Investigation into the possible synergistic apoptotic response to fludarabine and epigallocatechin-3-gallate in B-cell derived cell lines BJAB and I-83

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Abstract

The green tea polyphenol epigallocatechin-3-gallate (EGCG) has been shown to induce apoptosis in various cancers. Fludarabine is a highly effective treatment for B-cell chronic lymphocytic leukemia (CLL). This study examined the possibility of a synergistic apoptotic response in the B-cell derived cell lines BJAB and I-83 by combined treatment with EGCG and fludarabine. The average apoptotic cells observed in BJAB and I-83 cells after 48 hours of treatment with 5 μM dose of fludarabine were 46.52±16.45 and 34.18±3.45 respectively, which are expected levels of fludarabine induced apoptosis. EGCG failed to induce apoptosis in both cell lines, and there was no significant increase in apoptosis with combined treatment. It was concluded that combined treatment with EGCG and fludarabine does not cause a synergistic apoptosis in BJAB and I83 cell lines. Western blots were prepared to observe if cells treated with fludarabine showed an increase in cleaved caspase-3, which is a known response. It was found that all cells despite the treatment had the same level of un-cleaved caspase-3. These results may have been influenced by the contamination of the cell lines with *Mycoplasma*, the cell lines being processed too long in the lab, or the EGCG used may have been inactive.

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Introduction

Burkitt's Lymphoma

Lymphomas are responsible for 10% of the malignant diseases in children 15 years of age or younger, and 60% of these cases are non-Hodgkin's lymphomas (Shapira and Peylan-Ramu, 1998). Burkitt's lymphoma (BL) is a highly aggressive subgroup of non-Hodgkin's lymphoma presenting at extranodal sites or as an acute leukemia (Santini *et al*., 2008). It is composed of monomorphic medium-size B-cells with basophilic cytoplasm and numerous mitotic figures (Santini *et al*., 2008). BL occurs in children and young adults throughout the world (Shapira and Peylan-Ramu, 1998).

There are three clinical variants of BL, endemic, sporadic and immunodeficiency associated which are each different in clinical presentation, morphology and biology (Santini *et al*., 2008). Endemic BL occurs in high incidence in endemic regions such as equatorial Africa and northeastern Brazil, where it accounts for half of all childhood cancers (Shapira and Peylan-Ramu, 1998). Sporadic BL occurs elsewhere in the world at a much lower frequency, 20-40 times less than in edemic regions (Shapira and Peylan-Ramu , 1998). Immunodeficiency associated BL is associated with immunodeficiency syndromes such as HIV, and is likely due to deficient T-cell function (Shapira and Peylan-Ramu, 1998). BL is 1000 times more likely to occur in AIDS patients than in the general population (Shapira and Peylan-Ramu, 1998).

Endemic BL is extremely sensitive to chemotherapy; however, sporadic and immunodeficiency associated BL are not, and the prognosis used to be poor especially in adults (Ferry, 2006). Short-duration, high-intensity chemotherapy yields excellent survival in children: patients with the localized disease have a greater than 90% 5 year

survival rate; children with widespread disease achieve a greater than 90% complete response rate with an event-free survival rate at 4 years in 65% of patients with leukemic presentation; and 79% for those with stage IV lymphoma (Ferry, 2006). When similar aggressive chemotherapeutic treatments are administered to adults the complete response rates range from 65-100% and the overall survival rates are from 50-70% (Ferry, 2006).

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a disease that affects older adults, with more than 90% of cases occurring in adults over 50 (Inamdar *et al.*, 2007). CLL differs from most cancers as it is caused by the survival of the malignant clone, and not caused by an increase in proliferative activity (Pepper *et al.*, 1999). This disease is an accumulation of long-lived B lymphocytes that arrest in the G_0-G_1 cell cycle (Pepper *et al.*, 1999). CLL is initially responsive to many chemotherapy drugs, however this responsiveness decreases and many patients become resistant to treatment, which increases the difficulty of managing the condition (Pepper *et al.*, 1999). CLL symptoms develop gradually and it is difficult to pinpoint the onset of the disease (Inamdar and Bueso-Ramos, 2007). CLL is often discovered accidentally due to elevated lymphocyte counts observed during a routine visit to a physician, as 25% of patients do not show symptoms at the onset of the disease (Inamdar and Bueso-Ramos, 2007). The duration of this asymptomatic period varies greatly between individuals (Inamdar and Bueso-Ramos, 2007). In most cases CLL occurs as a monomorphic accumulation of small B-cell lymphocytes in the blood, bone marrow, lymph nodes, liver and spleen (Ayuk *et al.*, 2008, Keating *et al.*, 1989). The cells can be characterized by the surface membrane immunoglobulin, CD_5, surface antigen, and a predominance of kappa or lambda light

chains (Keating *et al.*, 1989). In vitro cells are prone to apoptosis indicating that there may be an extrinsic factor that plays a role in the maintenance of the disease (Pepper *et al.*, 1999)

Traditionally it was believed that CLL was an incurable disease of the elderly and that individuals will die with CLL but not because of the disease (Tam and Keating, 2007). The current view of CLL is that the majority of patients diagnosed with CLL will die due to complications related to the disease (Tam and Keating, 2007).

Apoptosis

Apoptosis, or programmed cell death, is an evolutionarily conserved process that occurs naturally by a series of events that lead to the death of unwanted cells, such as damaged or aged cells (Borner, 2003; Karp, 2005). Physiological features of a cell undergoing apoptosis are DNA fragmentation, cell shrinkage, blebbing of the cell membrane, mitochondrial dysfunction, and when the phospholipid phosphatidylserine is found on the outer side of the plasma membrane (Gulbins *et al.*, 2000). Resistance to apoptosis can result in autoimmune disorders or cancer (Borner, 2003). Enhanced apoptosis can lead to acute diseases such as ischemia, chronic pathologies such as neurodegenerative and neuromuscular diseases, and AIDS (Borner, 2003). Much of the apoptotic pathway has been evolutionarily conserved from worm to human (Budihardjo *et al.* 1999). Apoptosis can occur by an extrinsic pathway that is activated by external stimuli, and an intrinsic pathway that is a response to internal stimuli (Karp, 2005). There are three major components involved in apoptosis: the Bcl-2 family of proteins, the caspases, which belong to a family of cysteine proteases, and the Apaf-1/CED-4 protein,

which relays the signals that are incorporated by the Bcl-2 family of proteins to the caspases (Budihardjo, *et al.*, 1999).

Caspases

The caspases are cysteine proteases that cleave vital cellular substrates after aspartate residues (Borner, 2003). These enzymes are mostly inactive in healthy cells, existing as inactive enzymes that are activated in response to apoptotic stimuli through one or two sequential proteolytic events that cleave the inactive precursor into the large and small fragments that constitute the active enzyme (Borner *et al*., 2003, Budihardjo *et al*., 1999).

More than 14 caspases have been discovered due to the rapid expansion of the expressed sequence tag database and the presence of the conserved pentapeptide sequences at the caspase active site (Budihardjo *et al*., 1999). Caspase-1 and caspase-11 function primarily during cytokine processing, caspase-2, -8, -9, -10, -12 are the initiator caspases, and caspase-3, -6, -7 are effector caspases (Borner *et al*., 2003). The functions of the additional caspases are still unknown at this time (Budihardjo *et al*., 1999). The initiator caspase acts during an early stage of the apoptotic signaling pathway and is characterized by an extended N-terminal pro-domain, which interacts with a specific scaffold or adaptor protein that clusters the respective initiator caspases (Borner, 2003). This clustering enhances the autoproteolysis allowing for the formation of an active dimeric complex consisting of two small and two large subunits (Borner, 2003). The active initiator caspases amplify the apoptotic signal by activating effector caspases by cleaving at specific internal aspartic acid residues (Borner, 2003). The effector caspases cleave the substrates that are responsible for the destruction of the cells into apoptotic

bodies resulting in the subsequent phagocytosis by macrophages (Borner, 2003). The extrinsic and intrinsic apoptotic pathways are both regulated by caspase-activating cascades (Budihardjo *et al*., 1999).

The Bcl-2 Family of Proteins

The Bcl-2 family of proteins include antiapoptotic and proapoptotic proteins whose interactions will determine whether a cell with undergo apoptosis or survive (Borner, 2003, Henson, 2006). The proapoptotic proteins are Bax and Bak which trigger or sensitize a cell for apoptosis (Borner, 2003, Henson, 2006). The antiapoptotic proteins are Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1, and are most often increased in cancer cells, causing the cells to be resistant to apoptosis (Borner, 2003, Henson, 2006). The proapoptotic proteins induce the intrinsic pathway of apoptosis by translocating from the cytosol to the external membrane of the mitochondria (Karp, 2005). Once on the mitochondrial membrane the proteins increase the permeability of the membrane and promote the release of cytochrome c causing apoptosis (Karp, 2005).

