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Synergistic Interactions of Chlorambucil, DHA, and Trail in Jurkat and H460 Human Cancer Cells

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SYNERGISTIC INTERACTIONS OF CHLORAMBUCIL, DHA, AND TRAIL IN JURKAT AND H460 HUMAN CANCER CELLS

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by

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ABSTRACT

Synergistic Interactions of Chlorambucil, DHA, and TRAIL in Jurkat and H460 Human Cancer Cells
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Traditional chemotherapeutic drugs are prone to toxicity and may result in secondary cancers. In recent years much attention has been garnered by alternate methods of cancer treatment with fewer side effects, including immunotherapy and administration of ω-3 fatty acids, both of which have been shown to cause apoptosis in cancer cells. Docosahexaenoic acid (DHA), a fatty acid, is a normal component of cell membranes and is safe for systemic administration. The cytokine TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is able to induce apoptosis in cancer cells while sparing normal tissue. In this study, the alkylating agent chlorambucil (CLB) was combined with DHA and TRAIL in order to assess their ability to induce apoptosis synergistically in Jurkat human T-cell leukemia and NCI-H460 human non-small cell lung cancer cell lines. Dose response curves were created by treating each cell line with each agent individually and analyzing by a colorimetric cell proliferation assay. Sublethal concentrations of each agent were combined to determine the nature of their interaction, be it additive, sub-additive, or synergistic. Selected sublethal doses of each agent were combined and further analyzed by flow cytometry using Annexin V-FITC, a fluorescently-labeled molecule that binds to cells undergoing apoptosis. Jurkat was more sensitive than H460 to every combination tested, as well as to every individual agent except TRAIL. CLB combined with DHA proved synergistic in both cell lines by XTT. DHA is believed to increase fluidity of the membrane allowing CLB to diffuse more easily into the cell. CLB combined with TRAIL also was synergistic in both cell lines, but to a different degree perhaps due to TRAIL's ability to use different pathways in each, either receptor-mediated in Jurkat or mitochondrial in H460. By XTT, TRAIL and DHA combined in an additive manner at best. Flow cytometry indicated that in both cell lines DHA and TRAIL inhibited each other, more strongly in H460 than in Jurkat. The combination of all three agents resulted in synergy in both cell lines by XTT, with only 20% of Jurkat cells surviving, but appeared sub-additive by flow cytometry by the Annexin V-FITC assay, which is specific for apoptotic cells and does not stain those that have died by necrosis. The actual percentage of cell death may be higher than indicated by this analysis.

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Objective The aim of this study is to determine the ability of an omega-3 fatty acid, docosahexaenoic acid (DHA), to synergize with the alkylating anti-cancer drug chlorambucil (CLB). The capability of either or both of these agents to achieve synergy upon combination with TRAIL, a cytokine of the tumor necrosis factor family, will also be evaluated. Each treatment will be tested alone and in combination on Jurkat human T-cell leukemia and H460 human non-small cell lung cancer.

INTRODUCTION

Since the first use of poisonous nitrogen mustard gas derivatives as a cancer treatment in 1946, the field of chemotherapy has undergone vast and rapid growth. Advances in oncology have increased lifespan and improved quality of life for people afflicted with every variety of cancer, and has resulted in complete cures in many cases. Common varieties of cancer including leukemia and lymphoma, diseases that were always fatal in the past, now are routinely cured in many patients.

Early anti-cancer drugs such as the nitrogen mustard derivatives, while effective at shrinking tumors and treating leukemias, cause serious side effects including damage to the digestive system and bone marrow. Despite the remarkable progress in cancer treatment, the majority of modern chemotherapeutic agents still cause unpleasant, sometimes serious or even fatal, side effects. Nausea, vomiting, hair loss, skin rashes, digestive problems, hepatotoxicity, and bone marrow suppression are common occurrences during chemotherapy. The most egregious effect of the toxicity associated with some chemotherapeutic drugs is the onset of a secondary form of cancer resulting from damage to DNA caused by the treatment itself. This occurs with relative frequency in leukemias treated with alkylating drugs of the original class of nitrogen mustard

derivatives. For instance, treatment of chronic lymphocytic leukemia (CLL) with a drug designed to alkylate and cross-link genetic material, such as chlorambucil, may with long-term use cause damage to DNA that results in a far more serious form of cancer known as acute myelogenous leukemia (AML) (14).

Due to the obvious need to avoid side effects such as these, efforts to utilize other, less toxic means of treatment have become a primary concern. One method that shows great promise is the concept of immunotherapy, in which the body's own immune system is stimulated to fight cancer growth. This can be achieved in vitro by treating cancer cells with natural agents of immunity such as tumor necrosis factor-alpha (TNFα), interferon, Fas ligand (FasL), or the more recently discovered tumor necrosis factor-related apoptosis-inducing ligand, or TRAIL. While many cytokines such as these are very effective in vitro, they cannot be administered systemically due to the toxicity of high dosages of these substances. TNFα for instance is an inflammatory cytokine that results in fever and inflammation that make it unusable as a systemic treatment. FasL also displays toxicity when administered systemically (39).

TRAIL, however, has great potential for systemic use, as it works in a manner very similar to FasL but targets only cancer cells while remaining wholly nontoxic to normal cells (18, 36, 37). While some studies have indicated widespread hepatotoxicity caused by TRAIL (25), others demonstrate that this damage occurs only with use of a few varieties of the ligand that have been tagged or otherwise altered for research use, and illustrate that the native form of the molecule does not cause any liver damage in healthy individuals (23, 34). The only known exception is in subjects who are compromised by viral infection in which administration of TRAIL may cause liver damage (42). TRAIL

has also been shown to inhibit formation of metastases (37). Due to its apparent ability to effectively target cancer cells while sparing normal tissue, TRAIL is considered an excellent candidate for systemic treatment. TRAIL has also been proven to upregulate cyclooxygenase 1 in normal blood cells, resulting in an increase of prostaglandin secretion, indicating that the cytokine may have a more far-reaching impact into normal blood cell physiology. It has been shown to increase the lifespan of normal myeloid cells (31).

Another avenue of non-drug treatments currently being explored is the ability of ω-3 fatty acids to prevent and/or treat cancer. It has long been observed that people who consume a diet high in ω-3 fatty acids, found in fish and other marine sources, were less likely to develop some types of cancer (8, 32). Research into this phenomenon has determined that the same resistance is not conferred by ω-6 fatty acids, and that the overall length as well as the level of unsaturation of the fatty acid is positively correlated with its ability to induce apoptosis in cancer cells (8).

One of the most exciting possibilities to arise from research involving cytokines and fatty acids is the potential to combine these agents with established anti-cancer drugs in a synergistic fashion. Since a nontoxic cytokine such as TRAIL, or a fatty acid, has the ability to induce cancer cell death without damaging normal cells, it may be possible that combining one of these compounds with a drug will allow a smaller dose of the potentially toxic drug to be delivered while achieving the same benefit. In some cases, these interactions appear to be synergistic rather than merely additive. For instance, TRAIL has been shown to synergize with topoisomerase inhibitors including doxorubicin, also known as adriamycin (2, 26, 28, 40), and cisplatin (23). Adriamycin

and TRAIL together have in fact been shown to induce cell death effectively in a multiple myeloma line that is resistant to both treatments individually (17). TRAIL did not, however, show any evidence of synergistic interaction with the alkylating agent mitomycin (23). The ability to induce synergy seems to be at least in part a function of the stage of the cell cycle in which each component is most effective. The combination of agents that are active in the same stage of the cell cycle can reasonably be expected to interact in a positive manner.

The Alkylating Agents Despite the failure of mitomycin to synergize with TRAIL, the alkylating agents are still of great interest for potential interaction with cytokines or with fatty acids. The alkylating agents are the oldest class of chemotherapeutic drugs and work by alkylating DNA at the 7-N arginine. They have two reactive side-chains that are able to bind two nucleophilic groups simultaneously and cause them to cross-link, effectively disrupting nucleic acid function (1). The alkylating agents are considered to be non-phase specific, meaning they are active in all stages of the cell cycle. However, most are more effective later in the cell cycle, as cells exposed in early G_1 have longer to repair damaged DNA before synthesis begins (1). Drugs of this category are believed to be most effective in G_2 (21), and act to impair progression from this stage to mitosis (22).

The mechanism of action of the alkylating agents is such that they are intrinsically prone to causing potentially serious side effects. While bone marrow depression is the dose-limiting factor of most, it is temporary. The alkylating agents act on genetic material to substitute an alkyl group or a hydrogen ion for a covalent linkage. This alteration is quite cytotoxic, but with extended use may also become mutagenic (22).

While these drugs are valuable and effective tools in the treatment of cancer, they are also very dangerous and can produce side effects that are intolerable or even fatal. Because of this potential for systemic toxicity, these drugs could be made much more tolerable by reducing the overall effective dosage through synergistic combination.

Perhaps the most logical candidate to be assessed for synergism among the alkylating agents is chlorambucil (fig. 1). Chlorambucil, or CLB, is one of the original nitrogen mustard derivatives, and is routinely used to treat CLL. It is very effective in treating this disease; approximately 70% of patients show a significant response to treatment with CLB (14). It is however extremely toxic and therefore cannot be tolerated for long-term use or at very high doses. It commonly causes nausea and vomiting, may result in hepatoxicity, and in the long-term treatment that may be necessary in CLL, may result in secondary AML (30). The dose-limiting factor is bone marrow depression that can result in serious suppression of the immune system (28). Despite these undesirable effects, CLB is remarkably the mildest, least toxic, and slowest acting of the nitrogen mustards (22).

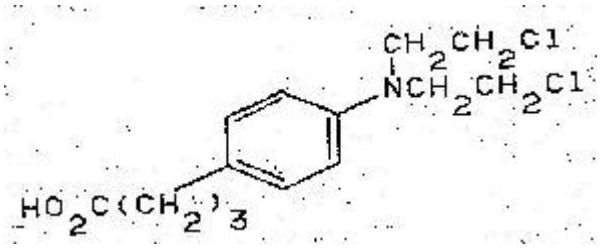


Figure 1. Molecular structure of chlorambucil (CLB). CLB enters the cells by simple diffusion (6).

Chlorambucil is administered orally and is absorbed completely from the gastrointestinal tract. It then binds to plasma proteins, primarily albumin (5, 22), and reaches peak plasma concentration in about an hour (22). The drug undergoes

biotransformation in the liver and is converted to a 1^{o} metabolite (5) that is itself as active a cytotoxic compound as CLB (22). The active metabolite reaches peak plasma concentration 2 to 4 hours after ingestion (5). The drug enters cells by simple diffusion (14) and proceeds to alkylate and cross-link DNA as previously described.

During the course of treatment, drugs of the nitrogen mustard family can induce a non-proliferative G_0 phase. These cells may undergo nuclear and cytoplasmic hypertrophy, but no further mitosis occurs (22). It may be possible to exploit this cytostatic effect by attacking these non-mitotic cells with a second anti-cancer agent that is active in the G_0 phase.

Omega-3 Fatty Acids The use of fatty acids as cancer treatment has shown great potential. It has long been known that individuals who eat a diet rich in ω–3 fatty acids are less susceptible to cancer than people who consume smaller amounts. The ω–3 fatty acids are an excellent option for systemic use because they are essential nutrients and natural structural components of cell membranes (32), thus they cause no ill effects upon administration. The effectiveness of fatty acids in inducing apoptosis in cancer cells is a function of the length and level of unsaturation of the molecule. Thus, a logical choice for use in cancer treatment is the very long chain polyunsaturated docosahexaenoic acid, or DHA (fig. 2). DHA is a 22-carbon molecule containing six double bonds that is capable of being incorporated into the cell membrane in high concentrations resulting in micelle formation and loss of membrane integrity (8).

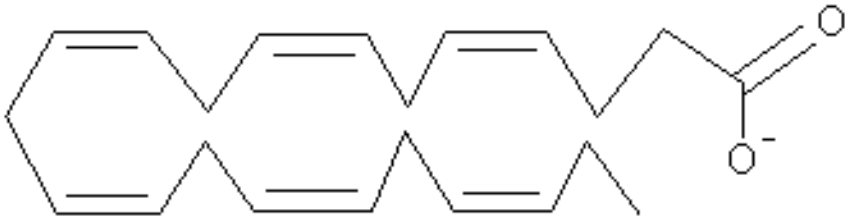


Figure 2. Molecular structure of DHA (3).

Once inside the cell, fatty acids can be incorporated into the mitochondrial membrane. Once there, they may be oxidized by reactive oxygen species, or ROS, that are liberated during mitochondrial respiratory burst. This oxidation ultimately results in a mitochondrial permeability transition state, which involves opening of large pores in the inner mitochondrial membrane (35). This results in depolarization and loss of membrane potential and leads to the release of cytochrome c (cyt c), which binds Apaf-1 and activates the caspase cascade of the mitochondrial pathway resulting in apoptosis (20, 26). The involvement of oxidative stress in fatty acid-induced apoptosis is underscored by evidence that this apoptosis can be at least partly prevented by administration of anti-oxidants, including tocopherol (20). There is some precedence for the concept of apoptosis induced by fatty acids found within the body itself. Direct production of fatty acids has been observed by target cells as part of the cell lysis procedure (29).

Siddiqui et al (32) in 2001 derived the method of apoptosis in Jurkat cells as caused by fatty acids. Jurkat cells contain protein phosphatase 1, or PP1, which stimulates release of cyt c and results in activation of the caspase cascade. PP1 is activated by ceramide, in a process that can be inhibited by phosphatidic acid, or PA,

which is also present within the cells. DHA has been shown in Jurkat cells to reduce the level of PA and thus prevent its inhibitory effect on PP1 (32).

Fatty acids also have great impact on the immune system, as they are components of prostaglandins, leukotrienes, and ceramides, and are involved in proliferation of leukocytes and cytokine production (20).

TRAIL The previously mentioned ability of TRAIL to synergize with many drugs, and its capacity to induce apoptosis in cancer cells while leaving normal cells unharmed, makes it a very useful tool with great potential for systemic treatment. It is stored in compartments similar to lysosomes in the cytoplasm of many cells, including T-cells, and may function to kill activated T-cells in order to prevent autoimmunity (24). TRAIL is unique among cytokines because it has multiple receptors. The ligand is able to interact with two signal-transducing receptors known as death receptors 4 and 5, or DR4 and DR5. These receptors are able to initiate a caspase cascade that ultimately leads to apoptosis of the cell (36). They are classified as type-1 transmembrane receptors, containing a cytoplasmic amino-terminus and an extracellular carboxy-terminus with two cysteine-rich domains (2). DR4 and DR5 are essentially homologous, and work in precisely the same manner and can be found in similar tissues. DR4 is expressed in most tissues, including the spleen, thymus, peripheral blood leukocytes, activated T cells, some tumor cell lines (13), and in the brain (4). DR5 is similarly expressed at high levels in the spleen and in peripheral blood leukocytes, and transcripts are present in both normal and cancer cell lines (13). Expression of these receptors can be upregulated by treatment with some chemotherapeutic drugs that cause damage to DNA (38).

The functional portion of the death receptors is the 80-amino acid cytoplasmic death domain (38). Upon binding of TRAIL, the receptors trimerize and recruit pro-caspase 8 to the death domain to form a death-inducing signaling complex, or DISC. DISC then recruits the Fas-associated death domain protein (FADD), a protein also involved in apoptosis induced by the Fas/FasL system (30). Pro-caspase 8 auto-activates to form the active caspase 8, which in turn activates the downstream caspases 3, 6, or 7 (36). Finally, NFkB is activated and enters the nucleus to activate transcription of genes associated with apoptosis (15) (fig. 3). Apoptosis however is not the only possible outcome of TRAIL-mediated cell death. In the event that receptor-interacting protein, or RIP, is recruited to DISC, the receptor pathway will alternately induce necrosis (34, 42) (fig. 4).

