Chapter 5: Synergistic Action of Chemotherapeutic Drugs and Membrane Fluidisers

5.1. Introduction

This chapter explores the synergistic action of low-dose membrane fluidising treatments and chemotherapeutic agents on Jurkat cells and primary CLL cells. Furthermore, the localisation of Hsp72, Hsp60, Hsp27 and Hsp90 in these treated cells is investigated.

Resistance to therapy by tumour cells is a major problem in cancer management. Therefore improving the efficacy of chemotherapeutic agents is of major importance in the development of cancer treatment. While increasing the chemotherapy dosage may help to alleviate this problem, this in turn may result in haematological cytotoxicity and increased risk of damage to non-transformed cells. Therefore, it would be of huge benefit if combination treatments utilising membrane fluidisers could allow more efficient delivery of such agents and become more mainstream in the clinical setting.

5.1.1. Cell Membrane Fluidity

The fluidity of a cell membrane is dependent upon several key factors. The first, involves the transverse diffusion of molecules from one monolayer to the other. For example, phospholipidmethyltransferases convert phosphatidylethanolamine to phosphatidylcholine and move this product from the inner to the outer monolayer (Goldstein, 1984). This movement results in changes to membrane fluidity. The flexibility of acyl chains within the bilayer also contributes to overall fluidity, as under normal circumstances, a gradient of flexibility in these chains exists from the surface towards the core of the bilayer, with increasing freedom of movement near the core. Cell membrane fluidising agents act to decrease the packing density of phospholipids, allowing all membrane components to move against each other more often but less strongly than normal (Goldstein, 1984).

The aliphatic alcohols BA, ethanol, PhA and heptanol have been shown to significantly increase membrane fluidity (Moulin et al. 2007a; Nagy et al. 2007; Balogh et al. 2005). Furthermore, the degree of membrane fluidity caused by these alcohols has been shown to be proportional to the length of the carbon chain (Goldstein, 1984). It is thought that
alcohols act to fluidise the cell membrane by intercalating between membrane lipids. This is assumed to cause a weakening of the van der Vaals forces between the lipid acyl chains, resulting in a disordering effect within the lipid bilayer (Shigapova et al. 2005; Balogh et al. 2005). Mild hyperthermic treatment has also been shown to increase the fluidity of the cell membrane in various cell types (Balogh et al. 2005; Moulin et al. 2007a).

In addition to alcohols and heat treatment, membrane fluidity has been shown to be affected by local anaesthetics. Bupivacaine is an anaesthetic agent used for epidural anaesthesia, brachial plexus anaesthesia and peripheral nerve block. The anaesthetic effects of this chemical, and other similar agents, have been shown, by a number of studies, to be caused by cell membrane fluidisation (Mizogami et al. 2002). Fluidisation of the membrane results in dysfunction of ion channels, receptors and enzymes associated with nerve cell membranes, contributing to local anaesthesia. It has been shown that cell membrane fluidisation can result from bupivacaine concentrations much lower than those used in the clinical setting. Similarly to alcohols, the degree of resultant membrane fluidisation appears to be dependent upon the structure of the anaesthetic agent and its hydrophobic property.

5.1.2. Combination Therapy

In an attempt to improve the efficacy of chemotherapeutic agents, researchers have introduced thermochemotherapy, the combination of a cytotoxic drug and hyperthermia (Hahn et al. 1975; Urano & Kim, 1983; Westermann et al. 2001; Bakhshandeh et al. 2003; Westermann et al. 2003 and Takemoto et al. 2003). This concept is based on the idea that heat shock, by fluidising the cell membrane, will result in more efficient delivery of the drug. The result would allow the use of much lower drug concentrations, therefore limiting the damage to non-transformed cells. Chemotherapy alone, involves a reduced or delayed administration of the next treatment cycle due to haematological cytotoxicity. This in turn results in a decrease in therapeutic efficiency (Debes et al. 2005). To date, many different families of cytotoxic drug have been combined with hyperthermia including alkylating agents, anthracycline antibiotics (Braun & Har-Kedar 1975) and platinum-based compounds (Robins et al. 1995; Westermann et al. 2001). Clinical trials have used various methods of applying hyperthermia treatment including local
hyperthermia, whole-body hyperthermia and perfusion heating (Bornstein et al. 1995). However, trials of thermochemotherapy have been limited due to the clinical shortcomings involved in heat delivery to tumours and the contradictory induction of HSPs and thermotolerance (Urano et al. 1999).

Early in-vivo studies on mice investigated the effect of treatment with different doses of Cyclo (Urano & Kim, 1983) or cis-DDP (Lindegaard et al. 1992) before immediate local heating at different temperatures. A thermal enhancement of cytotoxicity was seen in these studies. In-vitro studies have used a wide range of chemotherapeutic agents and have also observed synergism between hyperthermia and drug (Urano et al. 1985). However, this is not the case for all cytotoxic agents. Monge et al. (1988) showed that cytotoxicity of methotrexate and vincristine could not be enhanced by hyperthermia, while Urano et al. (1999) demonstrated that heat treatment did not affect the efficacy of 5-fluorouracil, mitomycin or Dox. In contrast, preliminary experiments performed by Hahn et al. (1975) demonstrated that combining hyperthermia with doxorubicin treatment increased the cytotoxicity of this anthracycline drug. Ohtsubo et al. (2000) showed that treatment of hamster V-79 cells with Dox immediately following heat shock at 44°C resulted in decreased cell survival when compared to heat shock or Dox treatment alone.

However, pharmokinetics appear to play a critical role in the phenomenon of thermal enhancement. Some cytotoxic agents have a reduced plasma half life compared to others while certain drugs such as Cyclo require prior activation in the liver and remain in the circulation significantly longer.