Extrinsic Pathway

The extrinsic pathway results in caspase activation by the binding of cell surface death receptors to their specific ligands (Budihardjo *et al.*, 1999). The cell surface death receptors are a family of transmembrane proteins that belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily (Budihardjo *et al.*, 1999). The mammalian death receptors all have a conserved cysteine-rich repeat at their extracellular domains (Budihardjo *et al.*, 1999). The ligands that activate these receptors are structurally related molecules belonging to the TNF gene superfamily (Budihardjo *et al.,* 1999). When the Fas receptor binds its ligand intracellular signals are released that lead

to the activation of caspases (Budihardjo *et al.*, 1999). This occurs by three defined steps: ligand-induced receptor trimerization, the recruitment of intracellular receptor-associated proteins, and the initiation of caspase activation (Budihardjo *et al*., 1999). An example of this is the binding of FasL to the Fas receptor induces trimerization of Fas (Budihardjo *et al.,* 1999). This Fas trimer is a death-inducing signaling complex (Budihardjo *et al*., 1999). The cytoplasmic region of Fas contains a death domain which recruits Fas-associating protein with death domain (FADD) which contains a death domain in its C terminus that binds to Fas through interactions between the death domains (Budihardjo *et al.*, 1999). If a single point mutation occurs within the death domain the apoptotic signal is completely destroyed, suggesting that the death domain is needed for the initiation of death signals inside the cell (Budihardjo *et al*., 1999). The N terminus of FADD is termed the death effector domain, and is critical in the recruitment of upstream procaspases such as procaspase-8 and/or procaspase-10 (Budihardjo *et al.*, 1999). There are two death effector domains found at the N-terminal domain of procaspase-8, which is used for binding FADD (Budihardjo *et al.*, 1999). The C-terminal domain contains a caspase homology region (Budihardjo *et al.*, 1999). Directly after recruitment, procaspase-8 is proteolytically processed to the active forms consisting of large and small catalytic subunits (Budihardjo *et al.*, 1999). As described above, the activation of caspase-8, an initiator caspase, results in the activation of the effector caspases which cleave many different cellular protein substrates vital for cell function resulting in the death of the cell (Borner, 2003).

There are three mechanisms by which the death receptor activity is regulated (Budihardjo *et al*., 1999). The first prevents the recruitment and/or activation of

procaspases at the death-inducing signaling complex. The second mechanism is through the expression of decoy receptors for tumor-necrosis related apoptosis-inducing ligand (TRAIL) (Budihardjo *et al.,* 1999). The decoy receptors are closely related to TRAIlL receptors DR4 and DR5, however, the decoys lack the cytoplasmic domain or contain a cytoplasmic region with a condensed death domain, inhibiting TRAIL-induced apoptosis (Budihardjo *et al.*, 1999). Normal human tissues express these decoy receptors more abundantly than tumor tissues (Budihardjo *et al*., 1999). The third mechanism is by direct inhibition of the proteolytic activation of the initiator procaspases (Budihardjo *et al.*, 1999).

Intrinsic Pathway

The intrinsic pathway begins with mitochondrial damage followed by cytochrome *c* release (Budihardjo *et al.*, 1999). This pathway is controlled by three main proteins, the first of which is cytochrome *c* (Budihardjo *et al.*, 1999). Cytochrome *c* is released by the mitochondria in cells that are undergoing apoptosis induced by a variety of stimuli, such as DNA damaging agents, kinase inhibitors, and activation of cell surface death receptors (Budihardjo *et al*., 1999). After cytochrome *c* is released it interacts with the other two essential proteins, Apaf-1 and procaspase-9, to activate caspase-3 (Budihardjo *et al*., 1999). Apaf-1 contains a caspase recruitment domain that binds caspases with a similar domain (Budihardjo *et al.*, 1999). The C-terminal half of Apaf-1 is composed of 12-13 WD-40 repeats, and the deletion of these WD-40 repeats causes Apaf-1 to be constitutively active, however inhibits the release of the activated caspase-9, which indicates that this domain functions to inhibit Apaf-1 activity and to help release the activated caspase-9 (Budihardjo *et al*, 1999).

Procaspase-3 is activated by Apaf-1 and caspase-3 (Budihardjo *et al.*, 1999). Apaf-1 binds ATP/dATP and hydrolyzes it to ADP/dADP, which has no functional consequence if cytochrome *c* is not present (Budihardjo *et al.*, 1999). In the presence of cytochrome *c* the hydrolysis of ATP/dATP induce the formation of a multimeric Apaf-1/cytochrome *c* complex, which functions to recruit and activate procaspase-9 (Budihardjo *et al*., 1999). The formation of the multimeric Apaf-1/cytochrome *c* is the commitment step in caspase activation (Budihardjo *et al.,* 1999). The formation of the multimeric complex recruits procaspase-9 and is activated by proteolysis (Budihardjo *et al.,* 1999). The active caspase-9 is released in order to cleave and activate caspases downstream such as caspase-3, -6, and -7 (Budihardjo *et al*., 1999). It is believed that the formation of the multimeric Apaf-1/cytochrome *c* complex may be to increase the local concentration of procaspases for intermolecular cleavage and to set a high threshold so that occasional leakage of cytochrome *c* will not result in the commitment to apoptosis (Budihardjo *et al.*, 1999).

It is believed that the main regulatory process for mitochondria-mediated caspase activation is the release of cytochrome *c* (Budihardjo *et al*., 1999). Of the three essential proteins described above only cytochrome *c* is not found in the cytosol, and is found in the intermembrane space of mitochondria (Budihardjo *et al*., 1999). Microinjection or electroporation of cytochrome *c* induces apoptosis in many cell types, indicating that the release of cytochrome *c* is the regulatory step in mitochondria-mediated caspase activation (Budihardjo *et al*., 1999). The release of cytochrome *c* is regulated by the Bcl-2 family of proteins (Budihardjo *et al*., 1999). The precise mechanisms by which the Bcl-2 family of proteins cause the release of cytochrome *c* are still unknown, however,

three theories have been put forth (Budihardjo *et al*., 1999). They are the permeability transition pore theory of the Kroemer group, the ion flow model of the Thompson group, and the BH3-containing protein model (Budihardjo *et al*., 1999). The first theory is founded on the concept that the permeability transition pore regulates the potential of the inner mitochondrial membrane, and that this pore is regulated by the Bcl-2 family of proteins (Budihardjo *et al*., 1999). The ion flow theory involves the possibility that the ratio of proapoptotic and antiapoptotic proteins influence the flow of ions and the flow of water (Budihardjo *et al*., 1999). The BH3-containing protein model is based on the hypothesis that BH3-containing proteins act through a separate pathway in the mitochondria inducing the release of cytochrome *c* (Budihardjo *et al*., 1999).

Fludarabine

When fludarabine was discovered it showed promise against CLL and was extensively studied for its effects on CLL with response rates of 50-60% in patients who had failed with traditional alkylating-agent therapy (Bernasconi *et al.*, 2007, Tam and Keating, 2007). This was followed by many studies that evaluated the effects of fludarabine on CLL and these studies observed major remission rates and prolonged remission rates compared to therapy with alkylating agents (Tam and Keating, 2007). Comparisons of chlorambucil and fludarabine and fludarabine-cyclophosphamide in chemotherapy-naïve CLL found superior complete response rates for fludarabine compared to chlorambucil (Tam and Keating, 2007). Fludarabine also has substantial activity against low-grade non-Hodgkin's lymphoma (Yu *et al*., 2005). Fludarabine is known to cause decreased immunosurveillance allowing for a high incidence of opportunistic infections and secondary cancers (Bernasconi *et al.*, 2007). Fludarabine is

often treated in combination with alkylating agents, rituximab or with both, causing improved response rates, complete remission rates, and increased remission duration (Kasamon *et al.*, 2007).

Fludarabine is a halogenated adenine analog that is resistant to deactivation by adenosine deaminase (Hood and Finley, 1991). The base in fludarabine is insoluble in water and therefore a monophosphate group was added to the 5' position causing the compound to be more soluble so that it can be administered intravenously (Hood and Finley, 1991). Fludarabine is rapidly dephosphorylated to 9-β-D-arabinofuranosyl-2-fluoroadenine (2F-ara-A), a reaction that is necessary for the cellular uptake (Hood and Finley, 1991). In the cell 2F-ara-A is rephosphorylated by deoxycytidine kinase to the active form 2F-ara-ATP (Hood and Finley, 1991). F-ara-A inhibits DNA synthesis by inhibiting DNA polymerases and DNA elongation by incorporation into the DNA molecule (Yu *et al*., 2005). The incorporation of fludarabine into DNA is the event that causes cytotoxicity in CLL cells (Yu *et al*., 2005). Fludarabine is mostly effective during the S-phase of cell division but is active against non-proliferating cells (BCCA Cancer Drug Manual, 2007).

Fludarabine causes severe demyelination when used at high doses to treat acute leukemia, which was not observed when lower doses were used to treat solid tumors and lymphoma (Keating *et al.*, 1989). The major dose-limiting toxic effect is myelosuppression and leucopenia (Keating *et al.*, 1989). Fludarabine causes lymphopenia by depleting normal T lymphocytes more than B lymphocytes (Keating *et al.*, 1989). Early studies found that 32% of lymphoma patients and 35% of CLL patients responded to fludarabine treatment (Keating *et al.*, 1989).

