

Thalidomide enhances cyclophosphamide and dexamethasone-mediated cytotoxicity towards cultured chronic lymphocytic leukaemia cells

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Received June 4, 2010; Accepted July 2, 2010

DOI: 10.3892/or_00000988

Abstract. Numerous chemotherapeutic regimens exist for the treatment of symptomatic or progressive chronic lymphocytic leukaemia (CLL). However, once the disease becomes refractory to nucleoside-based therapy the prognosis is poor. In this study we investigated the cytotoxicity of thalidomide in combination with dexamethasone, fludarabine and cyclophosphamide. Cells from a cohort of 25 CLL patients were incubated for 72 h with each of these three agents, at 3 concentrations, both with and without thalidomide. Cell viability was assessed using the Annexin V:FITC assay. Fludarabine was highly toxic to the cells, producing very high levels of cell death; however, thalidomide did not increase this effect. Cyclophosphamide combined with thalidomide showed a small, non-significant improvement in toxicity compared with monotherapy. Median cell death for 5 μ M dexamethasone monotherapy and for combination with thalidomide was 15% [interquartile range (IQR) 0-38%] and 17% (IQR 0-54%), respectively (Wilcoxon Signed Rank analysis, $p=0.034$). Cell death for 10 μ M dexamethasone monotherapy was 15% (IQR 0-45%) and 16% (IQR 0-62%) in combination with thalidomide (Wilcoxon Signed Rank analysis, $p=0.035$). At the highest doses tested 11 of 25 cases displayed an enhancement of cyclophosphamide-mediated cytotoxicity, and 14 of 25 cases showed enhanced dexamethasone-mediated cytotoxicity in the presence of thalidomide. Some CLL cells in which dexamethasone-mediated killing was enhanced were derived from patients with poor prognostic markers, including p53 mutations and unmutated IgV_H genes. In summary, thalidomide enhances cyclophosphamide- and dexamethasone-

mediated cytotoxicity of CLL cells *in vitro* in a proportion of cases.

Introduction

Chronic lymphocytic leukaemia (CLL) is the most commonly occurring leukaemia in the Western world, is more prevalent in men than women, and has a median age of diagnosis of 65 years (1-3). The disease is characterised by a proliferation and accumulation of small, homogeneous, mature B-lymphocytes in the blood, bone marrow, lymph nodes and spleen. In contrast to many malignancies, CLL proliferates at a relatively slow rate with the cells escaping the fate of programmed cell death (PCD or apoptosis) (4,5).

CLL is incurable, but symptomatic disease can be effectively treated with purine analogues, glucocorticoids, alkylating agents, monoclonal antibodies or combinations of these (6). The overall response and clinical outcome when using any therapeutic agent depends on several factors, including previous treatment, clinical stage and cytogenetic abnormalities making it very difficult to find a successful treatment for every patient (7). Patients with relapsed or refractory disease have limited treatment options, as few therapies are effective (8). Poor prognostic factors include unmutated immunoglobulin heavy chain variable regions (IgV_H), expression of CD38 and zeta-associated protein 70 (ZAP-70) (1).

Chlorambucil or fludarabine are usually incorporated into initial treatment regimens but rates of complete remission with these drugs as monotherapy is low. Cyclophosphamide has been evaluated extensively within combination regimens for CLL with both the cyclophosphamide, pentostatin, rituximab and fludarabine, cyclophosphamide, rituximab regimens producing excellent response rates (9,10). CLL cells display *in vitro* sensitivity to the cytotoxic effects of dexamethasone (6) and this agent has been incorporated into chemotherapy regimens found to be effective in the treatment of this disease (11,12).

Thalidomide is an immunomodulating agent that has been found to be an anti-angiogenic, antiproliferative and pro-apoptotic agent which acts through the inhibition of 'pro-

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Key words: CLL, thalidomide, dexamethasone, apoptosis, chemotherapy

survival' cytokines such as tumour necrosis factor α (TNF- α), interleukin-12 and other pro-inflammatory Th1 type cytokines (7,13,14). Thalidomide has been intensively investigated in multiple myeloma (MM), both as a monotherapy and in combination with other agents. Rajkumar and colleagues showed that thalidomide in combination with dexamethasone yielded superior response rates compared with dexamethasone, however, a greater toxicity was seen with the combination therapy (15). Fludarabine has also been assessed in combination with thalidomide for the treatment of CLL; a phase I clinical trial demonstrated an overall response rate of 100%, with 55% achieving a complete remission (13).