In clinical trials, attention has been focused on methods of heat delivery. Robins et al. (1993) and Wiedemann et al. (1994) have investigated the use of whole body hyperthermia (WBH), while Olieman et al. (1999), Klaase et al. (1994) and Fujimura et al. (1990) have studied the use of isolated perfusion. This method has proved highly effective, with one study on gastric cancer patients showing a recurrence rate of 27% after hyperthermic intraperitoneal perfusion (HIPP), compared with 94% in the drug-only treated patients (Fujimura et al. 1990). The use of a local hyperthermia delivery method such as HILP has many advantages over WBH. Firstly, the biological half life of the drug is longer due to limited intrusion into the systemic circulation and subsequent breakdown and excretion. Secondly, the isolated application of the drug allows the use of up to a 10-fold higher drug concentration without resulting in systemic damage (Lindegaard et al. 1992).
1992). Thirdly, it allows a relatively uniform temperature distribution throughout the target tissue. In the clinical setting, the blood supply to the tumour is a major factor in this combination therapy. Due to the rate of growth surpassing the rate of angiogenesis, the neoplasm often has insufficient vascular infiltration and so the diffusion distance for the drug is increased. It is proposed that mild hyperthermic treatment results in vasodilation and an increase in tumour oxygenation which in turn results in an improvement of the blood supply, allowing more effective delivery of the cytotoxic agent (Horsman & Overgaard, 1997; Song et al. 1996).

As mentioned previously, supplementary to hyperthermia, membrane fluidity has also been shown to be modified by alcohol compounds and local anaesthetics (Balogh et al. 2005; Nagy et al. 2007; Moulin et al. 2007a; Mizogami et al. 2002). Furthermore, BA, ethanol and hyperthermia have been shown to stimulate the cytotoxic effects of TRAIL against both Jurkat cells and primary CLL cells (Moulin et al. 2006; Moulin et al. 2007b). This stimulation was shown to be due to an increase in ceramide levels (Moulin et al. 2007a) which could be reversed by adding a ceramide inhibitor. Increased TRAIL receptor recognition was also observed after combined TRAIL and alcohol treatment, but not after TRAIL or alcohol treatment alone. This suggests that the enhanced cell death observed when treating with BA, ethanol or heat may be specific to TRAIL, or at least death-receptor ligands. Additionally, the concentration of TRAIL used in these studies results in approximately 50% cell death when used in isolation, and the concentrations of alcohol used to stimulate TRAIL also result in a significant reduction in cell viability when used in isolation (Moulin & Arrigo, 2006). Therefore, there would be no advantage to using this combination based therapy in the clinical setting.
5.1.3. Aims

This chapter will investigate whether compounds that result in fluidisation of the cell membrane can enhance the cytotoxicity of four chemotherapeutic agents. Sub-lethal doses of each treatment, that do not result in early-apoptosis or reductions in cell viability, will be determined and then combined in an attempt to reduce the chemotherapeutic dosage required for cell death to occur. Combination treatments will be performed on both Jurkat cells and primary CLL cells and will involve different families of cytotoxic drugs including a death receptor ligand (TRAIL), a topoisomerase inhibitor (Doxorubicin), an alkylating agent (Cyclophosphamide) and a member of the statin family (Lovastatin). This will establish if stimulation by fluidisers is drug specific. The effect of each membrane fluidiser on localisation of Hsp72, Hsp60, Hsp27 and Hsp90 will also be investigated.
5.2 Methods

Local research ethics committee approval (COREC - 05/Q1506/103) was obtained for this study and consent forms were completed by each patient. Blood samples were collected from patients affected by CLL (n= 40) by venepuncture in 7ml EDTA tubes.

Combination treatments were performed on both primary CLL cells and Jurkat cells and the degree of apoptosis was assessed using a caspase-3 assay and an Annexin V/PI assay. The localisation of HSPs in Jurkat cells was determined by flow cytometry and ELISA.

5.2.1 Cell Culture

Jurkat E6.1 cells were cultured according to the methods described in section 2.2.2.

5.2.2. Drug Treatment

Cells were treated with the following membrane fluidising and/or chemotherapeutic treatments according to the methods described in section 2.2.6:

- Benzyl Alcohol
- Ethanol
- Phenethyl Alcohol
- Bupivacaine
- Mild Hyperthermia
- Tumour Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL)
- Doxorubicin
- Cyclophosphamide
- Lovastatin

TRAIL, doxorubicin, benzyl alcohol, ethanol, phenethyl alcohol and bupivacaine treatments were performed over a period of four hours at 37°C. Cyclophosphamide and lovastatin treatments were performed over a period of 24 hours at 37°C. Once the sub-lethal concentrations of these drugs had been established using dose-response experiments, the chemotherapeutic agents were combined with heat shock and alcohol treatments. In experiments involving combination treatments of cyclophosphamide and lovastatin, benzyl alcohol, ethanol, phenethy alcohol and bupivacaine were removed after
four hours and cyclophosphamide and lovastatin alone were reapplied for the remaining twenty hours.

In experiments involving pre-treatments, cells were treated with 2.5mM methyl-β-cyclodextrin (mβcd) for two hours or mild hyperthermia (one hour at 42°C followed by a three hour recovery at 37°C) before being washed and either treated or not treated with doxorubicin and ethanol.

5.2.3 Caspase-3 Plate-based Assay
Levels of active caspase-3 were analysed following drug treatment to determine the degree of apoptosis occurring. This was done according to the methods described in section 2.2.7.

5.2.4. Propidium Iodide Assay
The Propidium Iodide assay was performed following drug treatment to determine the levels of necrosis in these cells. This was done according to the methods described in section 2.2.8.