Epigallocatechin-3-gallate

Epigallocatechin-3-gallate (EGCG) is a green tea extract derived from the *Camellia sinesis* plant (Carlson *et al.*, 2007). Traditional Chinese medicine has been using green tea extracts for centuries to treat and prevent chronic diseases, however recently there have been an increasing number of in vivo and in vitro studies investigating the possible anticarcinogenic effects of EGCG (Carlson *et al.*, 2007). EGCG is an antioxidant that can prevent oxidative damage in healthy cells, and also acts as an antiangiogenic agent, preventing tumors from developing their own blood supply, which would allow the tumor to grow larger (Carlson, 2007). EGCG also displays antibacterial properties, which could be involved in preventing gastric cancer (Carlson *et al.*, 2007). EGCG is a known tyrosine kinase receptor inhibitor (Lee *et al.*, 2004), as well as an inhibitor of the serine protease urokinase (Jankun *et al*., 1997). Apoptosis in cancer cells may be induced by EGCG by negatively regulating the cell cycle, preventing further divisions (Carlson *et al.*, 2007).

EGCG Antioxidation

The best studied mechanism of EGCG is as an antioxidant (Carlson *et al.*, 2007). Oxidative damage to cells can be caused by many factors such as UV light, free radicals and carcinogens (Carlson *et al.*, 2007). Oxidative damage to DNA, lipids and proteins may result in cancer development (Carlson *et al.*, 2007). DNA damage as a result of oxidative damage is a source of gene mutations that result in the modification of gene expression and cellular regulation (Carlson *et al.*, 2007). The oxidative damage to cells can be measured by measuring the byproducts of oxidative damage (Carlson *et al.*, 2007). EGCG has been found to reduce the plasma levels of biomarkers for oxidative damage of

lipids and DNA (Carlson *et al.*, 2007). The effect of EGCG on oxidative protein damage is not conclusive (Carlson *et al.*, 2007).

EGCG along with other antioxidants neutralize free radicals in the body (Carlson *et al.*, 2007). These antioxidants have a high affinity for some metal ions and behave as metal chelators and inactivate redox-active transition metal ions that would catalyze free radical formation (Carlson *et al.*, 2007). Antioxidants may also block some undesired agents such as bacteria and carcinogens from binding to cells (Carlson *et al.*, 2007). EGCG may also inhibit lipoxygenase, cyclooxygenaase, and xanthine oxidase enzymes which are able to cause oxidative damage in tissues by peroxidase activity (Carlson *et al.*, 2007).

EGCG may cause DNA damage in combination with Cu (II) and Fe (III) complexes by producing hydroxyl radicals from hydrogen peroxide (Carlson *et al.*, 2007). In vitro studies have observed EGCG causing DNA damage to human esophageal squamous cell carcinoma cell lines KYSE 510 and 150, and in a leukemia cell line HL-60 (Carlson *et al.*, 2007). In vitro research on healthy human lymphocytes found that at low concentrations EGCG protects DNA from strand breakage, and at high concentrations EGCG promotes DNA strand breakage (Carlson *et al.*, 2007).

EGCG Antiangiogenesis

Cancer research relies on the biochemical differences between cancerous and healthy cells (Carlson *et al.*, 2007). These differences become the targets of research to find a way of affecting cancerous cells without causing adverse affects to healthy cells (Carlson *et al.*, 2007). Preventing the formation of blood vessels in cancer cells is an area of increased interest (Carlson *et al.*, 2007). Angiogenesis is the physiological process

where cancer cells produce growth factors that cause the endothelial cells of surrounding blood vessels to proliferate and develop into new vessels (Karp, 2005). In the initial stages of tumor growth all nutrients and oxygen can be obtained by diffusion, once the tumor reaches 0.5 mm in diameter the tumor needs to create blood vessels to supply the growing tumor with oxygen and nutrients (Carlson *et al.*, 2007). Angiogenesis is a natural process that is turned on or off by biochemical molecules (Carlson *et al.*, 2007). Cancer cells override these signals and cause endothelial cell recruitment and proliferation (Carlson *et al.*, 2007). EGCG is an antiangiogenic agent that can be used against tumor growth (Carlson *et al.*, 2007). Antiangiogenic agents inhibit additional vascularization of existing tumors but also prevent angiogenesis in early stages of tumor development (Carlson *et al.*, 2007).

Angiogenesis is a process that is common to all solid tumors (Carlson *et al.*, 2007). Endothelial cells rarely undergo mutagenesis, making it unlikely that they will develop multidrug resistance mechanisms which would render the treatment ineffective (Carlson *et al.*, 2007). Angiogenesis is not required in mature adults and therefore inhibition of this process should not interfere with normal physiological activities (Karp, 2005). For these reasons cancer therapies that target angiogenesis are promising and of extreme interest.

There are many methods by which EGCG affects angiogenesis. EGCG has been found to inhibit angiogenesis induced by angiogenin, a growth factor that activates angiogenesis (Carlson *et al.*, 2007). In vitro, it has been observed that EGCG inhibits the activity of two types of proteases that degrade the extracellular matrix allowing tumors to invade surrounding tissues for angiogenesis to progress (Carlson *et al.*, 2007). EGCG has

been found to inhibit ephrin-A1-mediated endothelial cell migration and tumor angiogenesis (Bhar and Singh, 2008). EGCG may also affect angiogenesis by elevating the levels of interleukin 12, a molecule that causes antiangiogenesis in mice (Carlson *et al.*, 2007). In humans epithelial cells it has been observed to suppress the production of interleukin 8, a molecule that promotes angiogenesis (Carlson *et al.*, 2007).

EGCG may also affect angiogenesis by its effects on proteins, transcription factors, and gene expression (Carlson *et al.*, 2007). Research on the effects of EGCG on genes has found that it may influence cell-signaling pathways and can up-regulate or "knock down" the transcription of angiogenesis activators (Carlson *et al.*, 2007). By targeting extracellular-regulated kinase function and vascular endothelial growth factor (VEGF) EGCG suppresses angiogenesis (Carlson *et al.*, 2007). By selective suppression of critical phosphorylation of VEGF-R1 and R2 receptors EGCG promotes apoptosis in human chronic lymphocytic leukemia cells (Carlson *et al.*, 2007).

EGCG Induced Apoptosis

It is well established that a major characteristic of cancer cells is their ability to evade apoptosis (Carlson *et al.*, 2007). p53 is an important regulatory protein in apoptosis, and plays a critical role in preventing tumor development (Karp, 2005). In most human cancers p53 functions incorrectly or is non-functional (Karp, 2005). Carcinogen-induced DNA damage, abnormal proliferative signals, hypoxia and loss of cell adhesion are the most common signals that activate p53 (Karp, 2005). Because of the variety of signals that p53 responds to it can be concluded that it is involved in several stages of the carcinogenic process (Karp, 2005). Some of the important functions of p53 are the inhibition of abnormal cell growth, triggering of programmed cell death,

DNA repair, inhibition of angiogenesis and senescence (Karp, 2005). Tea polyphenols have been shown to increase the levels of p53 and increase apoptosis (Carlson *et al.*, 2007).

EGCG can activate p53 by changing the redox potential of the cell (Carlson *et al.*, 2007). An in vitro study suggests that low concentrations of EGCG promote cell cycle arrest and high concentrations induce apoptosis (Carlson *et al.*, 2007). By activating the p53 and Fas-Fas ligand pathways in HepG2 cells, a human hepatocellular liver carcinoma cell line, EGCG causes cell-cycle arrest and apoptosis (Nishikawa *et al.*, 2006). In the hepatocellular carcinoma cell line HLE, EGCG did not cause cell-cycle arrest, but did induce apoptosis (Nishikawa *et al.*, 2006). This may be because the p53 protein in HLE cells is mutated and therefore dysfunctional but is not mutated in HepG2 cells (Nishikawa *et al.*, 2006). This research suggests that EGCG gene regulation may be responsible for the effects EGCG has on cell cycle regulation (Carlson *et al.*, 2007).

EGCG may increase the half-life of p53 by inducing phosphorylation of its serine residues (Carlson *et al.*, 2007). Increased transcriptional activity of p53 can affect target genes and their downstream protein products causing an increase in pro-apoptotic proteins, trigger G1 phase arrest and apoptosis (Carlson *et al.*, 2007).

EGCG induces apoptosis in B lymphoblastoid Ramos cells by eliminating the mitochondrial transmembrane potential and increasing the generation of reactive oxygen species (Noda *et al.*, 2007). It is possible that EGCG increases the generation of reactive oxygen species leading to mitochondrial dysfunction or that mitochondrial dysfunction is caused by EGCG treatment and that this causes increased generation of reactive oxygen

species (Noda *et al.*, 2007). Treatment of Ramos cells with EGCG and the chemotherapy drug etoposide resulted in synergistic apoptosis (Noda *et al.*, 2007).

EGCG may induce apoptosis by inhibiting the activity of fatty acid synthatase (FAS), a metabolic enzyme that is involved in lipid synthesis (Carlson *et al.*, 2007). FAS is overexpressed in several types of human cancers (Carlson *et al.*, 2007). It has been found that EGCG inhibits the growth of both healthy and cancerous cells, however it only induces apoptosis in tumor cells (Carlson *et al.*, 2007). This may be due to the accumulation of toxic precursors in the process of lipid synthesis (Carlson *et al.*, 2007).