The mechanism(s) of action of thalidomide are still being investigated, as are the possible treatment options utilising this drug. Here we have assessed the synergistic effect of combining thalidomide with fludarabine, cyclophosphamide and dexamethasone *in vitro*, looking at cell survival to discover new drug combinations with clinical efficacy in CLL.

Materials and methods

Chronic lymphocytic leukaemia patients. The study utilised peripheral blood samples taken from 25 patients with typical CLL (mature lymphocytes expressing CD5, CD19, CD23 and clonally restricted surface immunoglobulin), all of whom possessed a white blood cell count $\geq 20 \times 10^9/l$. The study had Local Research Ethics Committee (Ref. 05/Q1104/33) and NHS Hull and East Yorkshire Trust approval. Informed consent was obtained from all patients prior to recruitment. Mononuclear cells were isolated from samples by centrifugation on a Ficoll-Hypaque gradient, using Histopaque®-1077 (Sigma), according to manufacturer's protocol, and cryopreserved in liquid nitrogen in the presence of 10% (v/v) dimethyl sulphoxide (DMSO). Before use, the cells were thawed at 37°C and the DMSO diluted to 0.5% (v/v) in media over a period of 20 min. Mean viability of the CLL-cells on thawing as assessed by trypan blue exclusion was 80.4%.

The patient cohort (Table I) comprised 13 males and 12 females, with a mean age of 72 years (range 37-87). The majority of patients were classed as early stage disease, either Rai 0 or Binet A (64 and 76%, respectively); however, a number of patients showed signs of poor risk markers such as the expression of ZAP-70 in 10% or greater of the malignant cells (23% of cases), unmutated IgV_H genes (26%), deletion of 17p or 11q (28%) and the expression of CD38 by 30% or more of the CLL-cells (24%). The average number of previous treatment cycles was 1.5 (range 0-5).

Reagents and media. Fludarabine (2-fluoroadenine-9- β -D-arabinofuranoside), cyclophosphamide monohydrate and dexamethasone crystalline were obtained from Sigma Chemical Co., Ltd. (Poole, UK); thalidomide was provided by Pharmion Corp. (Berkshire, UK). Roswell Park Memorial Institute (RPMI) 1640 culture medium with 300 μ g/ml L-glutamine (PAA; The Cell Culture Co., Somerset, UK) supplemented with 1% (w/v) bovine serum albumin (BSA; Fisher Scientific, Loughborough, UK) and penicillin-streptomycin solution (final concentration 100 U/ml penicillin and 100 μ g/ml streptomycin; Sigma). This medium was sterilized by filtering through a 0.2- μ m syringe filter (Anachem, Bedfordshire, UK).

Table I. Summary of clinical characteristics for the patient cohort.

Characteristics	No. of patients
Gender	
Male	13
Female	12
Age (years), mean (range)	72 (37-86)
Binet stage (at diagnosis)	
A	19
B	5
C	1
Rai stage (at diagnosis)	
0	15
I	3
II	4
III	2
IV	1
FISH	
Isolated deletion 13q	4
Trisomy 12	1
Deletion 11q	3
Deletion 17p	4
Normal	2
Not tested	11
IgV _H mutation status	
Mutated (<98%)	17
Unmutated (>98%)	6
Not tested	2
ZAP-70 (by flow cytometry)	
Positive	5
Negative	17
Not tested	3
CD38 expression	
Positive	4
Negative	13
Not tested	8
No. of previous therapies, mean (range)	1.5 (0-5)

Culture of cells. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were incubated at a concentration of 1×10^6 cells/ml for 72 h with 9, 18 or 36 μ M cyclophosphamide, 2.5, 5 or 10 μ M dexamethasone, 1, 2.2 or 4.4 μ M fludarabine, 400 μ M thalidomide or a combination of agents, as above, with thalidomide. Hydrogen peroxide (2.9 M) was used as a positive control for inducing cell death, by incubating an aliquot of cells for the final 30 min of the 72-h incubation period with 100 μ l of this solution.