TRAIL may also bind to a second set of receptors known as the decoy receptors, DcR1 and DcR2. These decoy receptors have an extracellular structure identical to that of the active death receptors (13), but differ in the cytoplasmic regions. In DcR2, the death domain is truncated, while in DcR1 it is absent (36). DcR1 is found in some normal tissues, but not in most cancer cell lines. DcR2 occurs in the fetal liver and adult testis, but rarely is seen in cancer cell lines (13). The presence of these decoy receptors may be the mechanism by which normal cells are able to avoid damage from TRAIL (fig. 4).

In 1998, it was discovered that TRAIL could also bind to a fifth receptor, osteoprotegrin, or OPG. OPG is a secreted molecule that is a structural homolog to the TNF receptor family and functions to inhibit osteoclastogenesis and increase bone density. Binding of TRAIL blocks the anti-osteoclastogenic function of OPG and could

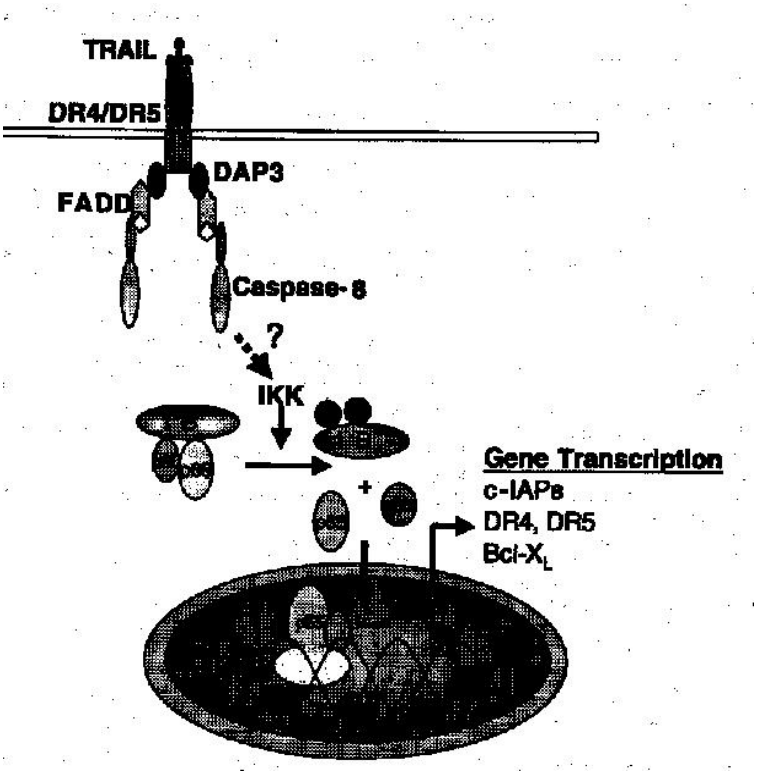


Figure 3. Diagram of the receptor- mediated apoptosis pathway used by TRAIL (34)

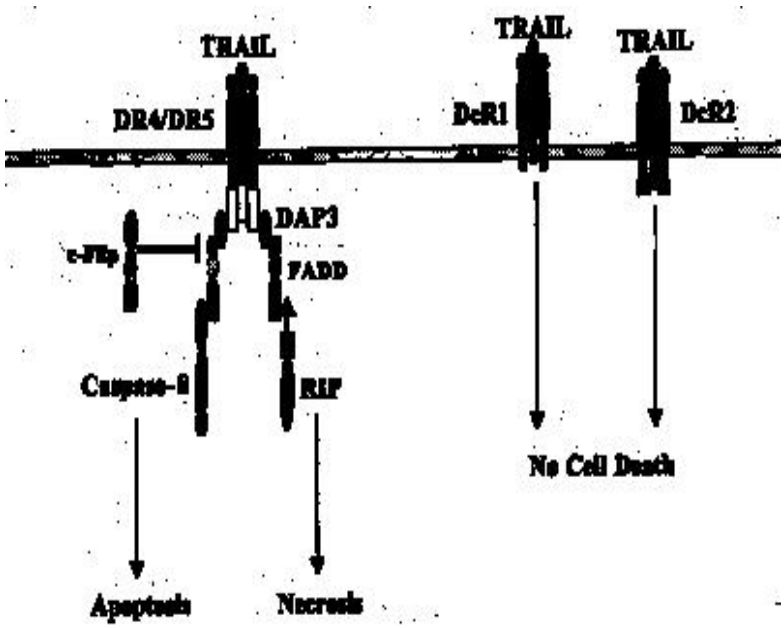


Figure 4. TRAIL death and decoy receptors and differential outcome of TRAIL-induced cell death (apoptosis or necrosis) (34).

therefore lead to increased activity of osteoclasts resulting in bone porosity (7).

In addition to the receptor-mediated apoptosis pathway, cell death induced by TRAIL may also be achieved through a separate mitochondrial pathway. This pathway is activated when the level of caspase 8 is insufficient to directly activate caspase 3 (31). The cascade can be initiated by binding of the receptor, but triggers the mitochondrial pathway at caspase 8 (38). The low level of caspase 8, insufficient to activate caspase 3, cleaves and activates the pro-apoptotic protein Bid to form t-Bid, or truncated Bid. tBid translocates to the mitochondria where it causes a loss of mitochondrial membrane potential (31) and stimulates release of cyt c and procaspase-9 into the cytosol (31, 38). Cyt c then ultimately activates effector caspase-3, which proceeds by the same manner as previously seen in the receptor-mediated pathway. This leads to membrane blebbing, cell shrinkage, and DNA fragmentation, all hallmarks of apoptosis (38). This pathway can occur alone or in tandem with the receptor-mediated pathway.

The ability of TRAIL to activate the same mitochondrial pathway used by chemotherapeutic drugs indicates that these agents could potentially be combined in such a manner as to achieve synergy. Administration of a genotoxic drug capable of upregulating the TRAIL receptors prior to addition of the cytokine can theoretically cause TRAIL to be much more effective, and has in fact been proven successful in laboratory studies (2). Some drugs, such as cisplatin, a platinum analog, when combined with TRAIL can upregulate p53, the quintessential apoptosis gene that is lacking in many cancer lines (23). In most cases, co-administration of agents appears to be less effective than pre-incubation, in which the drug is given first, then the cytokine is administered after a period of time sufficient for stimulation or upregulation of receptors (42). In

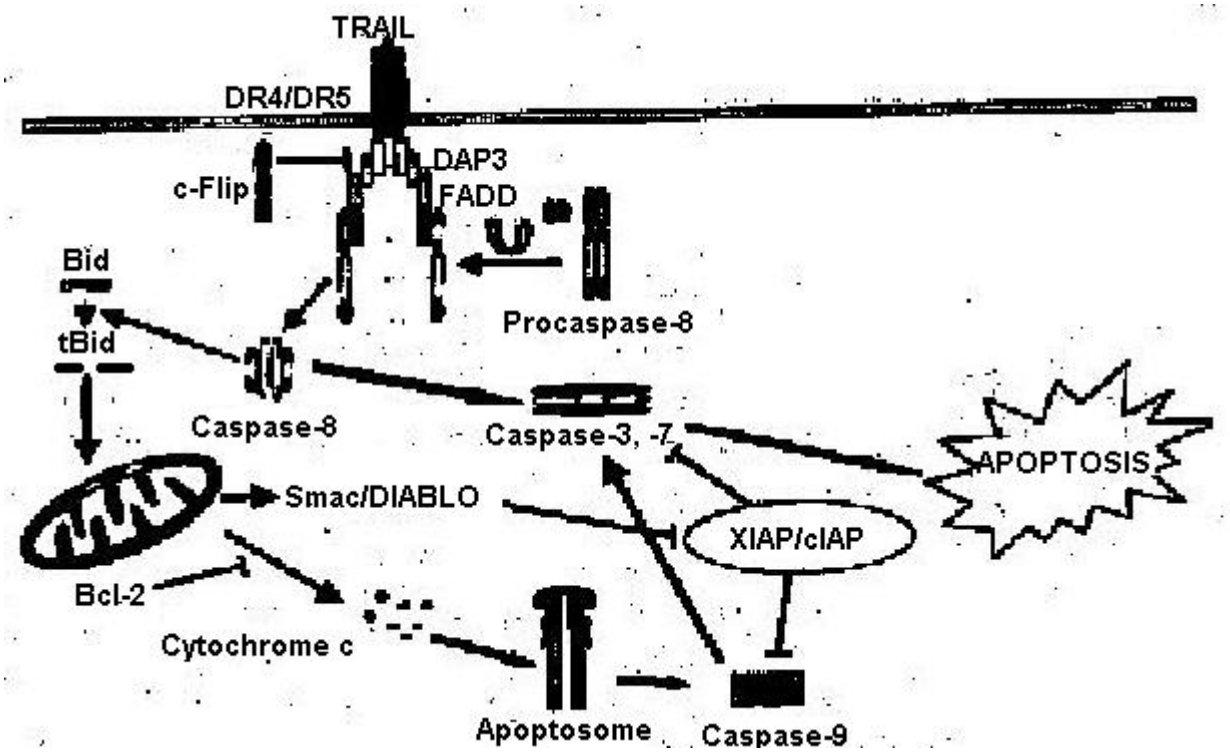


Figure 5. Receptor-mediated and mitochondrial pathways of apoptosis induced by TRAIL (34).

cancer cells that have retained a normal p53 gene, treatment with both a cytotoxic drug and TRAIL seems particularly effective (39).

Expected Outcomes This study will strive to evaluate the effect of the alkylating chemotherapeutic agent chlorambucil, the ω-3 fatty acid DHA, and the cytokine TRAIL in combination as compared to the observed effectiveness of each when administered alone when applied to Jurkat human T-cell leukemia cells or NCI-H460 human non-small cell lung carcinoma. It is known that TRAIL is most effective in the G_0 and G_1 phases of the cell cycle (16), and that while the alkylating agents are known to halt the cell cycle at G_2 (21), CLB is also capable of inducing a non-proliferative G_0 phase (22). DHA has

been shown in some studies to stop the cell cycle at G_1 (8). It has also been established that TRAIL can work by a receptor-mediated pathway or by a mitochondrial pathway, and that many drugs, as well as DHA, share many elements of this mitochondrial pathway resulting in apoptosis.

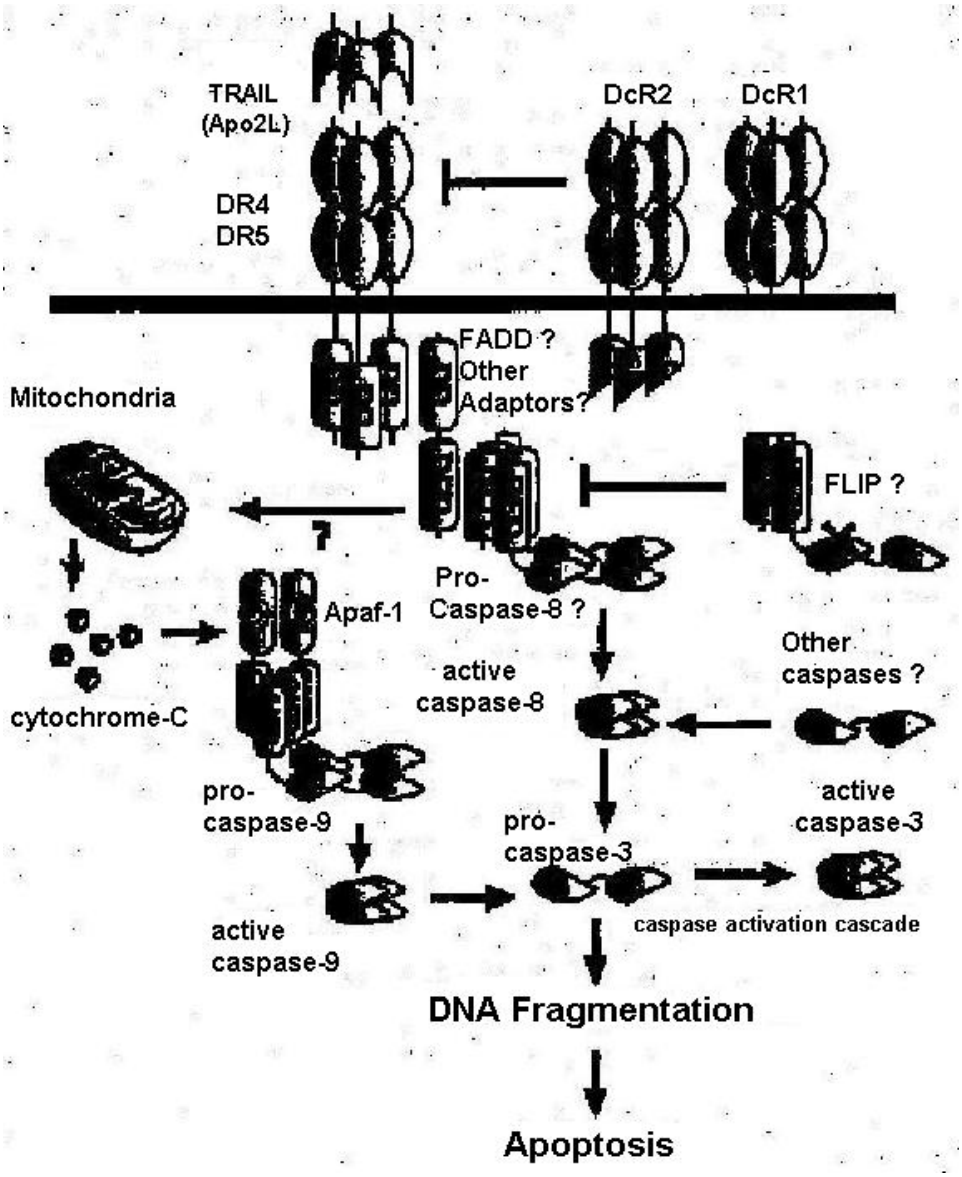


Figure 6. TRAIL receptors and pathways (2).

Based on this information, certain outcomes are anticipated. Since DHA is able to stop the cell cycle in G_1, one of the two stages in which TRAIL is most active, it is intuitive that these two agents should combine in a positive manner. If TRAIL activates its mitochondrial pathway, it will activate the same caspase cascade as DHA. Since there is no evidence that DHA will upregulate DR4 or DR5, the interaction is expected to be additive, but not synergistic. DHA preincubation however has previously been shown to induce apoptosis dramatically when pre-incubating cells before treatment with a cytokine from the same family as TRAIL, TNFα (29).

Since CLB is non-stage specific, it should prove to be active in any stage and to combine in a positive manner with either DHA or TRAIL by potentially activating the same caspase cascade. CLB enters the cell by simple diffusion. DHA is able to alter the cell membrane in a way that could potentially allow CLB to enter the cell more efficiently. Then both agents may potentially activate the same pathway. Some level of synergy may be seen with this treatment.

No previous studies have attempted to determine the effect of CLB when combined with TRAIL. It is possible that CLB may upregulate DR4 or DR5, thus making TRAIL more effective. Preincubation with CLB may cause cells to enter the G_0 phase, which is the phase when TRAIL is most effective. Subsequent administration of TRAIL after CLB preincubation could prove to be a synergistic combination.