EGCG exhibits cytotoxic effects on drug sensitive and drug resistant small-cell lung carcinoma (SCLC) by telomerase inhibition and subsequently apoptosis (Sadava *et al.*, 2007). EGCG caused increased activity of caspase 3 and caspase 9 which are important in the intrinsic pathway for apoptosis (Sadava *et al.*, 2007). EGCG also caused cell-cycle arrest in SCLC at the S phase which may be a result of the inhibition of telomerase (Sadava *et al.*, 2007).

The ability to induce apoptosis in cancerous cells and not in healthy cells has made EGCG an element of great interest for cancer therapy research (Carlson *et al.*, 2007).

EGCG Antimicrobial Activity

Green tea has been observed to display antibacterial activity (Carlson *et al.*, 2007). *Helicobacter pylori* is believed to be a risk factor for developing gastric carcinoma and along with additional antibiotic agents, green tea exhibits antibacterial activity against *H. pylori* in animal models (Carlson *et al.*, 2007).

Exposure Modification

One area in particular that green tea has been found to be extremely promising is by its protective effects against skin cancer caused by UV irradiation (Carlson *et al.*, 2007). Treating the skin with green tea polyphenols prior to UV-A or UV-B exposure prevents DNA damage (Carlson *et al.*, 2007). Topical and oral treatments with EGCG prevents UVB-induced skin tumor development in mice by; the induction of immunoregulatory cytokine interleukin 12, interleukin 12 dependent DNA repair following nucleotide excision repair mechanism, the inhibition of UV-induced immunosuppression through interleukin 12 dependent DNA repair, the inhibition of angiogenic factors, and the stimulation of cytotoxic T cells in a tumor microenvironment (Katiyar *et al.*, 2007). Oral treatment of green tea polyphenols was also observed to reduce UV-A radiation-induced oxidative DNA damage in mice (Carlson *et al.*, 2007).

EGCG treatment was also found to reduce the DNA damage and apoptosis in the oral cells of smokers (Carlson *et al.*, 2007). Consumption of green tea by smokers and nonsmokers has been associated with significantly reduced frequency of sister chromatid exchange, which often causes mutagenesis (Carlson *et al.*, 2007). A study investigating the harmful effects of the environmental pollutant pentachlorophenol (PCP) found that green tea reduced the development of hepatocellular and cholangiocellular tumors, and when tumors did develop their progression was decreased (Carlson *et al.*, 2007). EGCG may also accelerate the breakdown of the mutagenic heterocyclin amines, carcinogenic compounds produced from cooking meat and fish (Carlson *et al.*, 2007).

Epidemiological data

Much of the research that has been performed to associate drinking green tea to reduced risk of cancer has been conducted in Asia, as Asia has the highest green tea consumption rate (Carlson *et al.*, 2007). Research performed in China has indicated that there is a significantly reduced risk of esophageal cancer in woman associated with drinking green tea (Carlson *et al.*, 2007). The risk of gastric, lung, pancreas, rectal, and gallbladder cancers were reduced by 23-47% (Carlson *et al.*, 2007). It has also been found that green tea may have protective effects against breast, ovarian and prostate cancers (Carlson *et al.*, 2007). There was no association found between green tea consumption and decreased risk of lung cancer (Carlson *et al.*, 2007). Japanese studies have found that increased green tea consumption is associated with decreased risk of gastric and colorectal cancers (Carlson *et al.*, 2007). Another study in Japan found that drinking 10 Japanese cups (80-120 ml) of green tea a day delayed the average age of cancer onset in humans (Kuzuhara *et al.*, 2008). Increased consumption of green tea also delayed recurrence of human breast cancer (Kuzuhara *et al.*, 2008). Consumption of 10 Japanese-size cups of green tea, supplemented with green tea extract, prevented polyp formation after colorectal polypectomy in humans (Kuzuhara *et al.*, 2008).

Clinical studies

After oral administration of green tea 0.1% of the administered dose is absorbed into the gut (Carlson *et al.*, 2007). The maximum concentration of EGCG found in the plasma is after 1.4 to 2.4 hours from the consumption of tea solids, and 1.3 to 1.6 hours after consumption of green tea (Carlson *et al.*, 2007). Orally administered EGCG is excreted mainly in the feces and some is excreted in the urine (Carlson *et al.*, 2007).

Consuming green tea is safe, and a study has found that individuals with solid tumors can safely consume 1 g, or 900 ml, of green tea 3 times a day (Carlson *et al.*, 2007). Therefore green tea is a safe choice for cancer prevention and treatment (Carlson *et al.*, 2007).

Research has been performed that has found possible cancer preventing effects after a single dose of green tea (Carlson *et al.*, 2007). Prostaglandin E2 which may be involved in colorectal carcinogenesis was significantly reduced 4 hours after the ingestion of a single dose of green tea (Carlson *et al.*, 2007). Green tea derivatives were potentially effective in treating patients with premalignant conditions for cervical, prostate and hepatic malignancies without causing major toxicities (Carlson *et al.*, 2007). A study performed in China found no effect from treatment with decaffeinated green tea on esophageal squamous carcinomas (Carlson *et al*., 2007).

There are many benefits to EGCG over traditional cancer drugs. Drugs are expensive to produce in pure form and need to be administered intravenously or subcutaneously on a long term basis (Carlson *et al.*, 2007). EGCG is widely available, inexpensive and can be administered orally (Carlson *et al.*, 2007). Traditional cancer drugs will also often kill healthy cells at the same time as cancerous cells (Carlson *et al.*, 2007). EGCG appears to target biochemical and genetic features found only in cancerous cells (Carlson *et al.*, 2007). The binding of EGCG to DNA and RNA are reversible in a similar fashion as chemical chaperons (Kuzuhara *et al*., 2008). Many anticarcinogenic agents have toxic adverse effects, and EGCG has been found to be very safe (Carlson *et al.*, 2007).

Rationale for Experiment

Studies have shown that CLL cells over-express the antiapoptotic protein Bcl-2 (Pepper *et al.*, 1999), and fludarabine resistant CLL cells have increased levels of Mcl-1 which seems to have a large effect on the inhibition of apoptosis (Faria *et al.*, 2006). It has been suggested that high Bcl-2/Bax ratios may be indicative of a drug resistant phenotype in B-cell CLL cells, and that the levels of these proteins need to be modified in order to induce cell death (Pepper *et al.*, 1999). Therefore, if a treatment can be found that decreases the antiapoptotic members of the Bcl-2 family members, then cancer cells will be responsive to cellular signals for apoptosis (Henson *et al.*, 2006).

Research has already found that EGCG significantly increases apoptosis in chronic lymphocytic leukemia (CLL) B cells (Lee *et al.*, 2004). It was found that this increase in apoptosis was caused by EGCG suppression of Bcl-2 and Mcl-1 in CLL B-cells (Lee *et al.*, 2004). Treatment with Bcl-2 inhibitors has been observed to increase apoptosis in CLL cells and treatment in combination with chemotherapy is a promising area of study (Campàs *et al.*, 2006).

As EGCG decreases the levels of the antiapoptotic proteins Bcl-2 and Mcl-1 it may be able to amplify the apoptosis induced by fludarabine treatment. This study will look for this synergistic response.

Hypothesis

It was hypothesized that treatment of the Burkitt lymphoma-like cell line BJAB and the CLL-like cell line I-83 with fludarabine in combination with EGCG would result in a synergistic apoptotic effect.

Materials and Methods

Cell Culture

BJAB and I-83 cells lines were obtained from previously existing samples. These cell lines are free floating cells maintained in culture flasks. The cells were maintained in a humidified 5% CO_2, 37˚C incubator in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin.

Dose Curves

BJAB and I-83 cells were treated with 0 µM, 0.5µM, 1.25 µM, 2.5 µM, 5.0 µM, 10.0 µM, 15.0 µM and 20.0 µM doses of fludarabine and EGCG for 24 and 48 hours. The EGCG was purchased from Sigma-Aldrich. Approximately 1 x 10^6 cells were added to 8 wells of a 12 well dish and treated with the appropriate doses. The percentage of apoptotic cells was found by acridine orange/ethidium bromide staining. Fludarabine and EGCG dose curves were performed on BJAB cells 5 and 7 times respectively, and 5 times each for I-83 cells.

Time Trials

Based on the dose curves BJAB and I-83 cells were treated with 5 µM fludarabine and 20 µM EGCG. The trials were performed with no treatment, fludarabine, EGCG and combined treatment with EGCG and fludarabine. Approximately 1 x 10^6 cells were added to each well of a 12 well dish and given the appropriate treatment. Cells were treated for 24 and 48 hours and the percentage of apoptotic cells were found by acridine orange/ethidium bromide staining. The time trials were performed 4 times with BJAB cells and 3 times with I-83 cells.

Acridine Orange/Ethidium Bromide Staining

Cells were centrifuged for 5 minutes at 1200 rpm, and then placed on ice to prevent additional growth. The cells were resuspended in 100 µl of medium with 5 µl of a mixture of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide in phosphate buffered saline solution. 10 µl of this cell suspension was then applied to a microscope slide, covered with a coverslip, and viewed under a fluorescence microscope with a fluorescein filter. This staining method uses fluorescent dye that intercalates DNA (Jeon, 2002). Acridine orange stains DNA bright green allowing for visibility of the nuclear chromatin pattern, which allows for the observation of nuclear chromatin condensation which occurs during apoptosis (Jeon, 2002). Ethidium bromide stains DNA orange but is excluded from viable cells, staining only cells that have lost membrane integrity (Jeon, 2002). When viewing using a fluorescence microscope with a fluorescein filter, live and pre-apoptotic cells appear green and nonviable cells appear red. Random fields were scored until at least 200 cells had been counted and the percentage of apoptotic cells was calculated.