5.2.5 Annexin V/Propidium Iodide Assay
Levels of apoptosis and necrosis in drug-treated cells were also determined using the AnnexinV/Propidium Iodide assay on the flow cytometer. This was done using methods described in section 2.2.3.3

5.2.6 Flow Cytometry
5.2.6.1 Surface and intracellular Hsp27, Hsp60, Hsp72 and Hsp90 Analysis
Surface and intracellular HSP analysis was performed according to the methods described in sections 2.2.3.4 and 2.2.3.5

5.2.6.2 DR4/DR5 Analysis
DR4 and DR5 analysis was performed according to the methods described in sections 2.2.3.8
5.2.7 Fluorescence Microscopy

5.2.7.1 DR4/DR5 Staining

DR4/DR5 staining was performed on Jurkat cells to confirm the presence of functioning TRAIL receptors. This was done according to methods described in section 2.2.9.1

5.2.7.2 Hoechst Staining

Hoechst staining was performed on drug treated cells to assess the level of morphological damage caused by the combination treatments. This was done according to methods described in section 2.2.9.2

5.2.8 Statistical Analysis

Statistical analysis was performed using the one-way-ANOVA with Dunnet’s or Bonferroni’s post hoc tests depending on the data analysed: P values <0.05 were considered to be significant. * represents a p value < 0.05, ** represents a p value < 0.01 and *** represents a p value < 0.001

Graphs representing relative values were constructed by calculating a mean of the control values and dividing each individual treatment value by the mean of the control.
5.3 Results

5.3.1 Determination of Sub-Lethal Doses of Membrane Fluidising Treatments

The cells, at a concentration of $3 \times 10^5$ cells/well, were subjected to varying concentrations of benzyl alcohol (BA), ethanol, phenethyl alcohol (PhA) and the local anaesthetic bupivacaine for four hours. Jurkat cells were also exposed to varying durations of heat shock treatment at 42°C. Apoptosis and cell viability were then analysed using the Caspase-3 and Propidium Iodide fluorometric plate assays. The dose response curve shown in Figure 5.3.1 shows that a concentration of 20mM of BA is required to induce significant apoptosis ($p<0.01$), while concentrations above this result in necrosis. An ethanol concentration of 2.5% is sufficient to result in a significant increase in caspase-3 activity ($p<0.01$) and significant necrosis (Figure 5.3.2). A PhA concentration of 5mM resulted in a significant increase in caspase-3 activity, while concentrations above this resulted in significant necrosis (Figure 5.3.3). A significant increase in caspase-3 activity was observed when cells were treated with bupivacaine concentrations of 0.5mg/ml and 1mg/ml, while concentrations of 1mg/ml and 2mg/ml resulted in significant necrosis (Figure 5.3.4). Jurkat cells were also subjected to varying durations of heat shock treatment at 42°C followed by a three hour recovery period at 37°C. Heat shocking for two hours followed by a three hour recovery period resulted in significant apoptosis ($P<0.01$) and necrosis ($p<0.05$) (Figure 5.3.5). Based on these results, 5mM BA, 1.25% ethanol, 2.5mM PhA and 0.25mg/ml bupivacaine were considered sub-lethal. A one hour heat shock at 42°C followed by a three hour recovery period was also considered sub-lethal.

To confirm the sub-lethal nature of the BA, ethanol, PhA, bupivacaine and heat shock treatments, an Annexin V/Propidium Iodide assay was performed on the treated Jurkat cells (Figure 5.3.6). Greater than 90% of the total cell population in each treatment group was negative for both Annexin V and Propidium Iodide staining and therefore considered viable.
Figure 5.3.1: Analysis of apoptosis and necrosis in BA-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with benzyl alcohol for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01

Figure 5.3.2: Analysis of apoptosis and necrosis in Ethanol-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with ethanol for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01
Figure 5.5.3: Analysis of apoptosis and necrosis in PhA-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with phenethylalcohol for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01.

Figure 5.3.4: Analysis of apoptosis and necrosis in Bupivacaine-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with bupivacaine for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01.
Figure 5.3.5: Analysis of apoptosis and necrosis in heat-treated Jurkat cells. Caspase-3 and Propidium Iodide assays performed on Jurkat cells heat shocked at 42°C for varying lengths of time, followed by a three hour recovery period at 37°C. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical Analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. * represents a p value < 0.05. ** represents a p value < 0.01
Annexin V and Propidium Iodide assay was performed on Jurkat cells treated with (A) benzyl alcohol (5mM for four hours), (B) ethanol (1.25% for four hours), (C) phenethylalcohol (mM for four hours) and (D) bupivacaine (0.25mg/ml for four hours) or (E) heat shock (1 hour at 42°C followed by a 3 hour recovery at 37°C). Numbers indicate percentage of non-viable cells.

Figure 5.3.6: Cell viability analysis in fluidiser-treated Jurkat cells.
5.3.2 Determination of Sub-Lethal Doses of Chemotherapeutic Agents

Jurkat cells were exposed to varying concentrations of doxorubicin (Dox), cyclophosphamide (Cyclo) and lovastatin to determine sub-lethal concentrations for each drug. Apoptosis and cell viability were analysed using the Caspase-3 and Propidium Iodide fluorometric plate assays. The dose response curve shown in figure 5.3.7 shows that Dox concentrations of 15µg/ml, 20µg/ml and 25µg/ml resulted in a significant increase in caspase-3 activity, while Dox concentrations of 20µg/ml and 25µg/ml caused significant necrosis. Cyclo concentrations of 1000µg/ml resulted in a significant increase in caspase-3 activity (p<0.05) and a significant decrease in cell viability (p<0.05) when compared to control (figure 5.3.8). Lovastatin concentrations of 25µM and 50µM resulted in a significant increase in caspase-3 activity, while concentrations of 25µM and above resulted in a significant decrease in cell viability (Figure 5.3.9). Based on these results, Dox concentrations of 10µg/ml, Cyclo concentrations of 100ug/ml and lovastatin concentrations of 12.5µM were considered sub-lethal.

In contrast to Dox, Cyclo and lovastatin, target cells require the presence of the functioning cell-surface death receptors DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2) to be sensitive to TRAIL. Therefore, Jurkat cells were analysed for the presence of DR4 and DR5 using flow cytometry and fluorescence microscopy. The distinction between DR4 and DR5 and the presence of these receptors on the surface of Jurkat cells can be seen in Figures 5.3.10B, C, D and E. DR4 is expressed on 33.5% of the Jurkat cell population, while 92.5% of the population expresses DR5 (Table 5.3.1). Apoptosis and cell viability were then analysed in TRAIL-treated Jurkat cells. A TRAIL concentration of 50ng/ml resulted in a 7-fold increase in caspase-3 activity, while concentrations of 100ng/ml and above resulted in both significant apoptosis and necrosis (Figure 5.3.11). Concentrations of 25ng/ml and below were not significantly different from the control and were therefore considered to be sub-lethal.