Different sensitivities to certain treatments are anticipated between the two cell lines. Jurkat is considered moderately sensitive to TRAIL (33), while H460 is known to be highly sensitive (36). It has been observed that the anti-apoptotic protein Bcl_2, found in the mitochondrial membrane (11), can confer resistance to TRAIL to many cancer cell

lines, including breast cancer, cancers of the brain, and H460 cells (10). However, Bcl_2 was not able to block TRAIL-induced apoptosis in Jurkat cells (34). This implies that the two cell lines use opposing pathways to apoptosis. Since Bcl_2 is an anti-oxidant active in the mitochondria, and can block TRAIL apoptosis in H460, this suggests that TRAIL uses exclusively the mitochondrial pathway to cause apoptosis in this cell line. The inability of Bcl_2 to block apoptosis by TRAIL in Jurkat seems to suggests that Jurkat uses an alternate pathway. Indeed, it has been suggested by previous studies that TRAIL is able to activate components of both pathways in Jurkat (33). Bcl_2's activity as an anti-oxidant also allows it the potential to block apoptosis caused by oxidation by ROS of fatty acids in the mitochondrial membrane. The effect should be equivalent in both cell lines, as apoptosis induced by DHA occurs primarily by the mitochondrial pathway.

No previous studies have examined the ability of CLB to cause cell death in H460 as compared to Jurkat, thus it is not immediately clear how H460 will respond to treatment with CLB. It has been established that TRAIL-induced apoptosis in non-small cell lung cancer lines including H460 can be enhanced by treatment with a variety of chemotherapeutic drugs, sometimes resulting in synergy (9).

By use of colorimetric cell proliferation assays and analysis of apoptosis by flow cytometry, this study will determine the sensitivities of both Jurkat and H460 human cancer cell lines to CLB, DHA, and TRAIL, individually and in combination, and will evaluate whether the combination of these agents is sub-additive, additive, or synergistic. Both TRAIL and DHA possess the potential to synergize with a small dose of CLB in such a manner as to provide the same or greater benefit as that achieved by a larger dose of the drug alone. Because neither TRAIL nor DHA has been shown to cause any

systemic toxicity or intolerable side effects upon systemic administration, any combination of these agents bears potential to be a very effective and well-tolerated treatment with possible effectiveness in a wide variety of cancers.

MATERIALS

Cell Culture Jurkat and NCI-H460 cell lines were purchased from ATCC, Manassas, Va. (Jurkat clone E6-1, # TIB-152; NCI-H460 # HTB-177). RPMI-1640, #22400-089 was purchased from Gibco Invitrogen, Grand Island, NY, and stored at 4^{o} C. Medium was supplemented with 10% newborn calf serum (Gibco Invitrogen, #16010-159) and penicillin G/streptomycin, 100 units/ml (Gibco BRL Life Technologies, #1103562). Phosphate buffered saline, pH 7.2, was also from Gibco Invitrogen, # 20012-027. Trypan blue used for cell counts and viability was from Sigma, #T6146. Ethanol (E702-3) was purchased from Aldrich, Milwaukee, WI. Trypsin-EDTA solution used for removal of adherent cells from culture vessels came from Sigma, # T4049. Sodium azide was purchased from Sigma, # S8032.

Assays Colorimetric cell proliferation assays were performed by use of an XTT proliferation kit from Roche Molecular Biochemistry, # 1465015. Apoptosis assays were done by flow cytometry using Apo-Alert Enhanced Annexin V-FITC and Apo-Alert binding buffer (both from Clontech, Palo Alto, CA, catalog #s 8133-1 and 8134-1, respectively).

Anti-cancer agents DHA was purchased from Nu-Chek Prep, Elysian MN, # U-84-A. Chlorambucil, #C-0253 was purchased from Sigma, St. Louis MO. TRAIL was also from Sigma, # T5694.

Supplies

-Small cell culture flasks. Falcon 50 ml polystyrene tissue culture treated flasks with plug caps. Becton Dickinson Labware, Franklin Lakes, NJ. # 353014.
-Large cell culture flasks. 75 cm^2 cell culture treated flasks with plug caps. Corning Incorporated, Corning, NY. # 430720.
-Centrifuge tubes. 15 ml and 50 ml Fisherbrand polystyrene centrifuge tubes. Fisher Scientific, Pittsburgh, PA. #s 05-539-2 and 05-539-10.
-Capped culture tubes. Fisherbrand 75mm^2 polystyrene tubes. Fisher Scientific. # 202109428.
-5 ml glass test tubes. Fisher Scientific. # 14-961-26.
-Pipettes. Costar Stripette polystyrene pipettes. Corning Incorporated. 5 ml: #4487; 10 ml: # 4488; 25 ml: # 4489; 50 ml: # 4490.
-Pipette tips. Fisherbrand Redi-Tip general purpose tips. Fisher Scientific. Yellow 1-200 µl tips #21-197-8G; blue 101-1000 µl tips # 21-197-8F. Finepoint LTS tips, 1-250 µl for multichannel pipetter. Rainin, Oakland, CA. # GPS-L250. Eppendorf Biopur Combitips. Brinkman Instruments Ltd., Mississauga, Ontario. 1.25 ml tips # 022495101; 12.5 ml tips #022495208.

Equipment

-Pipetters. Rainin Pipetman. 1000 µl capacity, # P-1000; 200 µl capacity, # P-200, 20 µl capacity, # P-20. Rainin Pipet-lite 8-channel pipet, # L8-200. Eppendorf Repeater Pipette. Brinkman Instruments Ltd., # 022260006.
-Vortex. Fisher Genie 2. Fisher Scientific, # 12-812.

-Centrifuge. IEC Centra CL3R. International Equipment Company, Needham Heights, MA. # 3755.
-Flow cytometer. Epics Altra. Beckman Coulter, Inc., Miami, FL. # 6605563.
-Pipet pump. Drummond Pipet-aid. Drummond Scientific Company, Broomall, PA. # 4-000-110.
-Laminar flow hood. Safeaire biological safety cabinet. Fisher Hamilton L.L.C., Two Rivers, WI. # 54L392.
-Incubator. 50^{o}C humidified incubator, set to 37.5^{o}C with 5% CO_2. Heraeus Instruments, South Plainfield, NJ. # B-5060.

METHODS

Dose response curves were determined for DHA, CLB, and TRAIL for both Jurkat and H460 cell lines. Jurkat cells were cultured in 75mm^2 tissue culture flasks in sterile penicillin/streptomycin supplemented RPMI-1640 containing 10% v/v newborn calf serum and incubated in a humidified atmosphere at 37.5^o C with 5% CO_2. Jurkat cells were routinely passaged 1:4 (1 part cells: 4 parts growth medium) every 2 to 3 days. At about 3 days after passage, the cells were centrifuged 10 minutes at 21^o C and 1500 rpm, then re-suspended in 1 ml penicillin/streptomycin-supplemented RPMI (5 ml pen/strep solution per 500 ml RPMI) + 2% v/v newborn calf serum (hereafter known as 2% NCS). The cells were counted by the trypan blue exclusion method, in which a 25 µl sample of the cell suspension is removed to a glass tube and 25 µl trypan blue stain (0.2% w/v trypan blue dissolved in PBS and stored at 4^o C) and 50 µl PBS are added to it. The suspension was gently vortexed and 10 µl of the stained cells were pipetted onto a hemacytometer and counted. By this method, dead cells take up the trypan blue stain and thus appear blue, while live cells remain unstained. Percentage of viability may be determined by dividing the number of live, unstained cells by the total number of cells counted. Concentration was adjusted to 8x10^6 cells/ml in 2% NCS. Because Jurkat cells are prone to clumping, it is important to vortex the suspension to ensure homogeneity. The cells were then plated in 96-well tissue culture-treated microtiter plates at 50 µl per well, equivalent to 40,000 cells per well, before adding the appropriate concentrations of DHA, CLB, or TRAIL. All reagents were warmed to 37^o C prior to use to prevent shock to the cells.

Adherent H460 lung cancer cells were similarly cultured in sterile pen/strep supplemented RPMI with 10% calf serum and incubated as above. Upon confluence, the point at which a monolayer of adherent cells covers the entire surface of the flask, cells were harvested. The cell layer was first rinsed with warmed PBS, then 0.25% trypsin-EDTA solution was added to cover the surface of the cell layer. The flasks were placed in the incubator at 37^{o} C and left undisturbed until the cell layer detached from the flask, approximately 5 minutes. Upon detachment, the flask was removed from the incubator and 5 ml RPMI was added immediately to stop the action of the trypsin. The entire contents of the flask were pipetted into a sterile 15 ml conical centrifuge tube. The flask was then rinsed again with an additional 3 ml RPMI to remove any remaining cells. The contents were added to the conical vial and centrifuged at 1500 rpm for 10 minutes at 21^{o} C. The supernatant was then decanted and the cell pellet re-suspended in 1 ml 2% NCS in RPMI and passaged approximately 1:10, depending on cell density. H460 cells were counted by the trypan blue exclusion method as previously described, and cell concentration was adjusted to $2x10^{6}$ cells per ml. They were then plated at 50 µl per well in 96-well microtiter plates, equivalent to 10,000 cells per well. Because of the adherent nature of these cells, it is important to vortex the suspension before plating to ensure a uniform distribution. As with Jurkat cells, all reagents were warmed to 37^{o} C before use.

The appropriate concentrations of DHA, CLB, or TRAIL were then added to the plates. CLB and DHA are not soluble in water and must be dissolved in 100% ethanol. Upon dilution in RPMI, ethanol is added to each concentration in an appropriate volume to achieve a final concentration of no more than 0.5% ethanol in each, a concentration

which is not lethal to the cells. TRAIL, conversely, is water-soluble and therefore may be diluted directly in RPMI without any additional solvent.

Drugs were diluted to concentrations twice the concentration to be analyzed in 1-drug experiments, due to the dilution effect when combined on the plate with an equal volume of cell suspension. Drugs to be used in a 2-agent experiment were diluted to concentrations equal to 4 times the concentration to be tested, due to the dilution effect of the other drug and the cells. Similarly, agents used in a 3-drug synergy were prepared in concentrations six times the concentration to be examined, to account for dilution effect when plated with the other two drugs and cells. To determine presence of synergy, two or more agents were combined in sublethal concentrations. In experiments using 2 agents, a concentration of each was chosen which had been shown to bring about less than 50% cell death when used individually. This allows any interaction to be determined upon combination. If the total cell death appreciated is greater than the sum of cell death induced by the 2 treatments individually, the combined treatment is considered to be synergistic. Likewise for experiments requiring use of 3 agents, a concentration of each was chosen which induced less than 33% cell death when used alone.

For both cell lines, the plates containing the treated cells were incubated for 2 days in a humidified environment at 37^{o} C with 5% CO_2. At this time, XTT proliferation assay solutions were added to the plates. This colorimetric assay measures cell proliferation via reduction of XTT by the mitochondria. Healthy cells cause a change in the intensity of the color, while dead cells cannot reduce the XTT and thus result in no color change. This method allows an estimate of the number of live cells after the 2-day incubation relative to the number prior to incubation, thus healthy, proliferating cells

cause a greater color change than those cells that are not proliferating. While all the drugs are capable of causing cell death rapidly, the 2-day incubation allows surviving cells to continue to proliferate. The dead cells die quickly, but live cells continue to grow throughout the incubation period. This allows a greater difference in color intensity between the live and dead cells. If the cells survived but were not proliferating, the color change seen with the XTT assay would be much less distinct; there would be little difference among the treatments because the small number of cells originally plated would not give a strong color change. This method identifies concentrations of a given treatment that inhibit growth of the cells. This assay does not identify apoptosis or cell death.

XTT was frozen in individual 5 ml portions to prevent repetitive freeze-thaw cycles and stored at -30^{o}C. The solution was added to each plate at 50 µl per well and returned to the incubator. At 4 and 8 hours after XTT addition, plates were read with a spectrophotometric plate reader at 490 nm to measure absorbance in each well. Higher readings indicate greater proliferation, while lower absorbance readings show lack of cell proliferation. Background absorbance caused by the drug, growth medium, etc. were accounted for by including wells containing all the components of the treatment, but no cells. A negative control was created by adding XTT to cells that had been previously killed by addition of 100% ethanol. As a positive control, 50 µl of cells were plated as usual and 50 ml of a solution of 0.5% ethanol in RPMI was added without any cytotoxic agent.

DHA Stock DHA solution was prepared by adding the contents of one ampoule of 100% DHA to 10 mL of hexane to create a solution of 10 mg/ml and stored at –30° C in a brown glass vial sealed with Parafilm to protect from light and evaporation. One ml of this stock solution was further diluted to 1 mg/ml to inhibit concentration of the fatty acid due to rapid evaporation of the hexane upon opening the vial. This 1:10 solution was sealed in Parafilm and stored in the same manner as the stock solution. Under a stream of nitrogen to prevent oxidation, 67.7 µl DHA was placed in a microcentrifuge tube. It was then dried under nitrogen to evaporate the hexane, re-suspended in 10 µl 100% ethanol and added slowly dropwise to 990 µl RPMI while vortexing to achieve a concentration of 200 µM. It is critical that the hydrophobic DHA be added very slowly to the RPMI while vortexing to ensure a homogeneous suspension. This 200 µM DHA solution was then serially diluted to make concentrations of 100, 80, 60, 40, and 20 µM in RPMI with 0.5% ethanol. These dilutions were then plated at 50 µl per well in replicates of six. Cells were added to the plates at the appropriate concentration and incubated as previously described. Effective inhibitory concentrations of DHA were determined by XTT analysis.

Chlorambucil Powdered chlorambucil, stored at 4°C, was carefully weighed using an analytical scale and dissolved in 100% ethanol to a concentration of 100 mM. Note that facemask, gloves, and eye protection are required while handling this drug to prevent risk of inhalation or absorption. Ten µl of this solution was added to RPMI to create a solution of 1.0 mM. Some fizzing occurs upon addition. The 1.0 mM solution was then serially diluted to create a range of concentrations of from 1 µM to 500 µM in RPMI,

with ethanol added to create a final concentration of 0.5%. These dilutions were then plated in 96-well tissue culture treated plates in replicates of six at 50 µl per well. An equal volume of cells was added to the plates in the appropriate concentration and incubated as previously described. Effective inhibitory concentrations of chlorambucil were determined by XTT assay.

TRAIL Stock TRAIL solution of 20 µg/ml was created by mixing powdered soluble TRAIL, stored at –30^{o}C, with distilled water. The solution was divided into 20 µl aliquots in small sterile microcentrifuge tubes, wrapped in Parafilm to prevent evaporation, and stored at -70^{o} C. One 20 µl aliquot was mixed with RPMI to yield a 1 ml solution of 400 ng/ml. This solution was then serially diluted in RPMI to create a range of concentrations from 1 ng/ml to 400 ng/ml. These concentrations were placed in 96-well microtiter plates in replicates of six at 50 µl per well. 50 µl of cells were then added to the plates in the appropriate concentration and incubated as previously described. Effective inhibitory concentrations of TRAIL were then determined by XTT assay.

Combinations All three agents were tested in all possible combinations against both cell lines. Possible outcomes were inhibition, additive effect, or synergy. Two-agent experiments were plated at 25 µl of each agent per well; three-agent combinations were plated at 16.7 µl of each per well to achieve a total volume of 50 µl of the treatment solution, and 50 µl of cells at the appropriate concentration were added to each well. In all cases, plates were incubated as previously described and then analyzed by XTT assay.

CLB/DHA Chlorambucil was mixed and diluted as above in the correct concentrations to be plated at 5, 10, 15, and 20 μM. DHA was prepared in the appropriate concentrations to be plated at 10 and 20 μM. Each drug concentration was plated alone and in combination to total 50 μl per well before addition of cells.

CLB/TRAIL Chlorambucil was mixed and diluted to the correct concentrations to be plated at 2.5, 7.5,and 12.5 μM. TRAIL was diluted to achieve concentrations, when plated, of 7.5, 25, 50, and 100 ng/ml. Each final concentration was plated individually and in every possible combination of CLB and TRAIL to a final volume of 50 μM per well and an equal volume of cells was added.

TRAIL/DHA TRAIL was prepared in appropriate concentrations to be plated at 7.5, 25, 50, and 100 ng/ml. DHA was diluted to achieve final plating concentrations of 10 and 20 μM. TRAIL and DHA were plated alone and in combination to a final volume of 50 μl per well, and an equal volume of cell suspension was added.