Western Blot Analysis

Protein lysates were prepared by centrifuging cells for 5 minutes at 1200 rpm. All medium was removed and the pellets were placed on ice. According to the size of the pellet a 50:1 ratio of NP-40:PMSF was added to the pellet and vortexed for 10 seconds. NP-40 is a detergent that lyses cells by solubilizing membrane proteins, and PMSF is a protease inhibitor used to prevent the degradation of proteins. The suspension was incubated on ice for 5 minutes, vortexed for 10 seconds, then incubated for an additional 5 minutes on ice. The suspension was then centrifuged for 5 minutes at 13000 rpm in a

cold room (4˚C) to remove additional cell debris. The lysates were kept in -80˚C until needed.

The protein concentration was determined by Bradford assay. Protein lysates were thawed on ice. Commercially available bovine serum albumin protein standards at 0.125, 0.25, 0.5, 0.75 and 1.0 µM concentrations, purchased from Bio-Rad Laboratories, mixed with 200 µl of Bradford reagent are used as standards. Samples were diluted in deionized water to 1:10 and 5 µl is mixed with 200 µl of Bradford reagent. The standards and samples were placed in a Titerlek Multiskan MCC/340 spectrophotometer and read at 620 λ. The absorbance values obtained from the spectrophotometer were entered into an excel spreadsheet. The standards were plotted as concentration (µM) vs. absorbance (Au) with y intercept = 0. The slope from the equation was used to calculate the concentrations of the samples by dividing the absorbance by the slope and multiplied by 10 due to the dilution. Then to calculate how much of each sample in µl was to be loaded so that even amounts of protein were added to each well, the desired protein concentration, 40 µg, was divided by the concentration.

These volumes of sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrotransferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% skim milk in tris buffered saline-Tween 20 (TBS-T) for one hour at room temperature with shaking. Nitrocellulose membranes have a high affinity for binding protein, therefore the membrane was blocked in 5% skim milk. The protein from the skim milk bound to the membrane so that when the membrane was treated with the antibody the membrane will not strongly bind the antibody in locations without caspase-3. Blots were performed with

a primary antibody, caspase-3 rabbit polyclonal IgG purchased from Santa Cruz Inc. The membrane was incubated in 1:100 primary antibody in 5% skim milk overnight at 4˚C with shaking. The membrane was washed in TBS-T three times for 10 minutes each at room temperature with shaking. TBS-T is a solution of pH stabilizers and salts that remove excess material without disrupting antigen-antibody binding. The membrane was incubated in 1.5 µl secondary antibody, goat-anti-rabbit IgG (H+L)-HRP conjugate purchased from Bio-Rad Laboratories, in 5 ml of 5% skim milk for one hour at room temperature with shaking. The membrane was washed with TBS-T three times for 10 minutes each at room temperature with shaking. Chemiluminescence was used to capture an image of the blot. ECL-plus western blotting detection system was added to the membrane and incubated at room temperature, in the dark, for one minute. The acridan substrate is converted to an acridinium ester intermediate when catalyzed by HRP causing the release of high levels of sustained output of light generating an intense chemifluorescent signal at 440 nm. The membrane was placed in a cassette and developed onto Amersham Hyperfilm^{HT} ECL high performance chemiluminescence film. The ester intermediate formed by HRP reacts with peroxide in alkaline conditions and emits light which is detected by the film.

The membrane was washed in TBS-T three times for 10 minutes at room temperature with shaking. The membrane was then stripped in 1:5 SDS reprobing buffer in TBS-T for 30 minutes at room temperature with shaking. This process disrupts the antigen/antibody interactions. The procedures described above were repeated to prepare a blot with the primary antibody rabbit-anti-actin IgG purchased from Sigma-Aldrich, and the same secondary antibody as above. The actin blot is to serve as a loading control.

Results

Dose curves were prepared for BJAB and I-83 cells for fludarabine and EGCG to see how the cells responded to various doses. The mean percent of apoptotic cells when BJAB cells were treated with 0-20 µM doses of fludarabine for 24 hours ranged from 8.69% to 22.3%, and from 6.07% to 47.1% after 48 hours (n=5) (Figure 1a). The mean percent of apoptotic cells when BJAB cells were treated with 0-20 µM doses of EGCG for 24 hours ranged from 8.21% to 14.4%, and from 8.96% to15.8% after 48 hours (n=7) (Figure 1b). The mean percent of apoptotic cells when I-83 cells were treated with 0-20 µM doses fludarabine for 24 hours ranged from 4.97% to 20.3%, and 8.07% to 42.6% after 48 hours (n=5) (Figure 2a). The mean percent of apoptotic cells when I-83 cells were treated with 0-20 µM doses of EGCG for 24 hours ranged from 5.73% to 10.8%, and from 6.75% to 9.36% after 48 hours (n=5) (Figure 2b). The percent apoptotic cells were higher after 48 hours for both cell types, especially with fludarabine treatment.

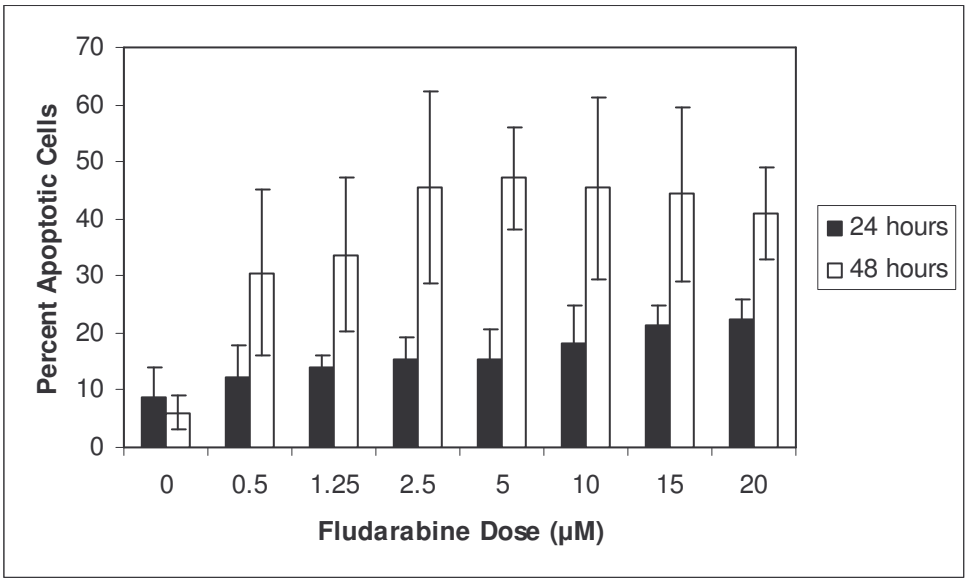
Based on the dose curves it was decided that the cells would be treated with 5 µM fludarabine and 20 µM EGCG for both BJAB and I-83 cells. This dose of fludarabine was chosen because it induced approximately 50% apoptosis. The highest dose of EGCG was used because there was no cell death observed with EGCG treatment. The mean background in the BJAB cells was 6.33% and 6.59% after 24 and 48 hours respectively (n=4). When BJAB cells were treated with fludarabine for 24 and 48 hours the mean percent of apoptotic cells ranged from 8.77% to 46.5%, EGCG failed to induce cell death, and combined treatment did not increase the percent of apoptotic cells (46.13%) (n=4) (Figure 3a). The background in the I-83 cells was 7.93% and 17.4% after 24 and 48 hours respectively (n=3). When the I-83 cells were treated with fludarabine for 24 and 48

hours the mean percent of apoptotic cells ranged from 17.7% to 34.2%, EGCG failed to induce cell death, and combined treatment did not significantly increase the percent of apoptotic cells (40.38%) (n=3) (Figure 3b).

The BJAB cells were more responsive to the 5μM dose of fludarabine after 48 hours than I-83 cells with mean percent of apoptotic cells at 46.5%, as appose to 34.2% in I-83. The backgrounds observed in the BJAB cells were consistent. The backgrounds in the I-83 cells increased greatly from 24 to 48 hours, and were observed to increase from the time when the dose curves were prepared to when the time trials were prepared.