To confirm the sub-lethal nature of the Dox, Cyclo, Lov and TRAIL treatments, an Annexin V/Propidium Iodide assay was performed on the treated Jurkat cells (Figure 5.3.12). Greater than 90% of the total cell population in each treatment group was negative for both Annexin V and Propidium Iodide staining and therefore considered viable.
Figure 5.3.7: Analysis of apoptosis and necrosis in Doxorubicin-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with Doxorubicin for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01.

Figure 5.3.8: Analysis of apoptosis and necrosis in Cyclophosphamide-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with Cyclophosphamide for twenty four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. * represents a p value < 0.05.
Figure 5.3.9: Analysis of apoptosis and necrosis in Lovastatin-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with Lovastatin for twenty four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. * represents a p value < 0.05. ** represents a p value < 0.01.
Figure 5.3.10: Expression of DR4 and DR5 on Jurkat cells.
DR4 and DR5 analysis of Jurkat cells was performed using the flow cytometer (A, B and C) and fluorescence microscope (D and E). (A). Histogram of no stain control sample. (B). Histogram of DR4-associated fluorescence (C). Histogram of DR5 associated fluorescence. (D). DR4 staining (red) and DAPI staining (blue) of jurkat cells. (E). DR5 staining (red) and DAPI staining (blue) of jurkat cells.

Table 5.3.1: Percentage of Jurkat cells positive for DR4 and DR5 as determined by flow cytometry.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage of Jurkat cells positive for Protein Mean (± SD)</th>
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<tbody>
<tr>
<td>DR4</td>
<td>33.5 (4.4)</td>
</tr>
<tr>
<td>DR5</td>
<td>92.5 (2.65)</td>
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Figure 5.3.11: Analysis of apoptosis and necrosis in TRAIL-treated Jurkat cells. Caspase-3 and Propidium Iodide analysis of Jurkat cells treated with TRAIL for four hours. Data is represented as relative fluorescence when compared to the mean of the control group. Statistical analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant results are indicated. ** represents a p value < 0.01.
Figure 5.3.12: Cell viability analysis in drug-treated Jurkat cells.
Annexin V and Propidium Iodide assay was performed on Jurkat cells treated with (A). Doxorubicin (5µg/ml for four hours), (B). Cyclophosphamide (100µg/ml for twenty four hours), (C). Lovastatin (12.5µM for twenty four hours) and (D). TRAIL (25ng/ml for four hours). Numbers indicate percentage of non-viable cells.
5.3.3 Combination Treatment of Jurkat cells

Sub-lethal treatments of membrane fluidiser and chemotherapeutic agent were combined to determine if the efficacy of the drugs could be enhanced via fluidisation of the membrane by the alcohol compounds, local anaesthetic or heat treatment. As expected, TRAIL, BA, ethanol, PhA, bupivacaine or heat shock applied in isolation did not result in a significant increase in caspase-3 activity (Figure 5.3.13). However, when the sub-lethal TRAIL treatment was combined with a sub-lethal dose of BA, ethanol, PhA, bupivacaine or heat shock, significant apoptosis was observed (p<0.001). A sub-lethal dose of Dox was then combined with sub-lethal doses of BA, ethanol, PhA, bupivacaine or heat shock to determine if the efficacy of this anthracycline drug could be enhanced via fluidisation of the membrane. Combination treatments involving Dox resulted in significant apoptosis (p<0.001) (Figure 5.3.14). To determine if this synergism between TRAIL/Dox and membrane fluidisers applies to the other anti-cancer agents, four hour treatments of alcohol or heat shock were used in combination with twenty four hour treatments of Cyclo and lovastatin. When the sub-lethal dose of Cyclo was combined with a sub-lethal dose of BA, ethanol, PhA, bupivacaine or heat shock, significant apoptosis was observed (Figure 5.3.15). No stimulation of lovastatin-induced cell death by alcohols or bupivacaine was observed (Figure 5.3.16). However, heat shock treatment did appear to act in synergy with lovastatin. In the case of the alcohol treatment, the lack of synergism may be due to the alcohol treatment acting antagonistically to lovastatin and inducing HMG-CoA reductase.