Analysis of cell viability by Annexin V binding. Cell survival was assessed using the Annexin V:FITC assay kit (AbD Serotec, Oxford, UK). Briefly, following incubation, cells were washed in ice-cold phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 2.7 μ M potassium chloride, 137 μ M sodium chloride, pH 7.4; Sigma) resuspended in 200 μ l binding buffer, incubated with 5 μ l Annexin V:fluorescein isothiocyanate (FITC) for 10 min in the dark; and finally washed again in PBS. Cells were then resuspended in 200 μ l binding buffer and incubated with 10 μ l propidium iodide (PI) before sample analysis on a FACSCalibur flow cytometer (Becton-Dickinson, Oxford, UK). Ten thousand cells per sample were acquired and analysed using CellQuest software (Version 3.3; Becton-Dickinson). All experiments were done in duplicate.

Cell survival was investigated by dividing the flow cytometric data into viable, necrotic and apoptotic populations. Comparisons of the percentage of dead cells (apoptotic and apoptotic plus necrotic) demonstrated any effects of treatment with cyclophosphamide, dexamethasone or fludarabine either alone or in combination with thalidomide. The percentage of cell death given by the untreated cells incubated in similar conditions was defined as the baseline; hence all test conditions were normalised against this condition to allow comparison.

Analysis of IgV_H mutational status, fluorescent in situ hybridisation, ZAP-70 expression and proportion of CD19 positive cells. RNA for the IgV_H analysis was extracted using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK) and the Reverse-iT kit (ABgene, Epsom, UK) was used to synthesise complementary DNA. The IgV_H gene was amplified using PCR, as previously described (16), and the BIG dye terminator sequencing kit version 3 (Applied Biosystems, Warrington, UK) was used for sequencing. Sequences were compared with germline using IgBLAST (National Centre for Biotechnology Information, Bethesda, Maryland; <http://ncbi.nih.gov/igblast/>). Those with homology of $\geq 98\%$ were classified as unmutated and those with $< 98\%$ homology were classified as mutated (17).

Fluorescent *in situ* hybridisation for deletions in 13q, 11q and 17p as well as for trisomy 12 were performed according to published methodologies (18).

ZAP-70 and CD19 expression were assessed by flow cytometry with reference to an isotypic control (19). A threshold of 10% ZAP-70 positive cells was used to differentiate ZAP-70 positivity and negativity. Following a viable cell count, 4×10^5 cells were incubated with mouse anti-human CD19:RPE (AbD Serotec, Oxford, UK) for 30 min, after which cells were then incubated with 100 μ l fixation reagent (Reagent A, Leucoperm; AbD Serotec). Following washing with PBS permeabilisation reagent (100 μ l Reagent B, Leucoperm) was added to the cells along with the mouse anti-human ZAP-70 Alexa Fluor 488 (Caltag Laboratories, Paisley, UK). The flow cytometer was set to acquire 10,000 events for each sample and data were analysed as described above.

Statistical analysis. Distribution of data was analysed using tests for skewness and kurtosis. All data were found to be non-normally distributed. The significance of differences was

therefore evaluated by the Wilcoxon Signed Rank test at $p < 0.05$ level of significance. All statistics were performed using the SPSS 15.0 software package (SPSS Inc.).

Results

To assess the effects of the cytotoxic drugs with and without thalidomide, it was necessary to maintain the cells in culture for 72 h. CLL cells do not survive well *in vitro*, thus it was necessary to identify a cohort of patients with CLL cells whose survival was $\geq 30\%$ after 72 h that could be used for the cytotoxicity assays. Forty-five samples were analysed (median survival following 72 h culture was 31% (IQR 9-50%; range 1-79%) and it was only possible to utilise 25 samples for subsequent studies. This cohort was subsequently tested to assess the B-cell population using CD19 by flow cytometry; the median B-cell population was 96% (IQR 92-97%).

Thalidomide monotherapy. Dose ranging experiments with thalidomide were performed on a total of 10 independent patient samples using concentrations of 0.1-1 mM. Thalidomide showed some, limited effects on CLL cell viability when used as monotherapy, and a concentration of 400 μ M was selected for use in all subsequent experiments. This value represents a maximal level achievable in plasma levels *in vivo*. At this concentration cells from 12 of the 25 cases tested demonstrated an increase in cell death relative to untreated CLL-cells following 72 h incubation with 3 of these cases demonstrating a $> 20\%$ increase in cell death relative to untreated cells. An incubation period of 72 h was chosen as time course experiments demonstrated little increase in thalidomide induced CLL-cell death following culture for 24-48 h.