As there was no synergistic effect observed when BJAB and I-83 cells were treated with fludarabine in combination with EGCG, western blots were performed to observe if treatment with fludarabine cleaved caspase-3. Visual observation of the blot shows that the levels of un-cleaved caspase-3 were the same in cells that were treated with no treatment, fludarabine, EGCG, and fludarabine with EGCG in I-83 cells after 24 and 48 hours (Figure 4). An actin blot was prepared to serve as a loading control to ensure that all wells were loaded with even amounts of protein. The loading control indicated that the wells were loaded with even amount of protein (Figure 4). The western blot was prepared twice finding the same results.

a)



b)

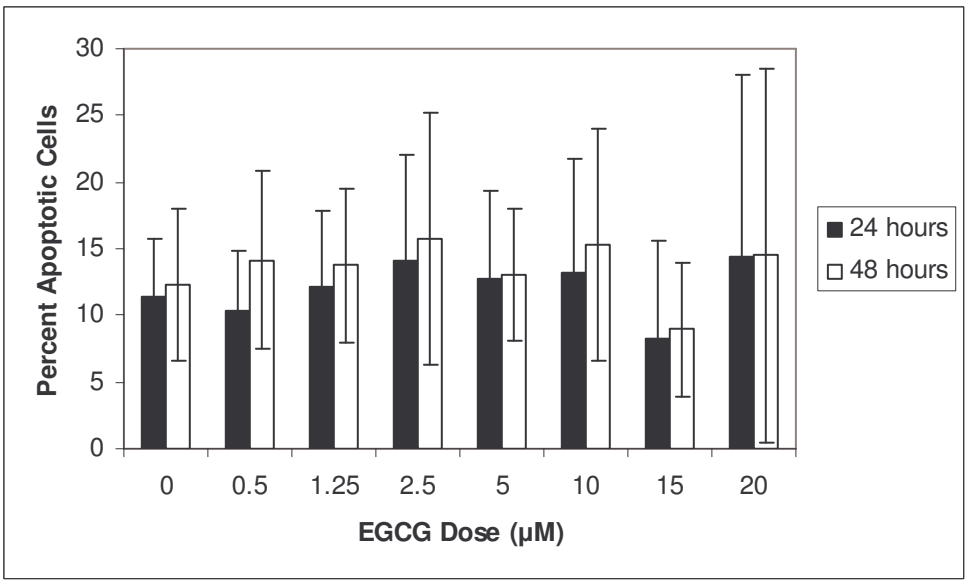
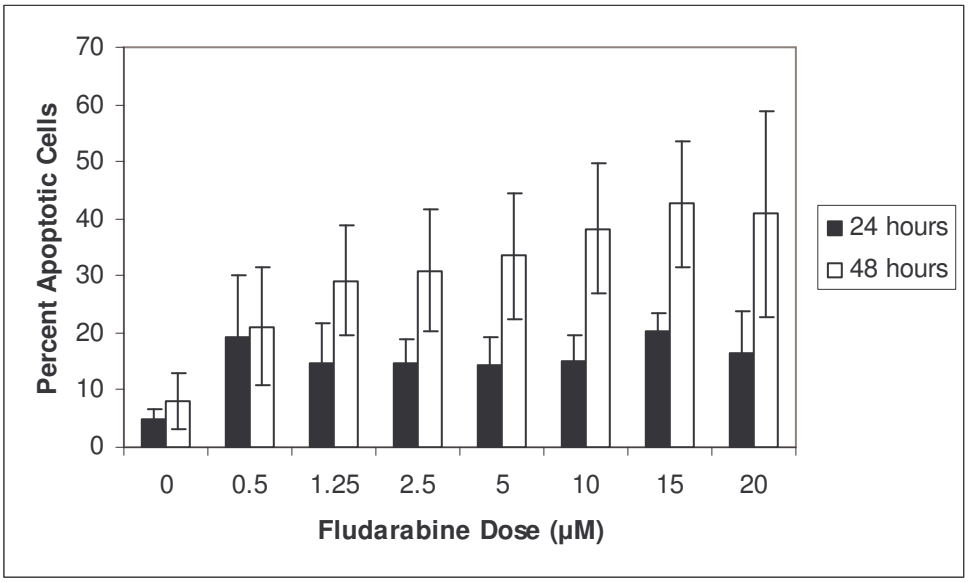


Figure 1: Dose curves prepared with BJAB cells treated with varying concentrations of a) fludarabine (n=5) and b) EGCG (n=7) that where incubated in a humidified 5% CO_2, 37˚C incubator for 24 or 48 hours. Cell death was assessed by acridine orange/ethidium bromide staining. The mean percentage of apoptotic cells is plotted with the error bars representing the standard deviation of the mean.

a)



b)

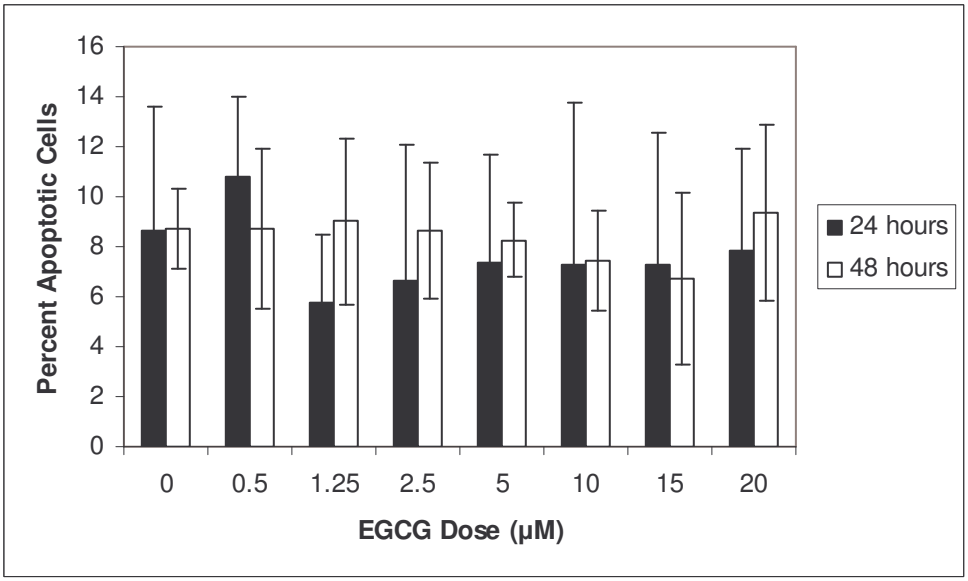
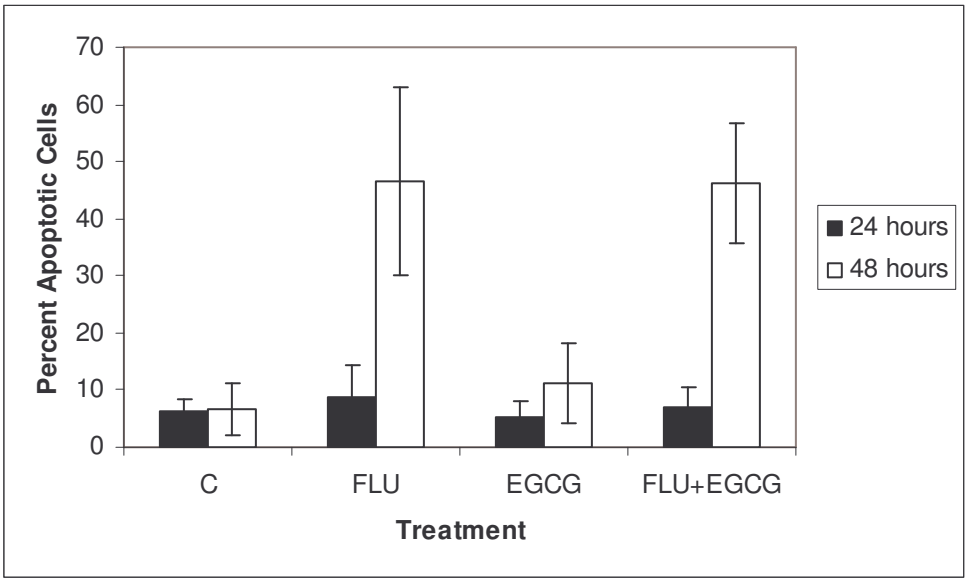


Figure 2: Dose curves prepared with I-83 cells treated with varying concentrations of a) fludarabine (n=5) and b) EGCG (n=5) that where incubated in a humidified 5% CO_2, 37˚C incubator for 24 or 48 hours. Cell death was assessed by acridine orange/ethidium bromide staining. The mean percentage of apoptotic cells is plotted with the error bars representing the standard deviation of the mean.

a)



b)

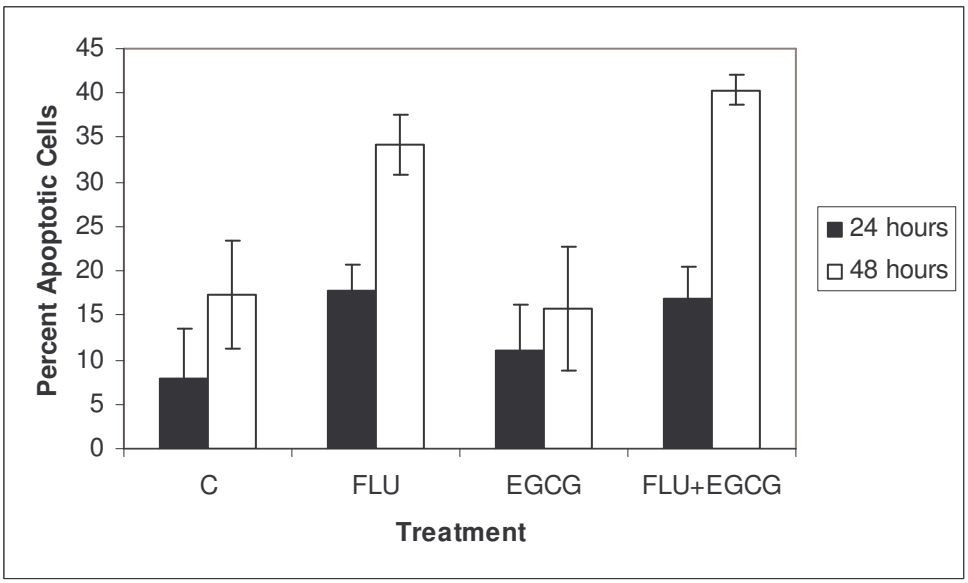


Figure 3: Time trials prepared with a) BJAB (n=4) and b) I-83 (n=3)cell lines that were treated with no treatment (C), 5 μM dose of fludarabine (FLU), 20 μM dose of EGCG (EGCG), and 5 μM dose of fludarabine and 20 μM dose of EGCG (FLU + EGCG) that where incubated in a humidified 5% CO_2, 37˚C incubator for 24 or 48 hours. Cell death was assessed by acridine orange/ethidium bromide staining. The mean percentage of apoptotic cells is plotted with the error bars representing the standard deviation of the mean.