The stimulation of TRAIL-/Dox- and Cyclo-induced cell death by the membrane fluidising treatments was also assessed by Annexin V/Propidium Iodide staining (Figure 5.3.17) Combining Dox with the alcohols or heat treatment results in 100% cell death following treatment. Stimulation of TRAIL-induced apoptosis was not as pronounced as that observed in Dox combination-treated cells, with approximately 50% of the Jurkat cell population remaining viable following treatment (Figure 5.3.18). All combination treatments involving Cyclo resulted in approximately 90% cell death (Figure 5.3.19). Hoescht staining of treated Jurkat cells further illustrated the toxic effects of combining these sub-lethal treatments (Figure 5.3.20). Nuclear fragmentation and chromatin condensation is clearly visible in the combination-treated cells, while Jurkats treated with single treatments did not display any signs of cell death.
Figure 5.3.13: Apoptosis in Jurkats treated with TRAIL combination therapy.
Caspase-3 assay on Jurkat cells treated with TRAIL (25ng/ml for four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours), Phenethyl alcohol (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of TRAIL & Heat Shock (25ng/ml for four hours added at the start of 1 hour at 42°C followed by 3 hour recovery at 37°C), TRAIL & BA (25ng/ml TRAIL + 5mM BA for four hours), TRAIL & Ethanol (25ng/ml TRAIL + 1.25% Ethanol for four hours), TRAIL & PhA (25ng/ml TRAIL + 2.5mM PhA for four hours) or TRAIL & Bupivacaine (25ng/ml TRAIL + 0.25mg/ml Bupivacaine). Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant differences from TRAIL treatment alone are indicated. *** represents a p value < 0.001.
Figure 5.3.14: Apoptosis in Jurkats treated with Doxorubicin combination therapy. Caspase-3 assay on Jurkat cells treated with Doxorubicin (5µg/ml for four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours), Phenethyl alcohol (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of Dox & Heat Shock (5µg/ml for four hours added at the start of 1 hour at 42°C followed by 3 hour recovery at 37°C), Dox & BA (5µg/ml Dox + 5 mM BA for four hours), Dox & Ethanol (5µg/ml Dox + 1.25% Ethanol for four hours), Dox & PhA (5µg/ml Dox + 2.5 mM PhA for four hours) or Dox & Bupivacaine (5µg/ml Dox + 0.25 mg/ml Bupivacaine). Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant differences from Dox treatment alone are indicated. *** represents a p value < 0.001.
Figure 5.3.15: Apoptosis in Jurkats treated with Cyclophosphamide combination therapy.
Caspase-3 assay on Jurkat cells treated with Cyclophosphamide (50µg/ml for twenty four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours) or combinations of Cyclo & BA (50µg/ml Cyclo for twenty four hours + 5mM BA for four hours), Cyclo & Ethanol (50µg/ml Cyclo for twenty four hours + 1.25% Ethanol for four hours) or Cyclo & Heat Shock (50µg/ml for twenty four hours added at the start of 1 hour at 42°C followed by 3 hour recovery at 37°C). Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant results are indicated. ** represents a p value < 0.01. *** represents a p value < 0.001.
Figure 5.3.16: Apoptosis in Jurkats treated with Lovastatin combination therapy. Caspase-3 assay on Jurkat cells treated with Lovastatin (12.5µM for twenty four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours, Phenethyl alcohol (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of Lovastatin & Heat Shock (12.5µM for twenty four hours added at the start of 1hour at 42°C followed by 3 hour recovery at 37°C), Lovastatin & BA (12.5µM Lov for twenty four hours + 5mM BA for four hours), Lov & Ethanol (12.5µM Lov for twenty four hours + 1.25% Ethanol for four hours), Lovastatin & PhA (12.5µM Lov for twenty four hours + 2.5mM PhA for four hours), or Lovastatin & Bupivacaine (12.5µM Lov for twenty four hours + 0.25mg/ml Bupivacaine for four hours), Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test and significant differences are indicated. ** represents a p value < 0.01.
Figure 5.3.17: Cell viability analysis in Jurkat cells treated with Doxorubicin combination therapy.
Annexin V and Propidium Iodide assay on Jurkat cells treated with (A). Doxorubicin & BA (5µg/ml Dox & 5mM BA for four hours) (B). Doxorubicin & Ethanol (5µg/ml Dox & 1.25% Ethanol for four hours), (C). Doxorubicin & PhA (5µg/ml Dox & 2.5mM PhA for four hours) (D). Doxorubicin & Heat (5µg/ml Dox added at start of a 1 hour heat shock at 42°C, followed by a 3 hour recovery) or Doxorubicin and Bupivacaine (5µg/ml Dox & 0.25mg/ml Bupivacaine for four hours). Numbers indicate percentage of non-viable cells.
Figure 5.3.18: Cell viability analysis in Jurkat cells treated with TRAIL combination therapy.
Annexin V and Propidium Iodide assay on Jurkat cells treated with (A). TRAIL & BA (25ng/ml TRAIL & 5mM BA for four hours) (B). TRAIL & Ethanol (25ng/ml TRAIL & 1.25% Ethanol for four hours), (C). TRAIL & PhA (25ng/ml TRAIL & 2.5mM PhA for four hours) (D). TRAIL & Heat (25ng/ml Dox added at start of a 1 hour heat shock at 42°C, followed by a 3 hour recovery) or TRAIL and Bupivacaine (25ng/ml TRAIL & 0.25mg/ml Bupivacaine for four hours). Numbers indicate percentage of non-viable cells.
Figure 5.3.19: Cell viability analysis in Jurkat cells treated with Cyclophosphamide combination therapy.
Annexin V and Propidium Iodide assay on Jurkat cells treated with (A). Cyclophosphamide & BA (100µg/ml Cyclo for twenty four hours & 5mM BA for four hours) (B). Cyclophosphamide & Ethanol (100µg/ml cyclo for twenty four hours & 1.25% Ethanol for four hours), (C). Cyclophosphamide & PhA (100µg/ml Cyclo for twenty four hours & 2.5mM PhA for four hours) (D). Cyclophosphamide & Heat (100µg/ml Cyclo added at start of a 1 hour heat shock at 42°C, followed by a 3 hour recovery), E. Cyclophosphamide & Bupivacaine (100µg/ml Cyclo for twenty four hours & 0.25mg/ml Bupivacaine for four hours). Numbers indicate percentage of non-viable cells.
Figure 5.3.20: Staining of nuclear material in Jurkat cells treated with Doxorubicin combination therapy. Hoechst 33342 staining of Jurkat cells treated with Fluidiser (PhA) or a combination of Doxorubicin and Fluidiser for four hours. Arrows indicate nuclear fragmentation and condensed chromatin. Images were visualised on a Nikon Inverted TE2000-U Fluorescence Microscope System at x40 magnification and analysed using IP Lab software.
5.3.4 Combination Treatment of Primary CLL cells

To determine whether these combination treatments may be useful in a clinical setting, primary CLL cells were subjected to treatments of TRAIL/Dox or Cyclo alone or combinations of drug and membrane fluidising treatment. Apoptosis and cell viability were then analysed using the Caspase-3 and Propidium Iodide fluorometric plate assays. Treating CLL cells with TRAIL and alcohol, TRAIL and heat shock or TRAIL and bupivacaine results in significantly greater apoptosis than treating with TRAIL treatment alone (p<0.001) (Figure 3.5.21). The cytotoxic effect of Dox and Cyclo was also enhanced by treating in combination with alcohol, heat shock or bupivacaine (Figures 3.5.22 and 3.5.23).
Figure 5.3.21: Apoptosis in Primary CLL cells treated with TRAIL combination therapy.