Fludarabine therapy in combination with thalidomide. Median cell death for fludarabine alone at the lowest dose tested, 1 μ M was 71% (IQR 48-92%) and in combination with thalidomide was 69% (IQR 35-91%). At 4.4 μ M the highest concentration of fludarabine used, cell death was 94% (IQR 86-98%) and in combination with thalidomide was 91% (IQR 70-96%). Fludarabine alone produced extremely high levels of cell death, which the addition of thalidomide did not further enhance.

Cyclophosphamide therapy in combination with thalidomide. Median cell death for cyclophosphamide at 9 μ M, the lowest concentration tested, was 2% (IQR 0-7%) and 1% (IQR 0-18%) when used in combination with thalidomide. At 18 μ M, median cell death with cyclophosphamide was 6% (IQR 0-13%) and 5% (IQR 0-22%) when combined with thalidomide. Finally at the highest concentration, 36 μ M cell death was 9% (IQR 2-22%) and 10% (IQR 0-25%) alone and combined with thalidomide, respectively. Of the 25 samples tested, thalidomide increased the proportion of dead cells in 8 cases when co-incubated with cyclophosphamide at 9 μ M, 12 cases when co-incubated with cyclophosphamide 18 μ M and 11 cases when co-incubated with cyclophosphamide at 36 μ M. Fig. 1 displays the data on the cytotoxic effects of thalidomide and cyclophosphamide monotherapy, and the combination of these two agents towards cultured CLL cells using the

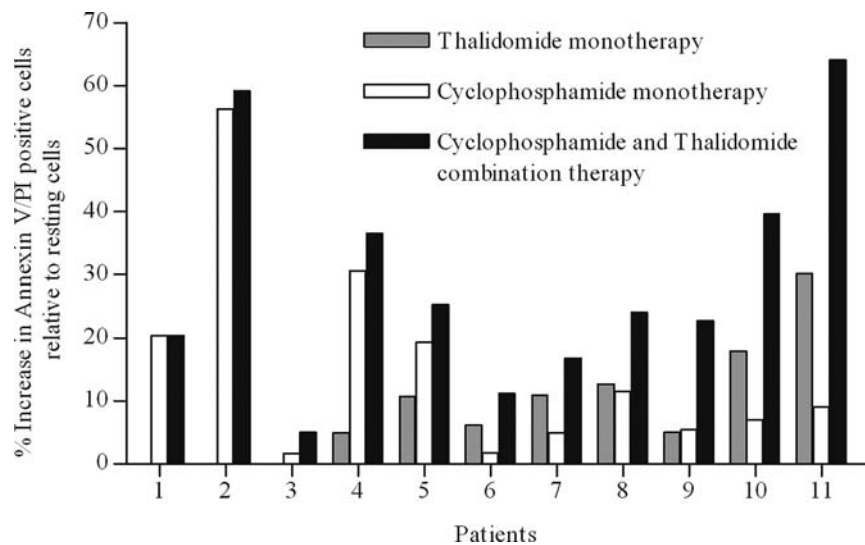


Figure 1. The percentage of dead, Annexin V/PI positive CLL cells from individual patient samples when treated with thalidomide (400 μ M), cyclophosphamide (36 μ M) or a combination of both are shown following incubation for 72 h. Cell death in response to cytotoxics is expressed as a percentage increase relative to the number of dead cells noted in the absence of exposure to cytotoxics under identical culture conditions. Results are arranged in order of increasing difference between treatment with cyclophosphamide alone and in combination with thalidomide. In patients 1-3 thalidomide alone caused no cell death. Only the 11 patients that showed an increase in cytotoxicity are displayed.