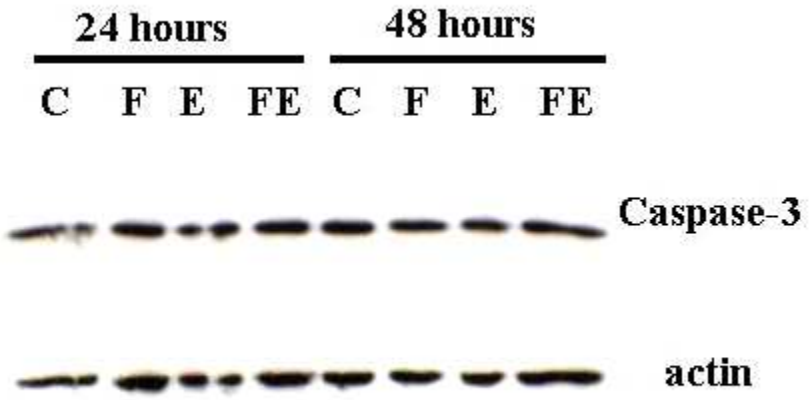


Figure 4: Results of the western blot prepared for caspase-3 in I-83 cells (n=2). It can be seen that there is no discernable difference in the caspase-3 bands for all treatments. There is also no difference in the actin bands indicating that even amounts of protein were added to each well. The samples were separated by SDS-PAGE (C=control, no treatment, F= 5 μM fludarabine, E= 20 μM EGCG, FE= 5 μM fludarabine and 20 μM EGCG), the primary antibody used was caspase-3 rabbit polyclonal IgG and the secondary antibody was goat-anti-rabbit IgG-HRP. The ECL-plus western blotting detection system was used to capture the image of the blot on Amersham Hyperfilm™ ECL high performance chemiluminescence film. The membrane was then stripped and an actin loading control was prepared with the primary antibody rabbit-anti-actin IgG, and the secondary antibody was goat-anti-rabbit IgG-HRP, and the membrane was developed as described as above.

Discussion

The results observed in this study were not expected. It is known that treatment with fludarabine cleaves caspase-3 (Yu *et al*., 2005), however the results found here indicate that the levels of un-cleaved caspase-3 were the same in cells treated with EGCG, fludarabine, and cells that were not treated. It is also known that EGCG induces apoptosis in CLL cells, however, this study did not observe EGCG induced cell death in the CLL-like cell line I-83.

This may be due to the fact that both BJAB and I-83 cell lines were contaminated with *Mycoplasma*. This was discovered after the research had been completed and there was no time available to repeat any of the tests performed. It was determined that this contamination was present in both cell lines prior to my arrival in the lab, therefore the cells were contaminated throughout this entire study.

The contamination was detected with the use of a MycoAlert *Mycoplasma* detection assay. The reagents in the detection kit cause the lysis of viable mycoplasma, then the enzymes from the *Mycoplasma* react with the mycoalert substrate catalyzing the conversion of ADP to ATP. The levels of ATP are then measured in samples from before and after the addition of the mycoalert substrate, and a ratio is obtained which is indicative of the presence or absence of mycoplasma. This test is sensitive to detect less than 50 colony forming units per milliliter of mycoplasma.

Most *Mycoplasma* contaminations come from human, bovine, and porcine sources (Cheng *et al.*, 2007). Mycoplasma contamination can occur by improper aseptic techniques, aerosols, and contaminated media ingredients, and spread rapidly through cultures within the lab as they get into the filters for the incubators and filter hoods.

Infection with *Mycoplasma* can cause the depletion of cell culture nutrients, alteration of cell growth characteristics, inhibition of cell metabolism, disruption of nucleic acid synthesis, production of chromosomal aberrations, changes to antigenicity of cell membranes and interference with virus replication (Cheng *et al.*, 2007).

More relevant to this study, and a possible reason for not observing caspase-3 cleavage by fludarabine, *Mycoplasma* can induce and enhance apoptosis in host cells (Sokolova *et al*., 1998). For this reason it has been suggested that *Mycoplasma* infections may present a serious problems when investigating apoptosis in cell cultures (Sokolova *et al.*, 1998). Many studies have found that *Mycoplasma* induces apoptosis in host cells, however the mechanisms by which this occurs are not clear (Sokolova, *et al*., 1998). *Mycoplasma* may induce apoptosis by providing the cell with enzymes that mimic components of the host cells apoptotic pathway, or by causing alterations within the interleukin network (Sokolova *et al.*, 1998).

Many of the dose and time trials prepared here were found to have a high background of apoptosis, the I-83 cells more so than the BJAB cells. It has been suggested that increased background levels of apoptosis may be due to spontaneous apoptosis caused by the infection (Sokolova *et al.,* 1998). Increased background levels may be caused by the involvement of mycoplasmal endonucleases in the host cells apoptotic pathways (Sokolova *et al.*, 1998). Ca/Mg-dependent endonuclease, DNase I and caspase-activated nucleases CAD and CPAN are metallodependent endonucleases that are ubiquitous and found in a variety of organisms (Sokolova *et al.*, 1998). *Mycoplasma* species produce similar enzymes that are active at neutral pH, are activated by Ca^{2+} and /or Mg^{2+} cations, inhibited by Zn^{2+}, and cleave double stranded DNA with of

5'P and 3'OH termini (Sokolova *et al*., 1998). *Mycoplasma* endonucleases are also capable of producing DNA laddering, an indication of apoptosis, suggesting that they preferentially recognize internucleosomal regions in chromatin (Sokolova *et al*., 1998). All of these features closely resemble the features of endogenous eukaryotic endonucleases involved in apoptotic DNA fragmentation (Sokolova *et al.*, 1998). A main difference is that *Mycoplasma* endonucleases produce more double-strand breaks, whereas eukaryotic enzymes usually cause single-strand breaks in DNA molecules (Sokolova *et al.*, 1998).

Based on this research the results observed in this study may have been due to the majority of the cells in culture having been killed by the contamination of *Mycoplasma*, and the cells that survived were more resistant to apoptosis. Cell lines change over time and may become more resistant to apoptosis, therefore another possibility is that the cell lines were passaged for too long in the lab. Cleavage of caspase-3 may not have been observed as the background cell death was so high that there was already a significant amount of caspase-3 cleaved in the untreated cells, causing the levels of un-cleaved caspase-3 to appear the same in all cells. The lack of cleavage of caspase-3 indicates that apoptosis was not occurring in the I-83 cells tested. The dose curves and time trials were prepared with BJAB cells first, then the I-83 cells were tested. The fludarabine in the lab was running low and had been in the lab for some time, therefore it may be possible that at some time during the four months between when the BJAB dose curves and the I-83 lysates were prepared the fludarabine may have become inactive. Another possibility is that, in this case, the 24 and 48 hour trials were not long enough for the cleavage of caspase-3 to occur.

In BJAB cells fludarabine induced good levels of cell death and the EGCG induced none, therefore there may have been a problem with the EGCG used. The EGCG used was purchased from Sigma-Aldrich and prepared just prior to use, therefore it is unlikely it had time to become inactivated in storage. It may have been beneficial to test the EGCG to observe if it was viable by performing an EGCG control. EGCG is a known inhibitor of the enzyme urokinase, a proteolytic enzyme used by human cancers to invade cells (Jankun *et al*., 1997). EGCG inhibits urokinase by binding in such a way that it blocks the His-57 and Ser-195 of the catalytic triad, interfering with its ability to bind substrate (Jankun *et al.*, 1997). The binding of EGCG to urokinase can be monitored with spectrophotometric analysis by observing the appearance of the spectrozyme cleavage product, para-nitroanilide, absorbs strongly at 405 nm (Jankun *et al*., 1997). If the EGCG is actively binding, and therefore inhibiting urokinase, there would be a decrease in product produced over time (Jankun *et al*., 1997). This method could be used to see if the EGCG in the lab is active and able to bind urokinase.