Caspase-3 assay on primary CLL cells treated with TRAIL (25ng/ml for 4 hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours), PhA (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of TRAIL & Heat Shock (25ng/ml for four hours added at the start of 1 hour at 42°C followed by 3 hour recovery at 37°C), TRAIL & BA (25ng/ml TRAIL + 5mM BA for four hours), TRAIL & Ethanol (25ng/ml TRAIL + 1.25% Ethanol for four hours), TRAIL & PhA (25ng/ml TRAIL + 2.5mM PhA for four hours) or TRAIL & Bupivacaine (25ng/ml TRAIL + 0.25mg/ml Bupivacaine). Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant differences from TRAIL treatment alone are indicated. *** represents a p value < 0.001.
Figure 5.3.22: Apoptosis in Primary CLL cells treated with Doxorubicin combination therapy.

Caspase-3 assay on primary CLL cells treated with Doxorubicin (5µg/ml for four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours), Phenethyl alcohol (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of Dox & Heat Shock (5µg/ml for four hours added at the start of 1 hour at 42°C followed by 3 hour recovery at 37°C), Dox & BA (5µg/ml Dox + 5mM BA for four hours), Dox & Ethanol (5µg/ml Dox + 1.25% Ethanol for four hours), Dox & PhA (5µg/ml Dox + 2.5mM PhA for four hours) or Dox & Bupivacaine (5µg/ml Dox + 0.25mg/ml Bupivacaine for four hours). Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant differences from Doxorubicin treatment alone are indicated. *** represents a p value < 0.001.
Caspase-3 assay on primary CLL cells treated with Cyclophosphamide (50µg/ml for twenty four hours), Heat Shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C), Benzyl Alcohol (5mM for four hours), Ethanol (1.25% for four hours), PhA (2.5mM for four hours), Bupivacaine (0.25mg/ml for four hours) or combinations of Cyclo & Heat Shock (50µg/ml for twenty four hours added at the start of 1 hour at 42°C followed by 23 hour recovery at 37°C), Cyclo & BA (50µg/ml Cyclo for twenty four hours + 5mM BA for four hours), Cyclo & Ethanol (50µg/ml Cyclo for twenty four hours + 1.25% Ethanol for four hours), Cyclo & PhA (50µg/ml Cyclo for twenty four hours + 2.5mM PhA for four hours) or Cyclo & Bupivacaine (50µg/ml Cyclo for twenty four hours + 0.25mg/ml Bupivacaine for four hours). Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant differences from Cyclophosphamide treatment alone are indicated. *** represents a p value < 0.001
5.3.5. Localisation of HSPs Following Membrane Fluidising Treatments

Jurkat cells, at a concentration of $3 \times 10^5$ cells/well, were treated with the pre-determined sub-lethal treatments of BA, ethanol, PhA, bupivacaine and heat shock for four hours. Intracellular levels of Hsp72, Hsp60, Hsp27 and Hsp90 were analysed by flow cytometry.

Expression of iHsp72 significantly decreased in response to a four hour treatment with BA, ethanol and bupivacaine, but not in response to PhA. In contrast, a one hour heat shock followed by a three hour recovery resulted in a significant increase in iHsp72 (Figure 5.3.24A). Similarly, levels of iHsp60 also significantly decreased in response to BA, ethanol and bupivacaine, but not PhA (Figure 5.3.24B). However, in contrast to iHsp72, heat shock treatment resulted in a significant decrease in iHsp60. Levels of iHsp90 were unaffected by any of the alcohol or bupivacaine treatments but were reduced in response to heat shock treatment (Figure 5.3.24C). iHsp27 expression was also significantly decreased in response to heat shock and was also reduced in response to PhA. BA, ethanol and bupivacaine failed to affect iHsp27 levels after four hours. (Figures 5.3.24D).

The significant decrease in iHsp72 and iHsp60 observed after treatment with alcohols and bupivacaine was thought to be attributed to movement of these HSPs to the surface of the cell. Surface-Hsp72 and surface-Hsp60 were therefore analysed in these treated cells. Figure 5.3.25A illustrates the resultant increase in sHsp72 after treatment with BA, ethanol and bupivacaine. Simultaneously to the decrease in iHsp60, an increase in sHsp60 was observed after treatment with BA and ethanol, but not after treatment with bupivacaine (Figure 5.3.25B). Although a decrease in iHsp72 and iHsp60 was observed after treatment with PhA, an increase of this protein at the cell surface was not observed.
Figure 5.3.24: Intracellular HSP analysis in fluidiser-treated Jurkat cells.
Intracellular HSP analysis on Jurkat cells treated with benzyl alcohol (5mM for four hours), ethanol (1.25% for four hours), phenethyl alcohol (2.5mM for four hours), heat shock (1 hour at 42°C, followed by a 3 hour recovery at 37°C) or bupivacaine (0.25mg/ml for four hours. (A) iHsp72 analysis (B) iHsp60 analysis (C) iHsp90 analysis (D) iHsp27 analysis. Statistical Analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant differences from the control group are indicated. n=9 in each experiment. * represents a p value < 0.05. ** represents a p value < 0.01
Figure 5.3.25: Surface HSP analysis in fluidiser-treated Jurkat cells.
Surface HSP analysis on Jurkat cells treated with benzyl alcohol (5mM for four hours), ethanol (1.25% for four hours), phenethyl alcohol (2.5mM for four hours), heat shock (one hour at 42°C, followed by a three hour recovery period at 37°C) or bupivacaine (0.25mg/ml for four hours. (A) sHsp72 analysis and (B) sHsp60 analysis. Statistical Analysis was performed using the one-way ANOVA with Dunnet’s post hoc test and significant differences from the control group are indicated. n=9 in each experiment. * represents a p value < 0.05. ** represents a p value < 0.01.
5.3.6. Manipulation of HSP Localisation Prior to Combination Treatments

It was proposed that the ability of membrane fluidising agents to enhance the killing potential of cytotoxic agents may be due to their effect on iHsp72, in addition to fluidising the cell membrane and allowing more efficient entry of the drugs. By causing the movement of intracellular Hsp72 to the membrane, the drugs may be able to exert their cytotoxic effects more effectively without being hampered by the anti-apoptotic properties of Hsp72.