TRAIL/CLB/DHA In previous dose-response assays, TRAIL showed activity above the 50 ng/ml range. For this reason, TRAIL was prepared in concentrations appropriate to be plated at 50 ng/ml. CLB was diluted to give a final plating concentration of 7.5 μM, a level that had been determined to be sublethal alone in dose response studies. Likewise, DHA was prepared to final plating concentrations of 10 and 20μM. As in all other combinations, drugs were added to a total volume of 50 μl per well and an equal volume of cell suspension was added.

Determination of Apoptosis Samples of Jurkat cells having undergone treatment with each cytotoxic/cytostatic agent individually and in combination were analyzed by flow

cytometry using Expo 32 software to determine if the cells were apoptotic as indicated by fluorescence. Cell concentration was adjusted to $8x10^6$ cells per ml for Jurkat cells and $4x10^6$ cells per ml for H460 cells and plated at 500 µl per well in large 24-well plates. Each well was then treated with a total volume of 500 µL of chlorambucil, DHA, TRAIL, or a combination of two or all three agents in sublethal concentrations and incubated for 48 hours at 37° C. A sample treated only with 0.5% ethanol in RPMI served as a live control, and cells killed by addition of 500 µl of 100 mM sodium azide were used as a negative control. After incubation, wells containing like treatments were pooled and centrifuged at 1500 rpm for 10 minutes at 21° C, then washed twice with cold PBS. Cells of each treatment were then re-suspended in 500 µL of cold binding buffer. A 100 µL sample of each suspension was removed to a glass tube and 5 µL of stock Annexin V-FITC (stored at 4°C) was added to each. The samples were allowed to incubate at room temperature in the dark for 15 minutes, and then 400 µl of binding buffer was added to each and vortexed. Samples were analyzed by flow cytometry. The Annexin V-FITC fluorophore binds to phosphatidylserine, a membrane phospholipid found on the surface of cells only during apoptosis.

Adherent H460 cells were treated in much the same way; however, removal of the cells from the wells required use of trypsin. The fluid medium was aspirated from each well and trypsin solution was added to cover the attached cell monolayer on the bottom surface of each well. The plate was then placed in an incubator for approximately 5 minutes or until the cell layer began to detach from the surface. RPMI was added to each well to neutralize the effect of the trypsin. The cells were then pooled, washed, and stained by the same procedure used for Jurkat cells, as described above.

Graphs were created for each experiment using SigmaPlot software and statistical significance was determined using Student's two-tailed t-test.

Each treatment was analyzed by flow cytometry using an argon laser. Live cells do not bind the fluorophore and thus do not fluoresce, while apoptotic cells effectively bind Annexin V-FITC and thus produce a higher level or fluorescence. A logarithmic graph of the fluorescence of cells having undergone each treatment was created, and then each was superimposed onto the live (negative) controls to determine amount of fluorescence, representative of apoptosis, relative to the controls.

RESULTS

Jurkat human T-cell leukemia cells and H460 human non-small cell lung carcinoma cells were treated with chlorambucil, a common chemotherapeutic alkylating agent known to have significant activity in lymphoid malignancies; the unsaturated fatty acid DHA; and the cytokine TRAIL, which has been previously shown to induce apoptosis in both cell lines. Cells were treated with each cytotoxic agent either alone or in combination. Initially dose response curves were created using the XTT assay, a colorimetric test that indicates relative numbers of respiring cells. Experiments were then repeated and analyzed by flow cytometry to gain a more accurate picture of the effect of each combination. The use of Annexin V-FITC allows apoptotic cells to be preferentially visualized.

Dose Response Curves

Chlorambucil For Jurkat cells, CLB is effective in low doses, with ED_{50} observed at approximately 5 μM by XTT analysis. Little difference in cell death was seen among samples treated with doses of CLB above 125 μM, presumably because all cells are dead at this dose (fig. 7a). Higher doses were required to obtain similar effect in H460 cells, in which ED_{50} occurred at around 25 μM. As with Jurkat, there was no significant difference in cell killing among concentrations greater than 125 μM in this assay (fig. 7b). Cells treated with concentrations of this level and higher are presumed to all be dead, despite an absorbance reading that would seem to indicate some survival. This is believed to be an artifact of the XTT assay, which gives a color change based on coupling of electrons in the mitochondria of live cells. XTT could conceivably couple electrons

from the atmosphere giving a false appearance of cell survival. This mechanism is suspected due to the increase in absorbance over time in cells that have previously been killed with 100% ethanol and are therefore obviously not respiring.

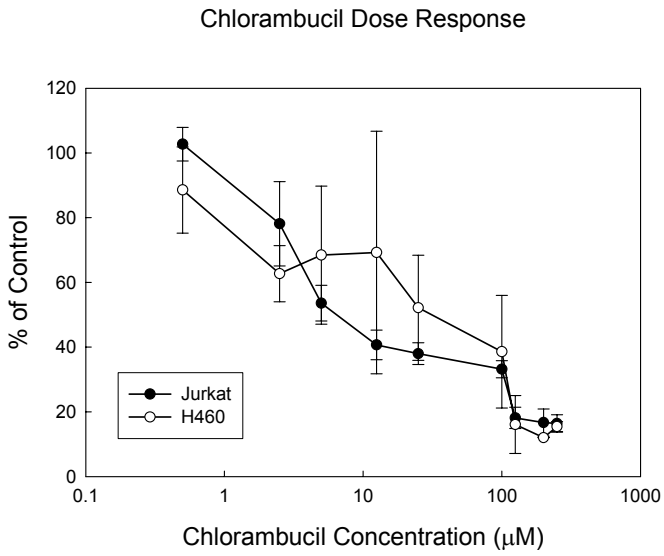


Figure 7. Dose response curves for cell lines treated with chlorambucil. Cell survival is shown as percent of control (untreated cells). Jurkat cells were plated at $8x10^5$ cells per ml on 96 well microtiter plates and treated with varying concentrations of DHA, then incubated for 48 hours at 37^o C with 5% CO_2 before adding XTT and incubating for 8 hours. H460 cells were plated at $2x10^5$ cells per ml and incubated as above. Error bars represent standard deviation. Graphs represent average of experiments (n=3).

Figure 8a) Overlay plot of Jurkat cells treated with 0.5 μM CLB as compared to live, untreated cells. Cells were treated with Annexin V-FITC. Fluorescence indicates apoptosis. Fluorescence is 13.60% greater than control.

b) H460 cells treated with 10 μM CLB. Fluorescence is 17.59% greater than control.

When cells treated with CLB were analyzed by flow cytometry, it was discovered that the apoptosis-inducing dose of the drug was actually much lower than XTT analysis would imply. In order to find sublethal doses, the concentration was reduced. A dose of only 0.5 μM was cytotoxic to 14% of Jurkat cells (fig. 8a), while 10 μM induced apoptosis in 18% of H460 cells (fig. 8b). Cells were stained with Annexin V-FITC, a fluorescent compound that stains phosphatidylserine, a membrane phospholipid that is exposed in the outer leaflet of the plasma membrane bilayer in cells undergoing apoptosis.

This discrepancy represents the limitation of the XTT assay as an indicator of cell death. XTT colorimetrically measures mitochondrial function by coupling free electrons from the respiratory chain. The basis of this concept is that healthy cells will actively respire, therefore liberating ample electrons and thus causing a more intense color signal than those cells which are unhealthy and respiring less efficiently, or those which are dead and thus not respiring at all. Flow cytometry, on the other hand, was carried out in order to obtain a clearer picture of the type of cell death, and gives a precise indication of whether each individual cell is undergoing apoptosis. The use of Annexin-V allows only those cells that are in the apoptotic process to fluoresce. Cells that have died by necrosis however may not be stained by this procedure. Flow cytometry is apparently much more sensitive than XTT assay. In addition, some dead cells measured by flow cytometry may have been killed by the preparation or the act of being run through the machine itself.

The results are shown here as overlay plots, constructed to compare cells treated with each agent or combination to a live, negative control. This allows background fluorescence to be accounted for and effectively subtracted out. A value is generated that

indicates the percent difference in fluorescence between the negative control and the treated sample.

DHA In both Jurkat and H460, DHA shows the same pattern of cell killing in which little or no effect is observed at low concentrations. However, an abrupt plunge in the number of surviving cells is observed when either cell line is treated with 30 μM DHA. This concentration appears to cause 100% cell death in both cell lines (fig. 9).

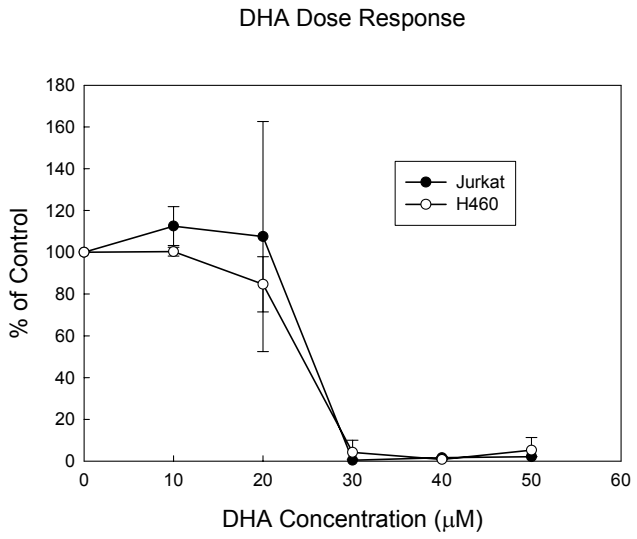


Figure 9. DHA dose response curves for Jurkat (n=2) and H460 cells (n=3). Cell survival is shown as percent of control (untreated, live cells). Cells were plated and incubated as previously described. H460 cells are somewhat more sensitive to low concentrations of DHA than are Jurkat by XTT assay, but both show complete inhibition of proliferation when treated with 30 μM DHA.

An unusual, but wholly reproducible phenomenon occurring in Jurkat cells, and to a lesser extent, H460 as well is the small increase in absorbance induced by low doses of DHA. Cells treated with concentrations of up to 20 μM in Jurkat, and 10 μM in H460, display greater absorbance indicative of cell proliferation than the untreated control cells when analyzed by XTT assay. It is thus far unknown why treatment with a low dose of

DHA should incur a protective effect or increase rate of proliferation in these cells. It is possible that the increase is not representative of proliferation but rather a technical artifact of the treatment and assay, to be discussed later.

Figure 10a) Jurkat cells treated with 10 μM DHA. Fluorescence is 44.98% greater than the control, indicative of % of apoptosis.

b) H460 cells treated with 10 μM DHA. Fluorescence is 13.63% greater than the control.

By flow cytometry, much lower concentrations were able to cause apoptotic cell death in Jurkat than was expected based on the previous XTT assays (fig. 10a and b). A concentration of 10 μM brought about a nearly 50% reduction in cell survival. Results of DHA treatment on H460 showed it to be less sensitive, with the same dose causing about 13.6% apoptosis.

TRAIL Jurkat cells, which are considered moderately sensitive to TRAIL, reached ED_{50} by XTT analysis when treated with approximately 80 ng/ml of the cytokine (fig. 11). However, by flow cytometry, doses as low as 15 ng/ml were cytotoxic to nearly 50% of cells, and 10 ng/ml caused apoptosis in nearly 20% of Jurkat cells (fig.12a).

H460, which is vastly more TRAIL-sensitive than Jurkat, responded much more dramatically. By XTT assay, ED_{50} was reached at about 60 ng/ml (fig. 11). A 10 ng/ml dose resulted in death of nearly 60% of H460 cells (data not shown), and 5 ng/ml induced apoptosis in greater than 20% of cells (fig. 12b).

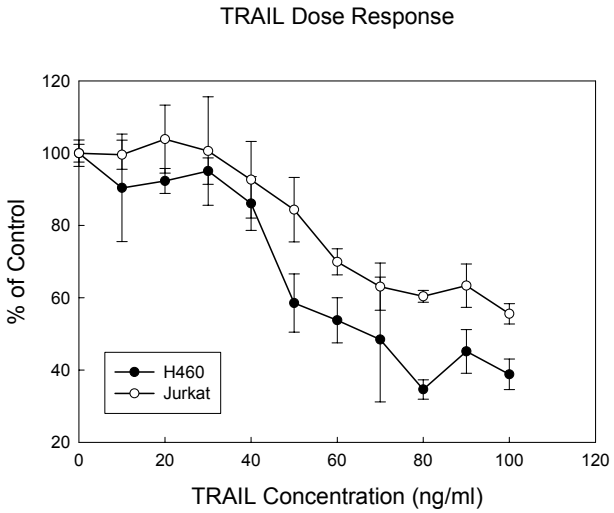


Figure 11. Dose response of Jurkat and H460 cells treated with TRAIL. Cells were plated and incubated as previously described. a) Jurkat cells, n=2. b) H460 cells, n=1. Cell survival is shown as percent of control (untreated cells).

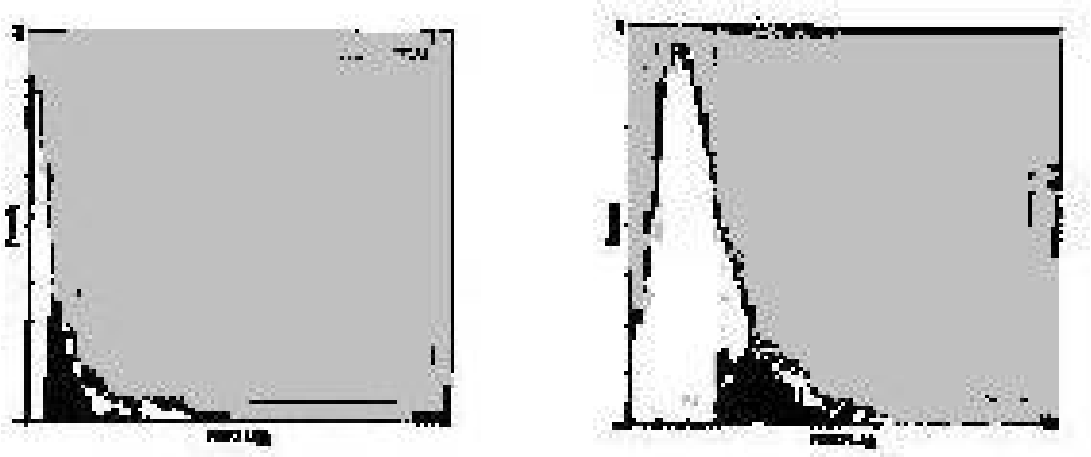


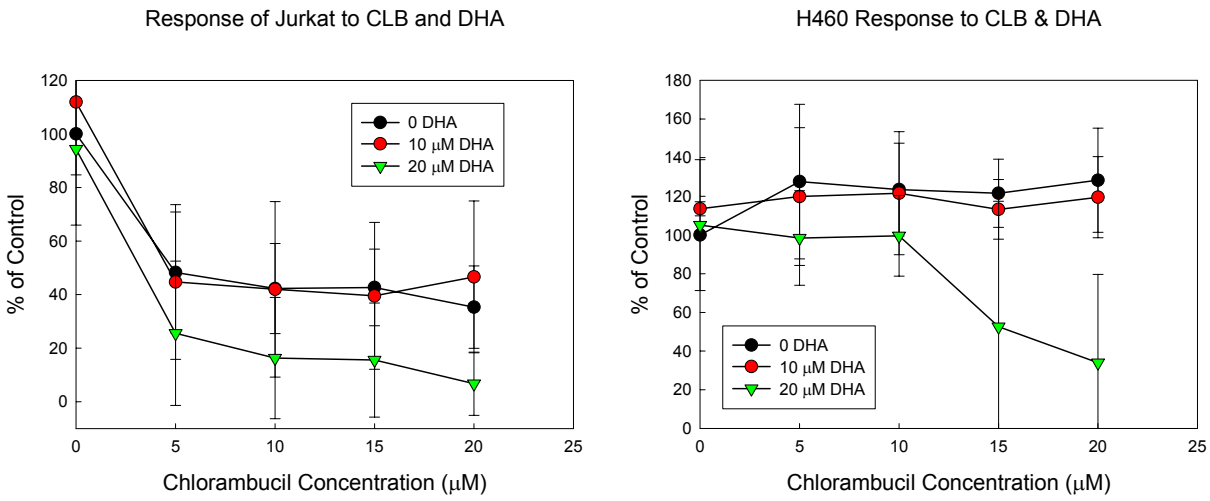
Figure 12a) Jurkat cells treated with 10 ng/ml TRAIL. Fluorescence indicates 18.14% apoptosis. **b)** H460 cells treated with 5 ng/ml TRAIL. Fluorescence indicates 21.91% apoptosis.

