph where the bottom panel represents total ion chromatograph. Peaks labeled with asterisks (*) were identified from Haijiang et al. (2003), (#) corresponds to a molecular weight match, iso refers to ginsenoside isomer.
Appendix A2.3. Representative chromatograph of HPLC-ESI-MS analysis of North American ginseng leaf (80% ethanol extraction). Top panel represents HPLC chromatograph where the bottom panel represents total ion chromatograph. Peaks labeled with asterisks (*) were identified from Haijiang et al. (2003), (#) corresponds to a molecular weight match, iso refers to ginsenoside isomer.
Appendix A3.1. Representative dot plots of annexin-V-FITC/PI flow cytometry of THP-1 cells after exposure to test compounds at LC50 @ 72 h (PD 13, PT 19, Rh2 15 and LFRh2 52 μg/mL) for different time intervals. The upper right outline box of each panel shows necrotic cells which are positive for annexin-V-FITC and propidium iodide (PI) (annexin-V+/PI+) and the lower right outline shows apoptotic cells, positive for annexin-V-FITC positive and PI negative (annexin-V+/PI−). LFRh2 = enriched Rh2 ginseng leaf fraction. Quantification of apoptotic and necrotic cells is listed in Table 6-2.