Jurkat cells were therefore pre-treated with either a sub-lethal heat shock or a two hour methyl-β-cyclodextrin (mβcd) treatment before incubation with a combination of Dox and ethanol. Pre-treatment with heat shock would hopefully increase internal levels of iHsp72 (shown earlier to significantly increase iHsp72 levels – Figure 5.3.24A) above a threshold that could not be sufficiently reduced by the ethanol to allow the Dox to cause cytotoxicity. Similarly, treatment with mβcd prevents the movement of HSPs to the outer surface of the membrane by depleting cholesterol and therefore interfering with lipid rafts (Dopico, 2007; Moulin & Arrigo, 2006; Broquet et al. 2003). This should consequently prevent ethanol from reducing iHsp72.

Pre-treatment with sub-lethal heat shock appeared to eliminate the synergistic effect of Dox and ethanol (Figure 5.3.26), with caspase-3 levels in these pre-treated cells emulating those seen in control cells. This suggests that the elevated iHsp72 levels observed after heat shock treatment (Figure 5.3.24) are sufficiently high to prevent ethanol from decreasing them below a threshold value that is required for the sub-lethal Dox to become toxic. Although sub-lethal doses of combined Dox and heat have been shown to be toxic to both Jurkat cells and primary CLL cells, treating cells with sub-lethal heat shock prior to Dox treatment also eliminates this synergistic effect (Figure 5.3.27) suggesting that the level of iHsp72 in the cells is vital in determining whether the drug is cytotoxic. A 30 minute pre-treatment with mβcd also significantly reduced the synergistic effect of Dox and ethanol treatment (Figure 5.3.28), although not to the degree observed by heat shock pre-treatment. iHsp72 levels were also shown to be higher in cells pre-treated with mβcd when compared to those treated with mβcd then ethanol (Figure 5.3.29), suggesting partial inhibition of movement to the membrane.
Chapter 5: Synergistic Action of Chemotherapeutic Drugs and Membrane Fluidisers

**Figure 5.3.26: Effect of heat shock pre-treatment on the cytotoxic effect of Doxorubicin combination treatment.**

Caspase-3 in Jurkat cells treated with a combination of sub-lethal Dox and ethanol for four hours, with or without a pre-treatment with sub-lethal heat shock (one hour at 42°C, followed by a three hour recovery period at 37°C). Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant results are indicated. ** represents a p value < 0.01. *** represents a p value < 0.001.

**Figure 5.3.27: Effect of heat shock pre-treatment or heat shock combination treatment on Doxorubicin-induced apoptosis.**

Caspase-3 in Jurkat cells treated with a combination of sub-lethal Dox and heat shock (one hour at 42°C, followed by a three hour recovery period at 37°C) or sub-lethal heat shock treatment prior to a four hour sub-lethal Dox treatment. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant results are indicated. ** represents a p value < 0.05. *** represents a p value < 0.001.
Figure 5.3.28: Effect of methyl-β-cyclodextrin pre-treatment on the synergistic action of Doxorubicin and Ethanol.
Caspase-3 in Jurkat cells treated with methyl-β-cyclodextrin (2.5mM for two hours), Doxorubicin (5µg/ml for four hours), a combination of sub-lethal Dox and Ethanol (5µg/ml Dox and 1.25% Ethanol) or a 2.5mM pre-treatment with methyl-β-cyclodextrin, followed by a combination of sub-lethal Dox and Ethanol. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant results are indicated. *** represents a p value < 0.001

Figure 5.3.29: Effect of methyl-β-cyclodextrin pre-treatment on intracellular Hsp72 levels in Ethanol-treated Jurkat cells.
iHsp72 analysis in Jurkat cells treated with methyl-β-cyclodextrin (2.5mM for two hours), Ethanol (1.25% for two hours), or a 2.5mM pre-treatment with methyl-β-cyclodextrin, followed by a two hour Ethanol treatment. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. Significant results are indicated. * represents a p value < 0.05. ** represents a p value < 0.01
5.4 Discussion

The efficacy of three chemotherapeutic drugs (TRAIL, Dox and Cyclo) was shown to be enhanced using cell membrane fluidising agents. Furthermore, the concentrations of drugs used in these experiments were sub-lethal when used in isolation. Membrane fluidisation has previously been shown to be affected by mild hyperthermia, BA, ethanol (Moulin et al. 2007a), heptanol (Balogh et al. 2005), PhA (Nagy et al. 2007) and the local anaesthetic bupivacaine (Mizogami et al. 2002). However, to date, these membrane fluidising agents have not been used to enhance the killing potential of sub-lethal doses of anthracyclines, alkylating agents or death receptor ligands.

Previous evidence has already shown that hyperthermia can either enhance or inhibit the cytotoxicity of specific apoptosis inducers depending on cell type, cytotoxic drug or the stage of drug treatment at which the hyperthermia is applied. Jaattela et al. (1992) and Mehlen et al. (1995) have shown that hyperthermic treatment of cells prior to treatment with TNF-α results in reduced cytotoxicity. In contrast, hyperthermic treatment performed after the beginning of treatment with TNF-α appears to enhance the cytotoxicity of the agent (Dubois et al. 1989) and (Robins et al. 1995). Fas-L and TRAIL killing have also been shown to be either enhanced or inhibited by hyperthermic treatment depending on stage of drug treatment in relation to heat shock (Tran et al. 2003; Ozoren & El-Deiry, 2002; Moulin & Arrigo, 2006). An in-vitro study by Roigas et al. (1998) demonstrated that subjecting prostate cancer cells to anti-cancer agents before a period of hyperthermia was more effective than subjecting them to hyperthermia before anti-cancer treatment, while simultaneous hyperthermia and cytotoxic drug treatment proved to be the most effective method.