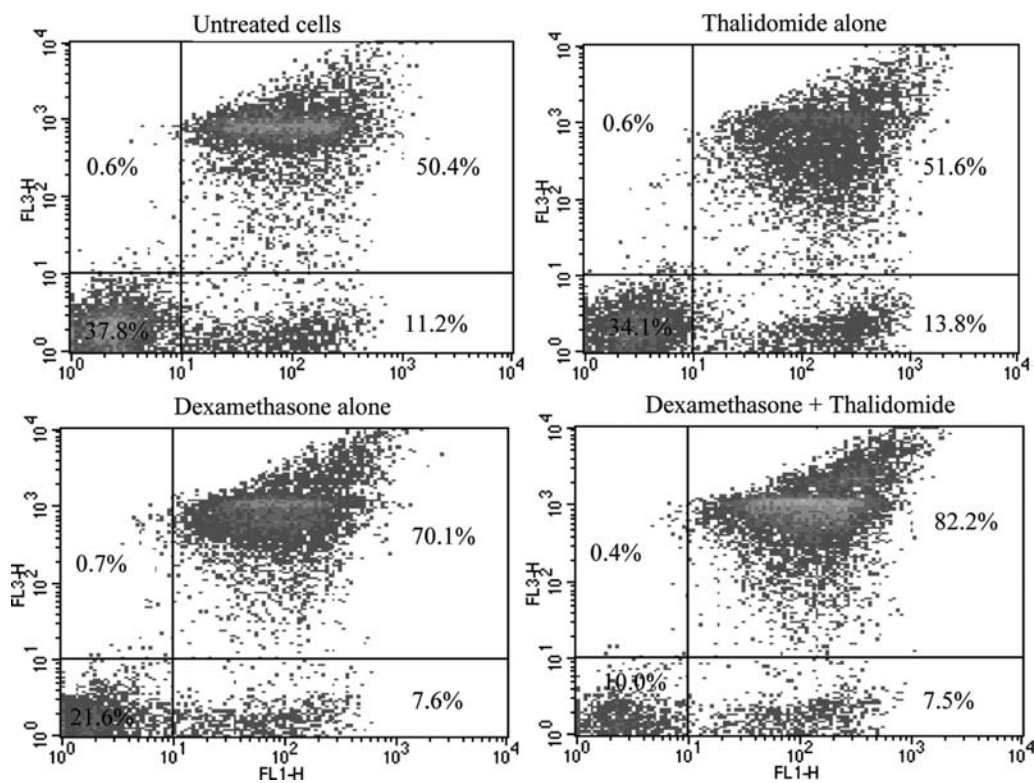


Figure 2. FACS plots of CLL-cells incubated for 72 h in the presence of media, thalidomide 400 μ M, dexamethasone 10 μ M or thalidomide 400 μ M and dexamethasone 10 μ M. The x-axis (FL-1) indicates Annexin V expression whilst the y-axis (FL-3) represents PI uptake. Events within the lower left quadrant of each plot represent Annexin V⁻/PI⁻ viable cells whilst events within the upper right quadrant of each plot represent Annexin V⁺/PI⁺ dead cells. Overall cell death was taken to be the total of Annexin V⁺/PI⁺, Annexin V⁺/PI⁻ and Annexin V⁻/PI⁺ cells. Percentages correspond to the quadrant where the figure is located. A total of 10,000 events were counted for each plot.

highest concentration of cyclophosphamide tested, in the 11 cases that showed an enhancement of the cytotoxic effect of cyclophosphamide by thalidomide.

Of the 11 cases in which thalidomide enhanced cyclophosphamide-mediated cytotoxicity towards CLL cells, in four cases the malignant cells expressed unmutated IgV_H