Determination of Interaction

CLB synergizes with DHA

Ranges of sublethal concentrations for each cytotoxic agent were selected to be combined and assessed for their ability to induce a synergistic response, defined here as cell survival of at least 10% less than that which would be expected if the interaction of the combined agents was additive. Variation among samples analyzed by XTT is quite common, and a day-to-day fluctuation of 10% is usual. Therefore, a result within 10% of the expected was considered normal. Cell survival greater than 10% higher than the expected was interpreted as "sub-additive" meaning the combination was less than the expected additive effect. The accuracy of flow cytometry does not leave such a wide margin for error however, as it is not subject to such fluctuation as is the XTT assay. Some variation however is always possible in any experimentation using a biological system. For XTT analysis of cells treated with CLB and DHA, CLB concentrations of 5, 10, 15, and 20 μM were combined with DHA at either 10 or 20 μM.

In both Jurkat and H460 cells, CLB combined synergistically with DHA (fig.13a). A low sublethal dose of DHA (10 μM) proved to be synergistic with the higher levels of CLB in some trials. Synergy occurred in two of two trials with 15 μM and in one of two with 20 μM in Jurkat cells. These combinations resulted in on average about 30% fewer cells surviving than if the combination had been merely additive. A higher dose of DHA, 20 μM, also showed synergy with all concentrations of CLB in one of two trials, resulting in at least 10% fewer cells surviving than expected.



a) b)

Figure 13. Jurkat and H460 cells treated with concurrent CLB and DHA. Cells were treated simultaneously a) Jurkat cells. b) H460 cells. Cell survival is shown as percent of control (untreated, live cells equal to 100%, not shown). Graphs represent an average of experiments (n=3).

Figure 14a) Jurkat cells treated with 0.25 μM CLB + 10 μM DHA. Fluorescence indicates 42.22% apoptosis.

b) H460 cells treated with 10 μM CLB + 10 μM DHA. Fluorescence indicates 41.82%. apoptosis.

H460 cells showed synergy with 10 μM DHA when combined with all concentrations of CLB, causing decrease in viability an average of about 15% greater than expected (fig. 13b). The higher dose of DHA however resulted in synergy only with 15 and 20 μM CLB, but proved to be exceptionally active in causing cell death in these concentrations. After treatment with 20 μM DHA with either 15 or 20 μM CLB, an

average of about 45% fewer cells survived than was expected if the interaction had been additive. Table 1 indicates a reduction of 85-90% when these concentrations were administered in H460 cells, but this extreme interaction was observed in only one of three trials. Other trials showed synergistic effects although less dramatic than that mentioned here.

No sub-additive effects were observed in any trial with either cell line treated with these two agents. Overall, in both cell lines there was no significant difference in average values of samples treated with 10 μM DHA with CLB and in those treated with CLB alone ($P>0.05$). Results of treatment with 20 μM DHA in combination with any concentration of TRAIL proved to be significantly different from CLB alone at every concentration ($P<0.05$). Trials resulting in synergy for this and all other combinations are summarized in Table 1.

By flow cytometry, sublethal concentrations of each agent were combined and overlay plots were created to compare the fluorescence of the treated cells to that of a live, untreated negative control. For Jurkat cells, 0.25 μM CLB was combined with 10 μM DHA. For H460, which is less sensitive to CLB than is Jurkat, 5 μM CLB was used. DHA concentration remained the same. In H460, this combination resulted in induction of apoptosis approximately 15% higher than anticipated based on the reactivity of each agent individually (fig. 14a). In Jurkat cells, no synergy occurred (fig. 14b).

TRAIL synergizes with low concentrations of CLB

TRAIL in concentrations of 7.5, 25, 50, and 100 ng/ml was combined with 2.5, 7.5, and 12.5 μM CLB for XTT assay. In Jurkat cells, 50 and 100 ng/ml TRAIL combined

synergistically with 2.5 and 7.5 μM CLB (fig. 15a). At these doses, viability ranged from 16 to 45% lower than anticipated. In Jurkat cells treated with 50 ng/ml TRAIL and 2.5 μM CLB, 69% cell survival was expected. The actual observed value of cells that survived in one trial, according to XTT analysis, was only 29.3%. There was no significant difference in viability between cells treated with 50 or 100 ng/ml TRAIL in combination with CLB at any concentration ($P>0.05$). Viability of Jurkat cells treated with these combinations however (50 or 100 ng/ml TRAIL + CLB) significantly different from that of cells treated with TRAIL alone.

H460 cells showed a similar profile, exhibiting synergy between 2.5 μM CLB and both 50 and 100 ng/ml TRAIL in one of two trials. Total cell survival was about 20% lower than anticipated in experiments using 2.5 μM CLB in combination with 50 or 100 ng/ml TRAIL. In one trial, 50 μM TRAIL also was mildly synergistic with 7.5 μM CLB, resulting in 11% less survival than expected. Figure 15b illustrates the overall pattern of cytotoxicity that occurs when these agents are combined, as shown by XTT assay.

A sub-additive effect, defined here as percent of surviving cells >10% higher than expected, occurred in two of two trials combining the lowest doses of each agent. Sub-additive interactions also were seen sporadically in other combinations, such as the highest level of CLB with low doses of TRAIL, and in one of two experiments combining the highest dose of TRAIL with the lowest of CLB.

For flow cytometry experiments combining TRAIL and CLB, a TRAIL concentration of 10 ng/ml was chosen. This level was only mildly cytotoxic to Jurkat, but caused a nearly 60% reduction in the more sensitive H460 cells. TRAIL was combined with CLB at 5 μM in H460, and with a much smaller dose of 0.25 μM in

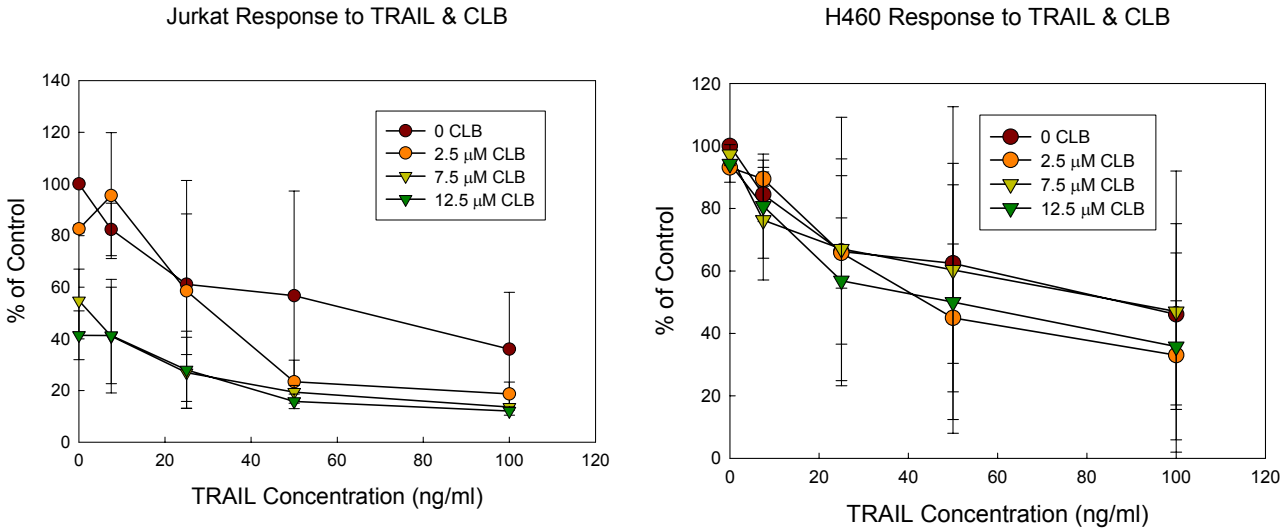
Jurkat, which is highly sensitive to CLB. Figure 16a shows that in Jurkat cells, the combination of TRAIL and CLB proved to be additive, showing cell survival within 5% of the expected value.

Table 1. Combinations resulting in synergy by XTT assay

Cell Line	CLB (μM)	DHA (μM)	TRAIL (ng/ml)	Expected Viability (%)	Observed Viability (%)	Difference (%)
Jurkat	15	10	---	70.4	53.9	16.5
Jurkat	15	10	---	64.9	36.6	28.4
Jurkat	20	10	---	67.3	39.5	27.8
Jurkat	5	20	---	62.1	45.3	16.8
Jurkat	10	20	---	51.2	26.7	24.5
Jurkat	15	20	---	48.9	23.6	25.3
Jurkat	20	20	---	47.1	21.6	25.6
H460	15	20	---	90.3	5.1	85.2
H460	20	20	---	90.4	0.03	90.4
Jurkat	2.5	---	50	69.1	29.3	39.8
Jurkat	2.5	---	100	44.8	14.5	30.3
Jurkat	7.5	---	50	48.7	21.2	27.5
H460	2.5	---	50	81.5	61.7	19.8
H460	2.5	---	100	63.1	45.3	17.7
H460	7.5	---	50	34.8	23.4	11.5
Jurkat	---	10	25	62.6	49.4	13.2
Jurkat	---	10	25	44.9	27.9	17.0
Jurkat	---	10	100	52.6	29.6	23.1
H460	---	10	100	74.3	48.6	25.7
Jurkat	7.5	10	50	32.8	21.5	11.4
Jurkat	7.5	20	50	43.4	22.0	21.4
H460	7.5	10	50	106.1	75.0	31.1
H460	7.5	10	100	73.1	54.0	19.2
H460	12.5	10	100	70.7	46.7	24.0

In H460 however, a sub-additive reaction was observed in which the total number of cells killed was less than the expected number if the interaction between the two agents had been merely additive (fig. 16b). Due to the highly cytotoxic effect created by the use of TRAIL at 10 ng/ml on H460 cells, the experiment was repeated using 5 ng/ml. This concentration caused apoptosis in approximately 21% of the cells tested, but still resulted in a sub-additive effect when co-administered with CLB, with cell apoptosis about 13% lower than anticipated.

Due to the ability of CLB to cause cells to accumulate in the G_0 phase, a stage of the cell cycle where TRAIL is believed to be most effective, an experiment was designed which attempts to exploit this concept. Cells were pre-incubated with CLB at 7.5 or 12.5 μM for 6 hours to induce the G_0 phase, washed, and then treated with TRAIL at 15 or 25



a) b)

Figure 15. Jurkat and H460 cells treated concurrently with a combination of TRAIL and CLB. Cell survival is shown as percent of control (untreated, live cells equal to 100%, not shown). a) Jurkat shows no difference in outcome of cells treated with 7.5 μM CLB and those treated with 12.5 μM. b) H460 shows no significant difference between any of the 3 concentrations of CLB. Data represent the average of experiments, n=2.

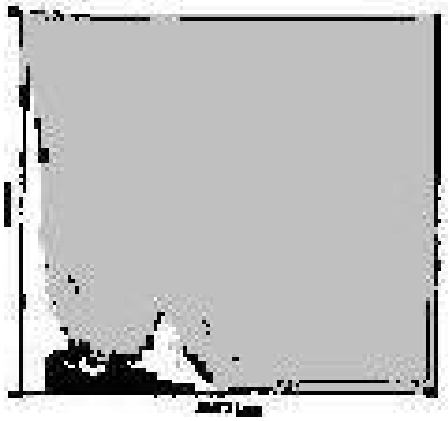
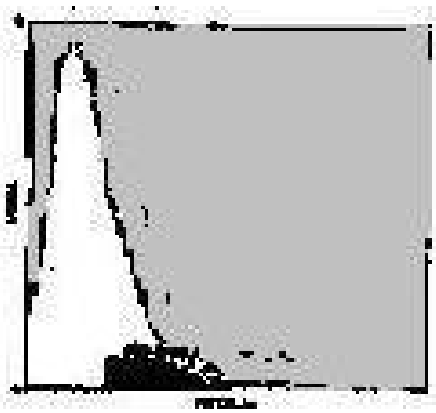


Figure 16a. Jurkat cells treated with 0.25 μM CLB + 10 ng/ml TRAIL. Fluorescence indicates 35.59%. apoptosis.



b) H460 cells treated with 10 μM CLB + 10 ng/ml TRAIL. Fluorescence indicates 26.84% apoptosis.

ng/ml. Samples were also treated with each agent individually or left untreated as a control and analyzed by XTT.

Jurkat and H460 cells showed very similar patterns in this experiment (fig. 17). In the samples treated with TRAIL only, the 15 ng/ml dose caused about a 20% reduction in cell viability in both cell lines. The higher dose however had no significant effect on viability in either cell line.

In Jurkat, there was no difference in cell survival in samples treated with 7.5 μM CLB alone or in combination with 15 ng/ml TRAIL. In cells treated with 12.5 μM CLB, addition of 15 ng/ml TRAIL increased cell survival ($P<0.05$). In both cell lines, the greatest effect was seen upon the combination of 25 ng/ml TRAIL with 7.5 μM CLB, resulting in viability more than 35% lower than that seen in cells treated with 15 ng/ml TRAIL and 7.5 μM CLB ($P<0.05$)

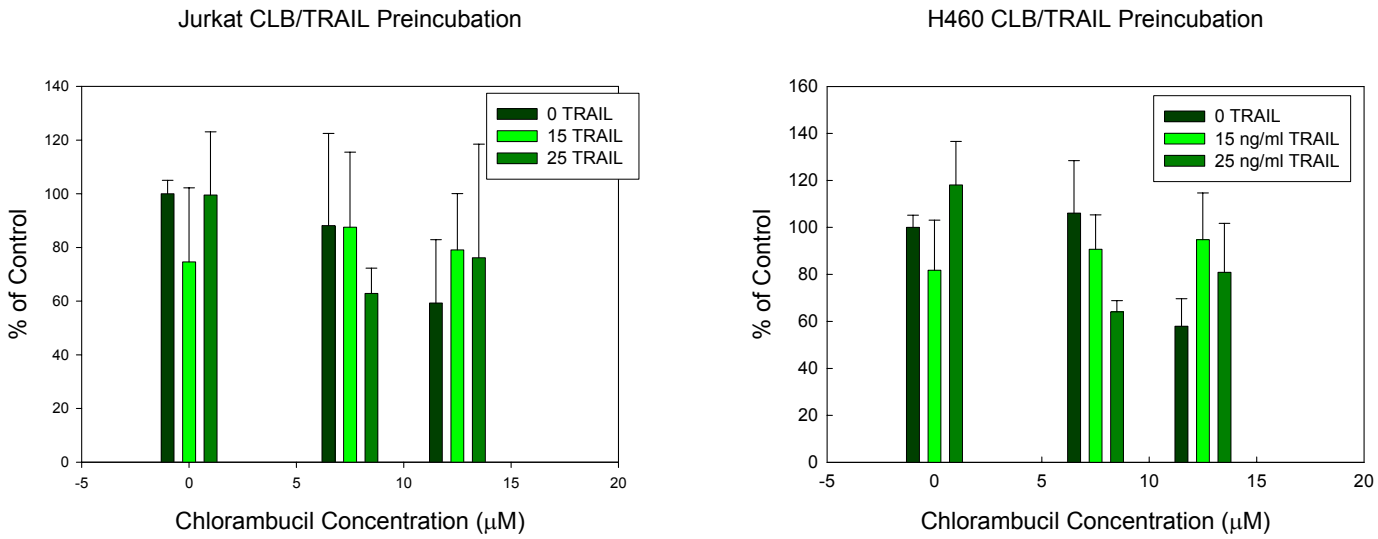


Figure 17. Response of Jurkat and H460 cells pretreated with CLB for 6 hours before administration of TRAIL. Cell survival is shown as percent of control (untreated live cells), n=2.