However, the stimulatory effect of hyperthermia upon TRAIL-induced apoptosis shown by Moulin & Arrigo (2006), was still observed when TRAIL was added twenty four hours after the initial one hour heat shock, suggesting that mild hyperthermia results in membrane modifications that allow a long lasting increased sensitivity to TRAIL. Heat shock stimulation of TRAIL killing was shown not to be dependent upon lipid rafts or newly synthesised HSPs, but was subsequently shown to be due to changes in membrane fluidity (Moulin et al. 2007a). Furthermore, fluidity measurements performed by this group showed that single ethanol or heat shock treatments resulted in significant increase in membrane fluidity, while treatment with 100ng/ml TRAIL did not affect fluidity.
However, combination treatments involving TRAIL and heat treatment or TRAIL and ethanol resulted in an increase in membrane fluidity that was much more intense than that observed in ethanol or heat treated cells alone. An enhanced antibody recognition of TRAIL receptors was also shown in TRAIL and fluidiser treated cells when compared to TRAIL or fluidiser only treated cells. This would account for the stimulation of TRAIL cytotoxicity but it does not explain why the killing potential of Dox and Cyclo, that do not operate via cell surface receptors, is also enhanced.

In support of the data presented in this chapter, Riehmann et al. (2005) have shown that treating a human pleural mesothelioma cell line xenotransplanted into mice with Cyclo and hyperthermia results in a significant decrease in tumour volume when compared to Cyclo treatment alone. The efficacy of Dox has also been shown to be enhanced by hyperthermia (Roigas et al. 1998), (Ohtsubo et al. 2000) and (Hahn et al. 1975). Inconsistencies between studies may be attributed to cell type, for example, Urano et al. (1999) showed that the efficacy of 5-fluorouracil could not be enhanced by hyperthermia when using mouse fibrosarcoma cells, while Roigas et al. (1998) presented contradictory data using prostate cancer cells.

The hyperthermic-associated inhibition of cytotoxicity observed in some studies is thought to be due to the increase in intracellular Hsp72 that functions to inhibit many of the key signalling proteins in the apoptosis pathway. A deeper understanding of HSP regulation is therefore critical if combination therapies are to become a main stream treatment regime and may explain the contradictory data in the literature. Analysis of Hsp72 and Hsp60 after four hour treatment with the membrane fluidising treatments revealed a change in the localisation of these proteins after certain treatments. BA, ethanol and bupivacaine all stimulated a movement of Hsp72 from the inside of the cell to the cell surface, an effect not observed after treatment with PhA. Similarly, BA and ethanol also stimulated movement of Hsp60 from the inside of the cell to the surface. Although a decrease in intracellular Hsp60 was seen after bupivacaine treatment, surface levels of this protein were not significantly different. As expected, levels of internal Hsp72 significantly increased in response to a one hour heat shock with a three hour recovery, but this treatment significantly reduced the levels of iHsp60. Expression of Hsp90 and Hsp27 remained unaffected by treatment with BA, ethanol and bupivacaine, while PhA appeared to reduce the levels of iHsp27 but not iHsp90. Expression of both iHsp90 and
iHsp27 were shown to decrease in response to heat shock treatment. All five membrane fluidising treatments are displaying the same stimulatory effect upon TRAIL-, Dox- and Cyclo-induced cell death, and yet after four hours of treatment, not all treatments show the same effect on HSP localisation. Further work is therefore necessary to clarify the relationship between membrane fluidisation and HSP localisation.

Increasing the levels of iHsp72 using a sub-lethal heat shock treatment prior to treating cells with a combination of Dox and ethanol eliminated the synergistic action previously observed. This suggests that ethanol is not able to decrease these previously elevated levels of iHsp72 enough for the sub-lethal Dox to become toxic. Furthermore, pre-treating cells with mβcd, a cholesterol sequestering agent also reduced the synergistic effect of Dox and ethanol, although not to the same degree as heat shock pre-treatment. However the effect observed after mβcd could either be due to a membrane stabilisation effect or as a result of mβcd reducing the movement of Hsp72 to the cell membrane. Indeed, analysis of iHsp72 in cells pre-treated with mβcd before ethanol treatment showed higher levels of iHsp72 than those in cell treated with ethanol alone, although this was not shown to be significant. This may suggest that the stabilisation of the membrane by mβcd may be more important in preventing ethanol enhancing Dox-induced cell death. However, Hsp72 may be moving to the cell membrane via alternative mechanisms such as exosome-associated transport (Clayton et al. 2005) or via ABC transporters (Mambula & Calderwood, 2006), mechanisms that cannot be blocked by mβcd. An alternative approach to preventing this movement of Hsp72 could be to treat with a combination of transporter pathway inhibitors such as glybenclamide (Mambula & Calderwood, 2006), an ABC transporter inhibitor, monensin (Hunter-Lavin et al. 2004), a classical pathway inhibitor and mβcd thereby preventing movement by alternative mechanisms.

The work presented here, demonstrates for the first time that combining sub-lethal doses of chemotherapeutic drugs with membrane fluidising agents results in a significant increase in drug efficacy without using large doses of drug. Whilst the use of the alcohol compounds in this way is clearly inappropriate in the clinical setting, the promising results shown by bupivacaine and hyperthermia do point to the possibility that manipulation of membrane fluidity, through the use of other agents, may have great therapeutic potential.
5.5 Summary

- The cytotoxicity of TRAIL, Doxorubicin and Cyclophosphamide can be enhanced by using in combination with the alcohols BA, ethanol and PhA, the local anaesthetic bupivacaine and mild hyperthermia.

- These combination treatments are effective in both Jurkat cells and Primary CLL cells.

- By using combination treatments in this way, sub-lethal doses of chemotherapeutic agents can be used, which in a clinical setting would help reduce damage to non-transformed cells and limit haematological cytotoxicity.

- The localisation of HSPs is affected by treatment of certain membrane fluidising treatment, with Hsp72 in particular moving from the inside of the cell to the membrane in response to BA, ethanol and bupivacaine.

- By preventing the decrease in internal HSPs before combination treatment, the synergistic effect of cytotoxic agent and fluidiser is significantly reduced.