Based on the results observed here it may be concluded that fludarabine and EGCG do not cause synergistic apoptosis in BJAB and I83 cell lines. Due to the contamination and the possible inactivity of the EGCG used, it may be worth while to reinvestigate the effects of EGCG, as well as the combination of EGCG and fludarabine, on these cell lines.

Conclusions

1. The EGCG used here was ineffective at inducing apoptosis in BJAB and I83 cell lines.
2. Combined treatment with fludarabine and EGCG does not cause a synergistic apoptotic response in BJAB and I-83 cell lines.
3. Due to the contamination, and possible inactivity of the EGCG used, it may be worth while to reinvestigate the effects of EGCG, as well as the combination of EGCG and fludarabine, on BJAB and I-83 cell lines.

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Appendix

Table 1: The percentage of apoptotic cells observed after treatment of BJAB cells with varying doses of fludarabine after 24 hours.

Dose (μM)	Percent Apoptotic Cells					Mean	Std Dev
0	4.19	4	6.74	15.96	12.56	8.69	5.34
0.5	4.42	16	17.5	15.2	8.57	12.34	5.59
1.25	13.4	15.09	10.12	15.61	15.38	13.92	2.29
2.5	14.96	21.82	15.59	13	11.32	15.34	3.99
5	14.22	23.11	16.05	15.35	7.62	15.27	5.52
10	8.57	16.02	24.06	24.75	18.26	18.33	6.61
15	\	\	22.65	24.12	17.26	21.34	3.61
20	\	\	25	18.09	23.94	22.34	3.72

Table 2: The percentage of apoptotic cells observed after treatment of BJAB cells with varying doses of fludarabine after 48 hours.

Dose (μM)	Percent Apoptotic Cells					Mean	Std Dev
0	7.5	5.16	8.41	7.87	1.4	6.07	2.89
0.5	22.77	16.81	42.86	49.32	21	30.55	14.53
1.25	41.06	14.5	48.53	38.92	25.51	33.70	13.57
2.5	69	45.62	36.17	52.88	24.66	45.67	16.79
5	58.6	51.44	40.36	48.82	36.2	47.08	8.92
10	63.24	46.44	44.7	52	20.28	45.33	15.77
15	\	\	49.07	56.65	27.27	44.33	15.25
20	\	\	48.32	42.5	32.38	41.07	8.07

Table 3: The percentage of apoptotic cells observed after treatment of BJAB cells with varying doses of EGCG after 24 hours.

Dose (μM)	Percent Apoptotic Cells							Mean	Std Dev
0	3.73	18.14	14.15	9.8	10.48	10.05	12.94	11.33	4.46
0.5	11.5	16.96	2.89	13.43	10.28	8.05	9.54	10.38	4.40
1.25	6.9	21.4	17.7	11.79	5.39	11.16	10.29	12.09	5.69
2.5	6.7	9.09	14.28	15.35	30.66	13.27	9.2	14.08	7.97
5	6	15.14	7.73	19.91	22.17	5.66	12.56	12.74	6.67
10	3.88	15.67	8.42	23.47	11.59	25	4.67	13.24	8.52
15	8.06	4.76	1.72	24.27	7.41	6.28	4.98	8.21	7.38
20	8.82	11.5	45.37	8.87	8.5	9.9	8	14.42	13.69

Table 4: The percentage of apoptotic cells observed after treatment of BJAB cells with varying doses of EGCG after 48 hours.

Dose (μM)	Percent Apoptotic Cells						Mean	Std Dev
0	12.15	14.85	15.31	12.87	13.57	5.08	12.31	5.77
0.5	22.06	15.61	12.45	9.66	15.42	9.61	14.14	6.70
1.25	15.46	18.41	12.25	8.64	16.22	11.54	13.75	5.74
2.5	19	20.4	29.54	6.34	10.57	8.91	15.79	9.45
5	15.35	13.94	19.51	7.8	9.13	12.55	13.05	4.94
10	4.94	29.58	22.71	10.29	8.82	15.62	15.33	8.73
15	11.39	10.73	16.75	6.4	4	4.44	8.96	5.04
20	15.92	44.35	9.5	6.44	3.98	6.86	14.51	14.01

Table 5: The percentage of apoptotic cells observed after treatment of BJAB cells with 5μM fludarabine and 20μM EGCG after 24 hours.

Treatment	Percent Apoptotic Cells							Mean	Std Dev
C	8.08	5.55	8.78	4.5	6.25	7.93	3.23	6.33	2.05
FLU	8.07	4.95	2.44	5.96	7.08	16.42	16.42	8.76	5.52
EGCG	1.3	8.17	4.31	8.56	3.43	3.52	6.83	5.16	2.73
FLU+EGCG	5.63	2.3	3.37	10.5	7.67	11.82	6.83	6.87	3.49

Table 6: The percentage of apoptotic cells observed after treatment of BJAB cells with 5μM fludarabine and 20μM EGCG after 48 hours.

Treatment	Percent Apoptotic Cells				Mean	Std Dev
C	4.98	13.22	3.62	4.53	6.5875	4.457708
FLU	32.5	42.74	40.54	70.3	46.52	16.45294
EGCG	1.91	15.38	9.35	17.7	11.085	7.056819
FLU+EGCG	34.28	53.27	40.59	56.36	46.125	10.43626

Table 7: The percentage of apoptotic cells observed after treatment of I-83 cells with varying doses of fludarabine after 24 hours.

Dose (μM)	Percent Apoptotic Cells				Mean	Std Dev
0	5.4	7.39	3.67	3.43	4.97	1.83
0.5	33.48	21.03	7.44	14.43	19.10	11.08
1.25	18.45	22.17	11.35	7.08	14.76	6.81
2.5	18.69	17.54	13.23	9.45	14.73	4.23
5	21.43	13.73	10	11.79	14.24	5.03
10	15.91	15.22	9.45	20.1	15.17	4.38
15	16.54	23.83	20	21	20.34	3.01
20	14.15	25.12	18.63	7.34	16.31	7.49

Table 8: The percentage of apoptotic cells observed after treatment of I-83 cells with varying doses of fludarabine after 48 hours.

Dose (μM)	Percent Apoptotic Cells					Mean	Std Dev
0	13.02	6.39	13.3	3.45	4.21	8.07	4.77
0.5	5	29.73	28.97	25	16.67	21.07	10.37
1.25	43.4	29.54	32.3	19.53	21	29.15	9.64
2.5	42.2	32.92	38.64	15.97	24.55	30.86	10.66
5	45.7	34.11	41.82	17.98	28.36	33.59	11.02
10	57.27	35.78	39.91	30	28.64	38.32	11.52
15	58.17	43.11	46.12	36.5	28.99	42.58	10.92
20	62	44.17	48.72	35.64	13.47	40.80	18.01

Table 9: The percentage of apoptotic cells observed after treatment of I-83 cells with varying doses of EGCG after 24 hours.

Dose (μM)	Percent Apoptotic Cells					Mean	Std Dev
0	7.8	14.83	12.08	2.25	6.38	8.67	4.92
0.5	12.2	13.14	13.06	5.5	10.05	10.79	3.21
1.25	4.57	9.85	7.14	3.83	3.28	5.73	2.73
2.5	6.57	15.84	5.31	2.15	3.23	6.62	5.44
5	5.74	13.82	9.5	3.5	4.23	7.36	4.29
10	5	18.8	4.23	3.38	4.98	7.28	6.48
15	5.98	16.59	5.8	3.9	3.98	7.25	5.31
20	7.42	14.81	6.9	4.76	5.29	7.84	4.05

Table 10: The percentage of apoptotic cells observed after treatment of I-83 cells with varying doses of EGCG after 48 hours.

Dose (μM)	Percent Apoptotic Cells					Mean	Std Dev
0	10.75	7.35	9.86	8.44	7.07	8.69	1.59
0.5	8.74	5.69	13.33	5.8	9.95	8.70	3.18
1.25	8.37	6.31	14.62	9	6.85	9.03	3.31
2.5	8.5	4.83	12.5	8.8	8.5	8.63	2.71
5	6.9	8.51	10.14	6.67	9.05	8.25	1.47
10	4.5	8.21	9.76	6.5	8.23	7.44	2.01
15	3.78	3.98	12.22	7.62	6.13	6.75	3.45
20	7.36	6.51	15.42	8.99	8.53	9.36	3.52

Table 11: The percentage of apoptotic cells observed after treatment of I-83 cells with 5μM fludarabine and 20μM EGCG after 24 hours.

Treatment	Percent Apoptotic Cells					Mean	Std Dev
Control	2.87	3.78	16.33	5.69	11	7.93	5.65
FLU	15.67	15.24	18.93	16.03	22.5	17.67	3.06
EGCG	3.98	16.53	11.45	7.69	15.5	11.03	5.27
FLU+EGCG	14.56	12.32	20.09	17	20.75	16.94	3.59

Table 12: The percentage of apoptotic cells observed after treatment of I-83 cells with 5μM fludarabine and 20μM EGCG after 48 hours.

Treatment	Percent Apoptotic Cells			Mean	Std Dev
C	22.52	10.73	18.91	17.39	6.04
FLU	38.09	32.93	31.53	34.18	3.45
EGCG	19.25	7.65	20.28	15.73	7.01
FLU+EGCG	41.74	40.85	38.55	40.38	1.65