H460 cells treated with 7.5 μM CLB, unlike Jurkat, showed a small but significant difference when combined with 15 ng/ml TRAIL. There was no difference in viability between cells treated with 12.5 μM CLB and 15 ng/ml TRAIL and those treated with 12.5 μM CLB and 25 ng/ml TRAIL. Despite the drop in viability seen upon combination of 25 ng/ml TRAIL with 7.5 μM CLB, the combination of this concentration of TRAIL with 12.5 μM CLB proved to actually increase cell survival relative to 12.5 μM CLB alone ($P<0.05$).

Jurkat is more sensitive to combined TRAIL/DHA than H460

TRAIL in concentrations of 7.5, 25, 50, and 100 ng/ml was combined with 10 or 20 μM DHA for XTT analysis. This combination proved to be synergistic in some cases (at all concentrations, 5 of 15 experiments showed synergy in Jurkat and 5 of 24 in H460), but not as frequently as did the previous combinations of TRAIL with CLB or CLB with DHA. The treatment consisting of 100 ng/ml TRAIL and 10 μM DHA, however,

resulted in synergy in one of two trials involving Jurkat, and two of two H460 experiments.

Jurkat cells showed significant responsiveness to TRAIL/DHA only in low doses. The combination of 25 ng/ml TRAIL with 10 μM DHA showed a reduction in cell viability more than 10% greater than anticipated in two of two trials. Use of a higher dose of DHA did not improve the potential for synergy. In general, this combination was not considered to consistently provoke a synergistic cell death response in other concentrations (fig. 18a).

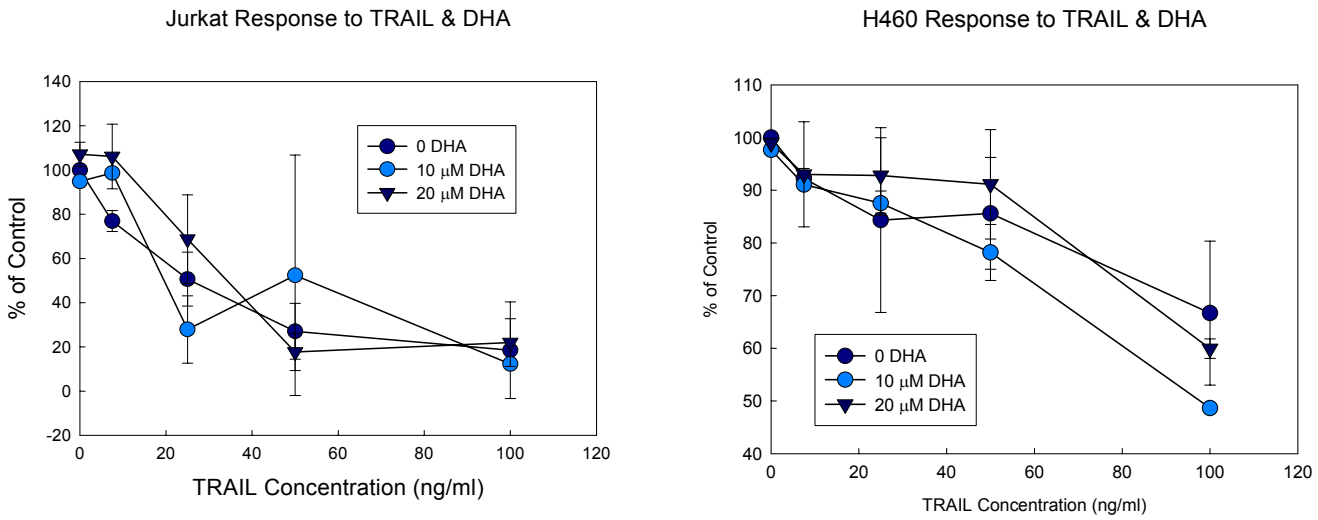
H460 was overall less sensitive than Jurkat to the combination of these cytotoxic agents (fig 18b). Low doses of TRAIL did not synergize with either level of DHA, and high TRAIL doses (50 and 100 ng/ml) synergized only sporadically with either concentration of DHA. The only exception was the aforementioned 100 ng/ml TRAIL with 10 μM DHA, which resulted in restriction of cell proliferation averaging nearly 20% greater than expected.

Sub-additive effects, cell survival more than 10% higher than expected, resulted from this treatment in each of two experiments combining the lowest levels of each agent in Jurkat, as well as in both trials involving low dose DHA with 50 ng/ml TRAIL. In one of two trials, the two lowest levels of TRAIL also were sub-additive in combination with 20 μM DHA in Jurkat.

In H460, 25 ng/ml TRAIL was sub-additive when combined with either concentration of DHA in one of three trials.

For both Jurkat and H460 cells, 10 ng/ml TRAIL was co-administered with 10 μM DHA and cells were stained with Annexin V-FITC for flow cytometry (fig. 19). The

effect in both cell lines was slightly less than additive, as the previously mentioned XTT analysis would indicate. As above, due to the extreme effect of this dose of TRAIL on H460, the experiment was repeated with a lower concentration of the cytokine. Again, a sub-additive effect of approximately the same capacity was observed at the higher dosage.



a) b)

Figure 18. Jurkat and H460 cells treated with TRAIL and DHA. In both cell lines, 10 μM DHA caused a greater response than 20 μM DHA. a) Jurkat shows a greater sensitivity to this treatment than H460. n=2. b) H460 shows a significant response at the highest level of TRAIL with both concentrations of DHA (n=2). Cell survival is shown as percent of control (untreated, live cells equal to 100%, not shown).

Jurkat is more sensitive to TRAIL/CLB/DHA than H460

TRAIL in concentrations of 50 and 100 ng/ml was combined with both CLB and DHA. CLB was used at 7.5 and 12.5 μM, and DHA in concentrations of 10 and 20 μM.

Figure 19a) Jurkat cells treated with 10 ng/ml TRAIL + 10 μM DHA. 42.5% apoptosis is indicated.

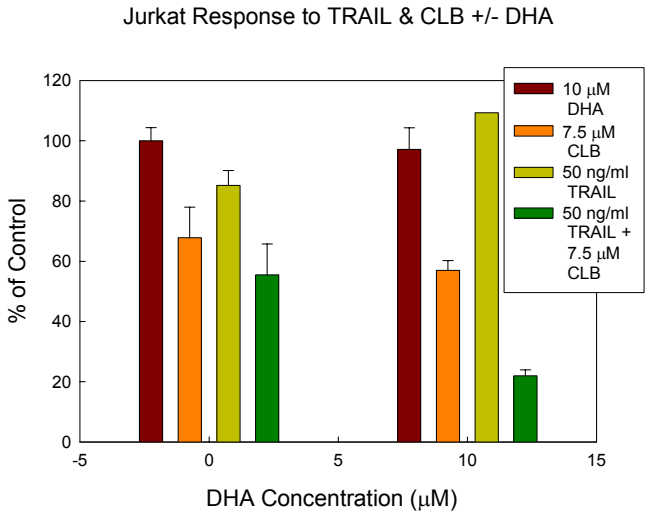
b) H460 cells treated with 5 ng/ml TRAIL + 10 μM DHA. 14.02% apoptosis is indicated.

In Jurkat, the combination of 50 ng/ml TRAIL with 7.5 μM CLB was mildly to moderately synergistic with both concentrations of DHA (12 and 21% less proliferation than expected, respectively). Every combination resulted in at least an additive effect; no sub-addition occurred in Jurkat cells treated with all three agents in any concentration (fig. 20a).

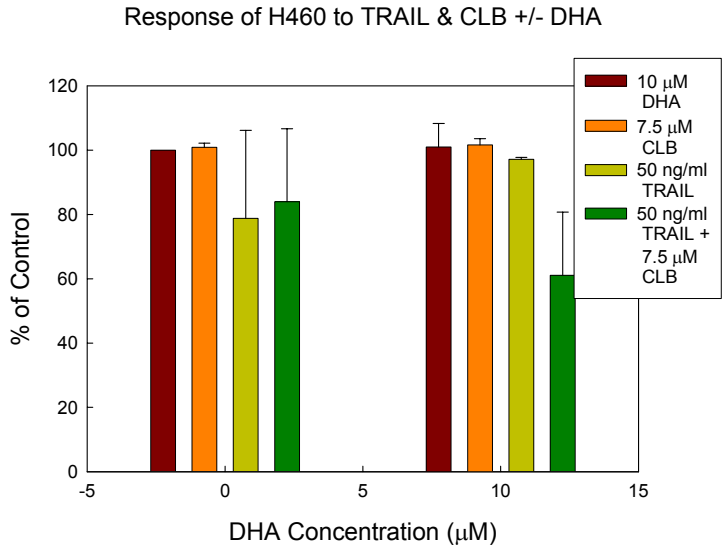
H460 cells interestingly responded in a strongly synergistic fashion to combinations of 7.5 μM CLB and 10 μM DHA, regardless of whether TRAIL concentration was at 50 or 100 ng/ml (20 and 30% greater cell death than anticipated, respectively) (fig. 20b). This is to be expected, since combinations of CLB and DHA in this range previously were shown to be synergistic without the presence of TRAIL. The lower CLB dose (7.5 μM) resulted in a more strongly synergistic effect than did the higher dose (12.5 μM). 50 ng/ml TRAIL, however, resulted in a sub-additive effect (12-15% less cell death than expected) when combined with 20 μM DHA, regardless of CLB concentration. It is of some importance to note that the addition of DHA to the

combination of TRAIL and CLB greatly increased cell death in both cell lines, as shown in figure 20.

In preparation for apoptotic analysis by flow cytometry, Jurkat cells were treated with 10 ng/ml TRAIL, 10 μM DHA, and 0.25 μM CLB. Cell viability was approximately 25% higher than anticipated compared to the expected additive effect (fig. 21a). In H460, accurate results could not be obtained with the combination of 10 ng/ml TRAIL, 10 μM DHA, and 0.25 μM CLB because the additive combined effect of all three agents would have mathematically been expected to kill greater than 100% of the cells tested. This test was instead conducted using only 5 ng/ml TRAIL (fig. 21b). The combination resulted in less apoptosis than would be expected if the activity of the treatments had been additive.



a)



b)

Figure 20. Response to Jurkat and H460 cells to treatment with TRAIL, CLB, and DHA. In both cell lines, TRAIL + DHA is not shown to significantly increase cell death compared to TRAIL alone, and is in fact sub-additive in both cell lines. a) Jurkat is much more sensitive to treatment with all 3 cytotoxic agents than H460, with survival of just over 20%. b) H460 shows similar response to that seen in Jurkat to TRAIL alone or with DHA, but is much less sensitive to TRAIL + CLB and to the combination of all 3 agents. Both cell lines display more apoptosis when treated with all 3 agents as compared to TRAIL and CLB without DHA.

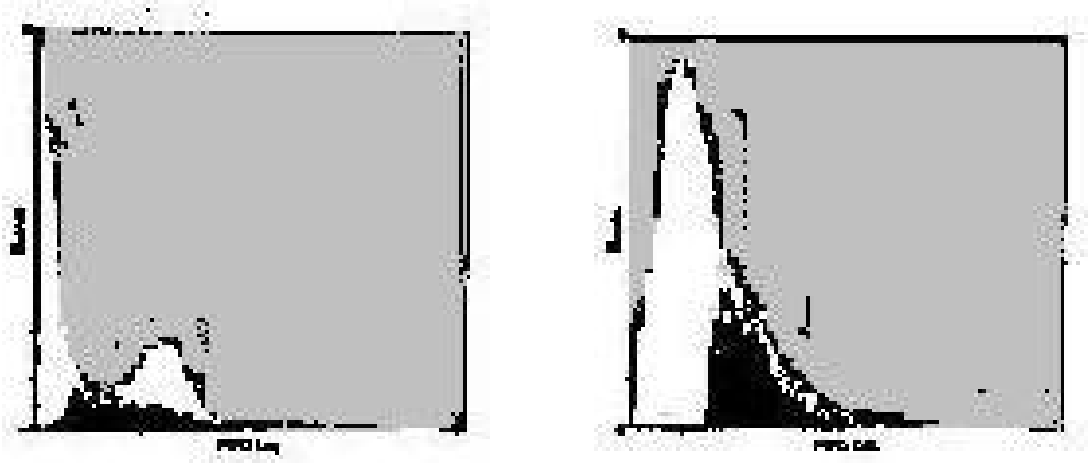


Figure 21a) Jurkat cells treated with 0.25 μM CLB + 10 μM DHA + 10 ng/ml TRAIL. Fluorescence indicates 40.76% apoptosis.

b) H460 cells treated with 10 μM CLB + 10 μM DHA + 5 ng/ml TRAIL. Fluorescence indicates 29.27% apoptosis.

Summary of Results

In summary, it is evident that combinations of the three cytotoxic agents examined here are able, in specific concentrations, to induce synergy in Jurkat, H460, or both. Jurkat reacted synergistically to 15 or 20 μM CLB combined with 10 μM DHA in, and to all tested concentrations of CLB when combined with 20 μM DHA. H460 also showed synergistic response to the combination of 15 or 20 μM CLB with 20 μM DHA, quite dramatically in one of three trials, showing 85-90% reduction in viability over the expected additive values.

Jurkat and H460 cells showed the same pattern of response to CLB combined with TRAIL, both showing synergistic reduction in viability when treated with 2.5 or 7.5 μM CLB with 50 ng/ml TRAIL as well as 2.5 μM CLB combined with 100 ng/ml

TRAIL. Jurkat was more sensitive to combined CLB and TRAIL, and both cell lines were more responsive to the lowest dose of CLB with 50 ng/ml TRAIL.

The combination of TRAIL with DHA was overall considered additive or subadditive, resulting in synergy only sporadically in certain trials.

When all three agents were combined, Jurkat was most responsive to the combination of 7.5 μM CLB, 20 μM DHA, and 50 ng/ml TRAIL, though synergy was also seen to a lesser degree with 10 μM DHA instead of 20 μM. H460 was most responsive to the combination of 7.5 μM CLB, 10 μM DHA, and 50 ng/ml TRAIL. H460 also displayed synergistic reduction of viability to combinations using 100 ng/ml TRAIL with 10 μM DHA and 7.5 or 12.5 μM CLB, while Jurkat did not show any synergistic interaction upon treatment with this higher concentration of TRAIL.

DISCUSSION

The purpose of this study was to determine if the alkylating agent chlorambucil could be combined with the omega-3 fatty acid docosahexaenoic acid and/or the cytokine TRAIL to reduce the dose required to cause significant cell death in Jurkat and H460 human cancer cells. Dose-response curves were created by XTT assay to determine the typical response to each of these three treatments individually for each cell line. Combinations of sublethal concentrations of each cytotoxic agent were then analyzed by XTT and selected concentrations were evaluated by flow cytometry to ascertain their ability to induce a synergistic cell death response in each cell line.

Chlorambucil Chlorambucil is a chemotherapeutic drug commonly used to treat lymphocytic leukemia. Briefly, CLB works by alkylating DNA; that is, by substituting an alkyl group for a covalent bond within the molecule. CLB is able to bind two nucleophilic groups simultaneously and cross-link them, disrupting nucleic acid function (22). It is therefore known as a bifunctional alkylating agent. CLB is most active in the G_2 stage of the cell cycle, and may induce cells to enter and remain in a non-proliferative G_0 phase (22).

As expected, Jurkat T-cell leukemia cells showed great sensitivity to CLB, resulting in ED_{50} at 5 μM. H460 non-small cell lung cancer was much less sensitive, with ED_{50} at about 25 μM when incubated with the drug for 48 hours and analyzed by the colorimetric XTT assay.

Flow cytometry, which provides a reliable method of evaluating apoptosis, demonstrated that the concentration of CLB required to induce apoptosis in each cell line was much lower than indicated by XTT. Cells were stained with Annexin V-FITC, which binds to phosphatidylserine, a phospholipid that is present in the outer membrane of cells beginning in the early stages of apoptosis. By flow cytometry, Jurkat showed a 50% increase in apoptosis when treated with 0.5 μM CLB, while H460 showed a similar response to 10 μM CLB.

It is believed that the discrepancy between XTT and Annexin V-FITC analysis is due to the nature of each test. They are in effect looking at different aspects of cell death or survival. While Annexin V binds exclusively to cells undergoing apoptosis, XTT essentially measures cell respiration. Annexin V-FITC will bind to cells that have just begun apoptosis but are still respiring. XTT on the other hand is colorimetric, giving the greatest absorbance in healthy, respiring cells. By this assay, if cells beginning apoptosis are still respiring they will cause a color change and higher absorbance, while this same subset of cells will be labeled and interpreted as apoptotic by Annexin V. Live cells are indistinguishable from necrotic cells by Annexin V, while necrosis can not be differentiated from apoptosis by XTT.

DHA DHA (22:6) is an ω-3 fatty acid that has been shown to confer protection against certain types of cancer in individuals who consume large amounts of dietary long chain ω-3 fatty acids. DHA is a natural component of cell membranes, and is known to induce apoptosis in many cell lines (32).

DHA may cause cell death by several avenues. It may incorporate into the cell membrane, disrupting membrane integrity (8). Fatty acids also may enter the cell and incorporate into the mitochondrial membrane where they are oxidized by reactive oxygen species in the mitochondria (35). This will in turn cause nonspecific pores to develop in the membrane resulting in depolarization and release of cyt c, which binds Apaf-1 and activates a caspase cascade resulting in apoptosis (20, 26).

By XTT assay, both Jurkat and H460 show a complete loss of viability when treated with 30 μM DHA. Interestingly, in both cases there is an apparent slight increase in absorbance among samples treated with higher doses. This is likely not indicative of increased cell survival, but is possibly a function of the ability of DHA to alternately induce necrosis rather than apoptosis in high concentrations. A cell that has died by necrosis is likely to have an extensively damaged, roughened membrane, which may cause increased absorbance when analyzed spectrophotometrically. There will be no color change in these cells upon XTT addition, as they are not respiring, but the increased surface area of the cells may cause the sample to appear dense or cloudy, resulting in a heightened absorbance value.

This study also shows the tendency for cells treated with low doses of DHA to display greater absorbance than untreated cells. This phenomenon occurred in both cell lines and was readily reproducible. The same effect has been reported by other researchers in this laboratory and others. It is unknown at this time if the low concentration of the fatty acid alters the membrane in such as way as to increase absorbance, or if it does indeed increase cell proliferation. It is possible that cells treated with low doses of DHA, while still healthy and actively respiring, are beginning to show

early signs of apoptosis including membrane blebbing. This irregular membrane surface, as previously described, could cause light to scatter thereby increasing absorbance as read by the spectrophotometer. On the other hand, it has not been ruled out that sublethal doses of DHA could potentially enter the nucleus and activate genes that could increase either proliferation or respiration.

One method of resolving this issue would be to incubate samples of cells in a range of concentrations of DHA and then stain with propidium iodide, which stains DNA within cells that it is able to enter through an extensively damaged membrane indicative of necrosis. Staining with Annexin V-FITC as well would allow the condition and type of cell death of cells treated with each concentration of DHA to be compared.

Another possible technique would be to read the absorbance of cells treated with a range of DHA concentrations without the addition of XTT. This would determine the absorbance caused by the cells alone, and resolve the matter of whether or not cells have a higher absorbance due to membrane damage when treated with low doses of DHA. Furthermore, a simple cell count could indicate if there are more cells present in samples treated with a low concentration of DHA after an incubation period than in the control.

TRAIL Tumor necrosis factor-related apoptosis-inducing ligand, or TRAIL, is a cytokine of the TNFα family. It is unique in that it possesses the ability to cause apoptosis in cancer cells but not in normal cells (18, 36). TRAIL possesses both death receptors and decoy receptors. These decoy receptors may be the mechanism by which normal cells avoid damage when exposed to TRAIL.

TRAIL may cause apoptosis in cancer cells by either of two pathways, the receptor-mediated or the mitochondrial. The initial steps of both pathways are identical, resulting in formation f DISC and auto-activation of caspase 8. If the amount of caspase 8 produced is sufficient, it will directly activate caspase 3 (36), which subsequently activates NF_kB (15). NF_kB then translocates to the nucleus and activates genes involved with apoptosis such as p53. This direct caspase cascade is known as the receptor-mediated pathway. If RIP is recruited to DISC, the result may be necrosis rather than apoptosis (34, 42).

In the event that the caspase 8 is insufficient to directly activate caspase 3, the mitochondrial pathway may alternately be triggered. The ability of apoptosis to be induced in a system containing low levels of caspase 8 indicates that minimal stimulation of the receptors is required to cause cell death in cells that are able to proceed by the mitochondrial pathway. This suggests that cells in which this mitochondrial pathway is selected will be more sensitive to the effects of TRAIL and respond to lower doses of it.

As expected, H460 showed greater sensitivity to TRAIL than Jurkat by both XTT and Annexin V-FITC. H460 is believed to undergo TRAIL-induced apoptosis by the mitochondrial pathway (10), a possible explanation of its heightened sensitivity as compared to Jurkat.

In H460, an interesting phenomenon occurs in which the greatest impact as seen by XTT appears in samples treated with a dose of 80 ng/ml. Doses higher than this appear to allow greater cell survival. It is believed that high levels of TRAIL may be able to induce necrosis rather than apoptosis and therefore cause the same kind of anomalous result as that seen in high doses of DHA. It is also possible that the apoptotic membrane

blebbing reaches such a degree at high concentrations of TRAIL that the membrane is able to cause a higher absorbance reading. It is not believed that the higher absorbance at these concentrations is indicative of greater cell survival.

CLB/DHA It was expected that the combination of CLB and DHA could result in synergy. DHA is able to alter the cell membrane causing nonspecific pores to open and increasing fluidity. CLB enters the cell by simple diffusion, and thus may be assisted in crossing the membrane if the membrane had been previously damaged or otherwise altered by the fatty acid. It is unknown if CLB activates a caspase cascade as observed with DHA, though some drugs have been proven to work in this manner. The combination of DNA damage by alkylation and the apoptosis cascade caused by DHA could also result in a synergistic interaction. Before experimentation began it was believed that this combination should result in synergy regardless of cell line.

At the conclusion of this study it was determined that high but sublethal doses (concentrations resulting in death of less than half of the sample) of each agent did indeed result in synergy, causing a greater than additive loss of viability by XTT analysis. Jurkat was somewhat more sensitive to this treatment than was H460, an expected result considering that Jurkat is vastly more sensitive to CLB alone than is H460.

For Jurkat, about 58% apoptosis was expected upon combination of CLB and DHA. The observed result was much lower at just over 42%, indicating an inhibition of the effect of the cytotoxic agents. This outcome is in direct opposition to that seen by XTT, in which synergy occurred in some concentrations.

This discrepancy is potentially due to the concentrations used. By XTT, synergy was only seen at certain concentrations that proved to be lethal by flow cytometry. It is possible that careful selection and analysis of other concentrations would also result in synergy by flow cytometry.

It is also possible that, as previously discussed, the combination will have resulted in necrosis, which would not be discernible by flow cytometry with Annexin V-FITC. Furthermore, phosphatidylserine relocates to the outer surface of the cell relatively early in the events of apoptosis. At this stage the cell may still be alive and capable of respiration, thus in this case a population of cells that appear apoptotic by flow cytometry may in fact appear alive by XTT. Patterns of forward and side scatter help to support this hypothesis, in that necrotic cells have more side scatter than live cells due to increased membrane complexity. In this study, cells treated with combined CLB and DHA were shown to have a slightly greater degree of side scatter than live cells. This discovery, combined with the lack of staining of this population, suggests that these cells were indeed necrotic (data not shown).

CLB/TRAIL Since CLB is non-phase specific, it is logical that its effect would be amplified by any cytotoxic agent working in any stage of the cycle. While no previous studies have attempted to combine CLB with TRAIL, other chemotherapeutic drugs have been shown to upregulate TRAIL receptors DR4 and DR5 and result in synergistic cytotoxicity (9).

It was suspected that the response to this treatment might be dissimilar between the two cell lines. Jurkat is highly sensitive to CLB, while H460's sensitivity at the

outset of this study was unknown. It has since been determined that it is much less sensitive than Jurkat to apoptosis induced by CLB. TRAIL, conversely, was known to be highly effective in causing cell death in H460 (36), while Jurkat was known to be only moderately sensitive to its effects (33).

TRAIL also apparently uses different pathways in causing apoptosis in each of these cell lines. Bcl_2, an anti-apoptotic protein active in the mitochondrial membrane (11), can protect H460 from TRAIL-induced apoptosis (10). This implies that TRAIL utilizes the mitochondrial pathway in H460 cells, a finding that is supported by H460's great sensitivity to the cytokine as previously discussed. In Jurkat cells, Bcl_2 conferred no such resistance, suggesting that the receptor-mediated pathway is selected (34). However, this selection does not appear to be absolute. Bcl_2 was able to slow apoptosis but not prevent it, and to inhibit release of cyt c in Jurkat (33). It appears that in the event that a step in the apoptosis cascade is blocked in the mitochondria, such as inhibition of cyt c release, Jurkat may be able to revert to a purely receptor-mediated pathway, indicating that mitochondria are not a critical component of the apoptotic pathway in this cell line.

It was expected prior to this study that CLB and TRAIL would combine in an additive manner due to their ability to function in the same phase of the cell cycle. Based on dose response curves for CLB and TRAIL individually, distinct patterns were evident. While there was a clear difference in sensitivity to TRAIL between the two cell lines, the difference was much greater in samples treated with CLB, with Jurkat being by far the more sensitive. Considering this sensitivity, Jurkat was expected to be more sensitive to treatment with combined CLB and TRAIL than H460.

As expected, in Jurkat, TRAIL combined synergistically with CLB resulting in cell viability 16-45% lower than the anticipated viability assuming only an additive effect. In one representative trial, an additive effect would have resulted in 69% viability; the observed viability was 29.3%. In H460 cells, synergistic effect was seen in the same concentrations as in Jurkat, but to a lesser degree. Overall, the prediction that Jurkat would display more sensitivity to combined TRAIL and CLB held true.

It is of some importance to note that Jurkat was made as sensitive to a dose of 50 ng/ml TRAIL as to a higher dose of 100 ng/ml by addition of CLB. This effect was not observed in H460. In Jurkat, in which TRAIL activates a receptor-mediated apoptotic pathway, perhaps DR5 was upregulated by addition of CLB resulting in greater binding of the receptor by the cytokine. In H460, in which TRAIL is thought to invoke the mitochondrial pathway, possible upregulation of the receptors may be inconsequential, as less binding of the ligand is required to activate this pathway. It also seems possible that the more direct receptor pathway results in apoptosis more quickly than the mitochondrial pathway, which requires several more steps that could be more time consuming. This might allow CLB and TRAIL to enter the nucleus in a time sequence that causes greater cell death. If the more involved mitochondrial pathway used in H460 were much slower, it could delay the arrival of the apoptosis signal from TRAIL, preventing it from combining with CLB with as dramatic an effect as was seen in Jurkat. The actual time sequence of each could be ascertained by treating samples of each cell line with a concentration of TRAIL known to induce apoptosis and at regular time intervals performing cell counts by trypan blue exclusion. This would allow the time required for

TRAIL to induce cell death in each cell line to be determined and the speed of the pathways compared.

In Jurkat, the synergistic effect observed previously by XTT was less apparent by flow cytometry., with total apoptosis slightly lower than what would be expected based on an additive model. The subtle synergistic effect H460 displayed by XTT assay also was not reproduced by flow cytometry, and the interaction was in fact sub-additive. As with the combination of CLB and DHA, the sub-additive effect seen here by flow cytometry is believed to be due to necrosis caused by the combined treatment.

Due to the previously determined ability of CLB to halt the cell cycle in the G_0 phase (22), and the knowledge that TRAIL is very active in the same phase (16), a small-scale experiment was designed to attempt to exploit this similarity. Samples from each cell line were incubated in the presence of varying concentrations of CLB, washed, and then treated with varying concentrations of TRAIL. The only significant restriction of proliferation was observed among cells pre-treated with 7.5 μM CLB and then incubated with 25 ng/ml TRAIL, in which a 30-40% decrease in viability was observed in both cell lines.

While this experiment indicated that pre-incubation was generally ineffective, potential remains for further investigation. Because a sample of cells growing in log phase are probably far from entering G_0, it is likely that the 6-hour pre-incubation was insufficient to allow all the cells to progress to this stage before being stopped there by the action of CLB. Hence, it is improbable that a sufficient percentage of the cells were in the desired G_0 stage when TRAIL was added. In addition, relatively low concentrations of CLB (15 and 25 ng/ml) were chosen. By XTT, synergy was seen in much higher

doses. It remains to be seen if a longer pre-incubation with CLB, followed by higher levels of TRAIL, would result in a more effective treatment.

TRAIL/DHA DHA is able to cause cell death in several ways, some of which may increase its capacity to synergize with TRAIL in Jurkat and/or H460. Both agents are capable of inducing both apoptosis in low doses and necrosis in high doses (34, 42). The receptor-mediated pathway TRAIL employs in Jurkat cells shares some later steps of its apoptotic pathway with DHA, beginning with the activation of caspase 3. TRAIL's mitochondrial pathway however is much more similar to that employed by DHA. Depolarization of the mitochondrial membrane and subsequent release of cyt c are the first in a series of steps leading to apoptosis that are identical in TRAIL and DHA.

While no previous studies have attempted to determine the interaction between DHA and TRAIL, pre-incubation with DHA resulted in an increase in apoptosis with a related cytokine, TNFα (29).

Based on the previously known information about each of these cytotoxic agents, certain outcomes were predicted. If TRAIL follows exclusively the mitochondrial pathway, this would permit the two agents to interact more efficiently by proceeding through the same mitochondrial steps and activating the same cascade. Co-administration of the two would allow DHA to enter the cell through the membrane while TRAIL simultaneously bound DR4 or DR5 and initiated apoptosis. TRAIL-induced apoptosis via the receptor pathway is expected to be somewhat less effective, because it allows less interaction as the pathways are somewhat different.

H460 is expected to be sensitive to the combined effect of TRAIL and DHA because the use of the same pathway by both agents would maximize the interaction. Jurkat, in which TRAIL apparently works by the receptor-mediated pathway but with components of the mitochondrial, is expected to be somewhat more sensitive than H460. While aspects of the DHA pathway will overlap with the mitochondrial portion of the TRAIL pathway, a separate, more direct receptor-mediated cascade may also be activated. The interaction in H460 is expected to be of a lesser degree than in Jurkat due to Jurkat's ability to utilize aspects of both pathways, potentially increasing the strength of the overall apoptotic signal.

By XTT assay, the prediction that Jurkat would be more sensitive to combined TRAIL and DHA than H460 was proven accurate; however, in the majority of experiments involving an array of concentrations, the result was additive or sub-additive in both cell lines.

H460 was overall slightly less sensitive to the effect of this combined treatment than was Jurkat, in some ways a surprising revelation considering H460's significantly greater sensitivity to TRAIL. The combination of 10 μM DHA with 100 ng/ml, however, did result in some level of synergy in both cell lines. This effect was not reproduced in any other combination.

A possible explanation for this result is that each cell has a limited amount of cyt c, caspase 3, and other components of the apoptotic pathways. While it is intuitive that administration of two agents that work in the same manner would increase apoptosis, it is made apparent by this study that this is not the case. To examine this theory, cyt c may be considered. In H460, DHA causes a release of cyt c from the mitochondrial

membrane. TRAIL, in its mitochondrial pathway, also induces release of cyt c from the same membrane. If the entire store of cyt c is released in response to only one agent, the other will not be able to cause the same release therefore its signal will not be conveyed. The same may be said in regard to other elements of apoptosis including caspase 3. Once the entire volume has been depleted by one agent, the effect of the second agent will be nullified.

In Jurkat cells, the same concept can be considered in which apoptotic molecules are consumed by one agent. However, it appears that in Jurkat, if the apoptotic signal is blocked at the mitochondria it can create a loop in which it returns to the initial stages of the cascade and initiates the receptor-mediated pathway. This would explain the slightly higher sensitivity seen here in Jurkat as compared to H460.

By flow cytometry, the combination of TRAIL and DHA seemed to inhibit apoptosis in both Jurkat and H460. In Jurkat, a reduction in proliferation of about 63% was expected based on addition of the effectiveness of each agent. The actual reduction was much lower, at only 42.5%, less than the effect caused by DHA alone. In H460, loss of viability of 35.5% was expected. The actual reduction was only 14%, lower than that caused by TRAIL alone.

It would appear based on these numbers that in Jurkat, TRAIL is inhibiting DHA, while in H460 the opposite is true. A difference in the rate of each pathway could explain this phenomenon. In Jurkat, TRAIL via the receptor pathway is able through a caspase cascade to quickly and directly convey its apoptotic signal. Meanwhile the signal produced in the mitochondria by DHA could be somewhat slower. It is possible then, that as previously suggested, TRAIL essentially is able to race ahead of the DHA signal, in

the process consuming some of the critical elements required to send that signal such as caspase 3.

In H460, it appears that DHA is able to enter the mitochondria and initiate its cascade ahead of TRAIL, which is involved in the process of cleaving Bid and the translocation of tBid to the mitochondrial membrane. It is possible that by the time tBid arrives, membrane depolarization and release of cyt c has already occurred, effectively causing TRAIL's apoptotic signal to be lost. This would imply that the effect of TRAIL would be abrogated by the presence of a sufficient concentration of DHA. This may not, however, completely inhibit TRAIL. It is possible that TRAIL will retain some ability to alternately cause cell death by necrosis rather than by apoptosis. This would allow the loss of viability to be greater than that caused by DHA alone, yet not as great as that of TRAIL alone, as is seen here.

The effectiveness of pre-incubation was not assessed in this study. It would be of interest to attempt to pre-incubate samples of each cell line in DHA followed by incubation with TRAIL. This procedure resulted in an increase in apoptosis with a cytokine from the same family as TRAIL, TNFα, against the human leukemia cell line U937 (41). It is unknown if DHA is able to upregulate the expression of the cytokine receptors, or if all the members of the TNF superfamily would exhibit the same interaction.

TRAIL/CLB/DHA To summarize what has been discovered to this point, it has thus far been determined by this study that CLB produces a synergistic effect when combined with DHA in Jurkat, and to a slightly lesser extent in H460 as well, probably due to

Jurkat's greater sensitivity to CLB. By flow cytometry, no synergistic effect was observed among the concentrations tested.

Synergy was also observed among samples treated with combined CLB and TRAIL when analyzed by XTT. Jurkat again was somewhat more sensitive to this combination. By flow cytometry, the effect was much less distinct. Synergy was still seen in Jurkat cells but to a lesser degree than was seen by XTT. In H460 the combination was sub-additive, indicating that one agent was possibly interfering with the activity of the other, or that they had induced necrosis, which could not be ascertained using Annexin V.

Upon combination of TRAIL and DHA, synergy was only observed sporadically in both cell lines by XTT assay. H460 was somewhat less sensitive to this combination, but overall TRAIL combined with DHA resulted in an additive or sub-additive effect in both Jurkat and H460. By flow cytometry, the combinations tested appeared to be inhibitory, in that the percent of apoptotic cells was lower in samples treated with both cytotoxic agents than in samples treated with just one of the two agents.

Considering all the information gathered here, expectations for the combination of all three agents were developed. It was expected that the positive effect seen when CLB is combined with either TRAIL or DHA would outweigh the negative impact of the interaction between TRAIL and DHA. Reduction in viability was anticipated to be greater in Jurkat than in H460, because Jurkat showed greater sensitivity in every combination, and was less effected by the inhibition of apoptosis seen with combined TRAIL and DHA.

In H460, the effect of inhibition that DHA appears to exert on TRAIL was expected to occur. DHA is likely to still synergize with CLB, but will inhibit the action of TRAIL, perhaps preventing TRAIL's synergistic action with CLB. In this cell line, the combination of CLB with DHA is more effective than CLB with TRAIL, thus the inhibition of TRAIL by DHA was not expected to be a major hindrance in overall reduction of viability. An additive effect was anticipated, but not expected to be much greater than the effect induced by CLB and DHA in combination.

In Jurkat, TRAIL appears to inhibit the action of DHA. It is possible that this could inhibit the ability of DHA to synergize with CLB. TRAIL and CLB are expected to combine normally, a combination that proved to be synergistic and more effective in reducing cell viability in Jurkat than in H460. Due to the high sensitivity of Jurkat to CLB and to the combination of CLB with TRAIL, the inhibition of DHA by TRAIL was not expected to be significant. It was anticipated that the combination of all three agents would combine in a synergistic fashion, and that the small amount of DHA would have little effect on the combined influence of TRAIL and CLB. It is also suspected that Jurkat will be more sensitive to the combined action of the three agents than H460.

When this experiment was carried out, it was observed that Jurkat was, as expected, significantly more sensitive to combined TRAIL/CLB/DHA than was H460. The combination of all three agents resulted in only about 20% viability by XTT, as compared to the approximately 60% viability seen when CLB is combined with TRAIL but without DHA. This effect was about 20% greater than expected, and proved to be much more effective than the combination of CLB with DHA or CLB with TRAIL.

By flow cytometry, the effect of the three agents was sub-additive and overall lower than the effect induced by DHA alone, again assumed to be due to a substantial percentage of necrotic cells.

It is proposed that despite the inhibition of DHA by TRAIL within Jurkat cells, the initial external effect of DHA on the cell membrane is unaffected by this inhibition. If DHA works with CLB by increasing the fluidity of the membrane and thus assisting CLB in its diffusion, the intracellular inhibition of DHA by TRAIL would have no effect on its extracellular actions that result in increased effectiveness of CLB. This would in effect result in more CLB inside the cell, therefore more CLB capable of combining with TRAIL, a collaboration that has proven to be synergistic. This increased amount of CLB inside the cell could heighten this effect, resulting in the overall lower viability in Jurkat cells treated with combined DHA, CLB, and TRAIL as seen here by XTT.

In H460 cells, the impact of treatment with all three agents was less profound than in Jurkat. H460 cells were shown in this study to be more sensitive to TRAIL but less affected by CLB, DHA, and combinations of CLB and DHA, as well as to combined TRAIL and DHA. Despite this seemingly imperturbable nature of H460, the combination of all three agents was effective. A viability of about 85% was seen when CLB was combined with TRAIL; addition of DHA reduced this viability to about 60%, 20-25% less than expected based on an additive model.

This effect is not believed to be due to combination of CLB and DHA, as previous experiments showed little impact with this combination in the concentrations used here. In fact, in this experiment the combination of CLB and DHA in the chosen concentrations appeared to be entirely inactive, while combined TRAIL and CLB caused

some reduction in viability. It is proposed that the synergistic impact seen here in the combination of all three agents is the same as that suggested for Jurkat. Extracellular DHA will increase the fluidity of the cell membrane, allowing CLB to cross the membrane freely, resulting in a higher level of CLB within the cell where its effects can combine with the apoptotic signal produced by TRAIL.

As mentioned previously however, intracellularly in H460 cells, DHA may be able to partially or completely inhibit TRAIL activity. This inhibition, as well as H460's mild sensitivity to CLB in general, could be responsible for the three-agent combination being overall less effective in H460 than in Jurkat despite the increase in CLB available to combine with the effect of TRAIL. DHA essentially provides more CLB to combine with TRAIL, but then partly inhibits the activity of TRAIL, apparently resulting in this case in greater apoptosis than in any combination of two cytotoxic agents, but less than is seen in Jurkat.

Upon examination by flow cytometry, a different picture emerged in which the combination of the three agents was sub-additive. The expected percentage of apoptosis was just over 53%, while the observed value was only about 29%, again attributed to the inability of the chosen fluorophore to stain necrotic cells.

Suggestions for further research

In order to clarify the results obtained in this study and to confirm or refute theories of interaction that have been set forth here, many research opportunities arise. The first of these is to attempt to consolidate the results obtained by XTT assay with those from Annexin V-FITC analysis. It is likely that the most viable and effective methodology

would be to repeat the experiments while including a second fluorophore, such as propidium iodide (PI), which will stain any cell that is dead regardless of whether it died by apoptosis or necrosis. PI binds to nucleotides within cells that it is able to enter via a damaged membrane, an indicator of cell death. It is not specific for apoptosis as is Annexin V, which binds only to phosphatidylserine in the outer leaflet of the plasma membrane. The use of a non-specific fluorophore like PI would allow all the cells that are dead to be visualized. Analysis with a fluorophore exclusively indicative of apoptosis leads to vastly different results than those obtained by XTT assay, which is essentially a colorimetric indicator of cellular respiration. It is likely that PI would give results much more similar to those seen in XTT. The use of both Annexin V and PI together is essential to visualize the proportion of cells that have died of apoptosis, a result which is clearly preferable in cancer research because in a living system, apoptosis does not lead to the undesirable inflammatory immune reaction that is caused by necrosis.

A more time-consuming but potentially useful technique for evaluating the accuracy of the XTT assay is trypan blue exclusion. This cell counting technique is simple and widely used, and is non-specific; that is, it stains dead cells regardless of the way in which they died. This method could be valuable especially for Jurkat cells, but somewhat less so for H460 which has shown some resistance to trypan blue staining. Other assays similar to XTT could also be used to confirm observed results; some of these include MTT and Aq One, both cell proliferation assays that work on the same basis as XTT. Other similar techniques include colorimetric assays that measure caspase levels, knowledge that could be useful in distinguishing apoptosis from necrosis and in determining a preferred apoptotic pathway in cell samples.

In the flow cytometry experiments performed in this study, a few sublethal concentrations were evaluated for synergy. It could be of some value to attempt to expand this study using other sublethal concentrations, as previously suggested, because it appears that in some cases, only certain very specific concentrations are able to combine synergistically. There is a distinct possibility that in situations that synergy was expected but not observed, other un-tested concentrations may actually combine in the anticipated synergistic manner.

To evaluate concepts proposed here regarding interaction between TRAIL, CLB, and DHA, several methods may be utilized. The suggestion for instance that TRAIL and DHA inhibit each other by consumption of the molecules of apoptosis could be examined using assays specific for those molecules, such as cyt c or caspase 3. Colorimetric assays exist that are capable of measuring levels of these molecules, and Western blotting using antibodies specific for the desired molecule would provide a very accurate measure of their presence or depletion.

The concept presented here that the timing of the arrival of the apoptosis signal in the nucleus has some bearing on the ability of two or more cytotoxic agents to synergize could be evaluated simply by measuring the time required for each agent individually to cause cell death in each cell line. This could be readily done by addition of the desired agent to the cells, followed by a range of incubation times after which each sample would be counted by trypan blue exclusion. This would answer the question, for instance, of whether the effect of combined TRAIL and CLB is less in H460 than in Jurkat because the mitochondrial path taken by TRAIL in H460 prevents the apoptotic signals of the two agents from arriving in the nucleus at the same time. This would also determine if one

apoptotic pathway induced by TRAIL is more time-consuming than the other, as has been proposed here.

The ability of the TRAIL receptors, DR4 and DR5, to be upregulated by CLB, DHA and combined CLB/DHA should also be explored. Some aspects of this study could be readily explained by the upregulation of the receptors in response to CLB. This could be carried out by Western blotting using lysate from each cell line and antibodies against the receptors.

Pre-incubation experiments for TRAIL with CLB varying the time of pre-incubation, as well as the concentration of each agent, would be of great significance in determining if the cells can be induced to enter the G_0 stage of the cell cycle, where TRAIL is most active. Pre-incubation using DHA and TRAIL would also be of interest, as it has been shown that DHA pre-incubation increases the action of a cytokine from the same family as TRAIL. Conversely, an inhibitory effect was seen here resulting from concurrent administration of DHA with TRAIL. It is unclear if this inhibition of apoptosis is due to cell line, simultaneous administration, or intrinsic differences between TRAIL and TNFα.

Finally, in order to determine precisely how each agent and each combination imparts its effects on the cells, micro array analysis would be invaluable in determining which genes are activated or de-activated in response to each treatment. It would be of great use in analyzing complex interactions such as the three-agent synergy observed in this study, as well as simpler effects such as the sudden plummet in viability seen in both cells lines when treated with 30 μM DHA.

Summary In summary, this study was designed to evaluate the ability of TRAIL, CLB, and DHA to synergize resulting in loss of viability in Jurkat and H460 human cancer cells. It was determined that synergy can occur among certain combined concentrations of CLB with DHA and CLB with TRAIL in both cell lines, but not in TRAIL with DHA in either cell line. Furthermore, synergy did occur to a greater extent upon simultaneous administration of all three cytotoxic agents, each in sublethal doses, than was observed in combinations of any two agents. The effect was greater in Jurkat than in H460, a theme that applied to every combination and to every agent individually except TRAIL. In many cases, H460 is proposed to be less sensitive to the effect of some combinations because in this cell line, TRAIL proceeds by a complex, potentially slower mitochondrial pathway rather than the much more direct receptor-mediated pathway used in Jurkat. Occasionally a single experiment resulted in an overwhelmingly synergistic response, such as the nearly 90% synergy seen in H460 cells treated with CLB and DHA.

It was theorized that synergy occurred upon treatment with all three agents because DHA first alters the cell membrane, facilitating diffusion of a greater amount of CLB than would otherwise be possible. Once inside the cell, CLB is able to then interact synergistically with TRAIL as was previously established.

TRAIL and DHA were proposed to inhibit each other because they share essentially the same pathway and require the same molecules of apoptosis in order to convey their death signals. Since there is a potentially limited supply of these molecules, one agent may consume all of a required component resulting in inhibition of the second agent.

Technical considerations were also explored, including the scope of the XTT proliferation assay as compared to the Annexin V-FITC assay. It was determined that the differences between the two are due to the fact that XTT can not differentiate between necrosis and apoptosis, while Annexin V binds only apoptotic cells leaving necrotic cells indistinguishable from live cells.

In general, it is believed that the work presented here provides significant evidence that the combination of TRAIL, CLB, and DHA as explored in this study has potential for clinical use. TRAIL and DHA are non-toxic to normal cells, and while CLB is very toxic, the concentration when combined with other agents may be reduced to a level that is within the tolerable range. This study provides a framework for additional research perhaps leading in the future to animal experimentation and eventually clinical consideration.

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