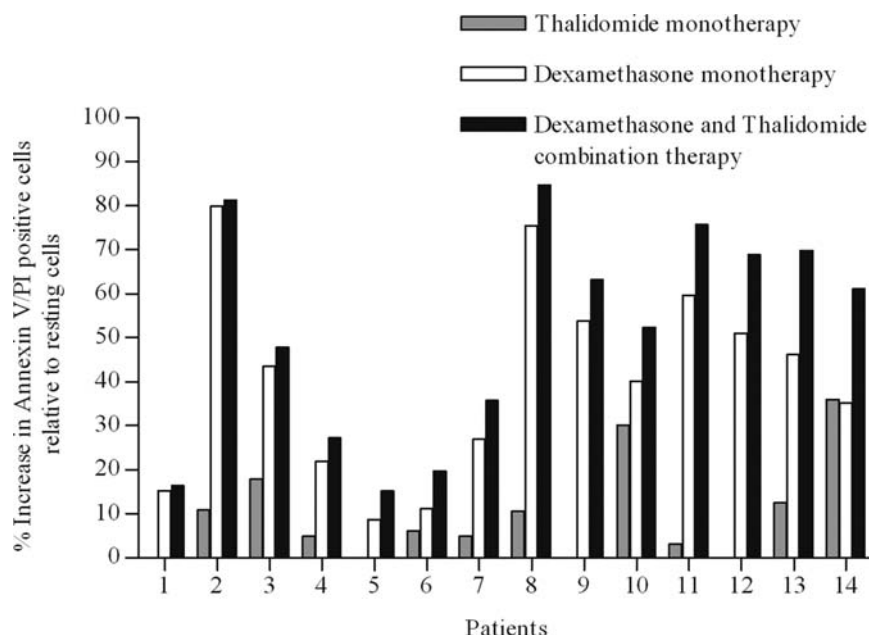


Figure 3. The percentage of dead, Annexin V/PI positive CLL cells from individual patient samples when treated with thalidomide (400 μ M), dexamethasone (10 μ M) or a combination of both are shown following incubation for 72 h. Cell death in response to cytotoxics is expressed as a percentage increase relative to the number of dead cells noted in the absence of exposure to cytotoxics under identical culture conditions. The results are arranged in order of increasing difference between treatment with dexamethasone alone and in combination with thalidomide. In patients 1, 5, 9 and 12 thalidomide alone caused no cell death. Only the 14 patients that showed an increase in cytotoxicity are displayed.

genes (data available for 10), three were ZAP-70 positive, one was CD38 positive (data available for 8) and two had a deletion 17p (data available for 14). The patients from which these samples were taken had received a mean of 1.4 previous courses of therapy (range = 0-3).

Dexamethasone therapy in combination with thalidomide. Thalidomide enhanced the cytotoxic effect of dexamethasone against CLL cells in 11 of 24 cases when co-incubated with 2.5 μ M dexamethasone, in 10 of 25 cases when co-incubated with 5 μ M dexamethasone, and in 14 of 25 cases when co-incubated with 10 μ M dexamethasone. Median cell death for dexamethasone at 2.5 μ M was 12% (IQR 0-42%) and 14% (IQR 0-51%) when in combination with thalidomide ($p=0.121$). In the presence of 5 μ M dexamethasone median cell death was 15% (IQR 0-38%) and 17% (IQR 0-54%) on co-incubation with thalidomide ($p=0.034$). At the highest concentration (10 μ M) median cell death with dexamethasone as a single agent was 15% (IQR 0-45%) and 16% (IQR 0-62%) on co-incubation with thalidomide ($p=0.035$; Figs. 2 and 3). Therefore significantly elevated levels of cell death were observed using a combination of 5 and 10 μ M dexamethasone and thalidomide, as compared with dexamethasone alone.

Of the fourteen cases in which thalidomide enhanced dexamethasone (10 μ M) mediated cytotoxicity towards CLL cells, the malignant cells from four cases expressed unmutated IgV_H genes (data available for 13), four were ZAP-70 positive (data available for 13), three were CD38 positive (data available for 11) and three possessed a deletion 17p (data available for 14). The patients from which these samples were taken had received a mean of 1 previous course of therapy (range 0-3).

Discussion

Fludarabine-based chemotherapy regimens are considered to be effective in the treatment of CLL, with a superior overall response compared with other treatment regimens, with overall response rates of 75-80% (20). However, some patients become refractory to fludarabine and form a unique group associated with shortened survival and a marked susceptibility to infections (21,22).

A phase I clinical trial was carried out to establish the safety and efficacy of combining fludarabine with thalidomide in previously untreated CLL patients. This yielded encouraging results, with an overall response rate of 100% and a complete remission rate of 55% (13). In an attempt to understand the mechanism by which thalidomide enhances fludarabine mediated cytotoxicity, Laurenti and colleagues measured the levels of TNF- α in five, heavily pre-treated, patients, undergoing combination therapy with cyclophosphamide, fludarabine and thalidomide (23). TNF- α is known to cause tumour proliferation of some malignancies, and thalidomide has previously been shown to inhibit production of this cytokine (24). However, serum TNF- α levels in these patient did not show any sign of decrease during treatment, but this may be due, at least in part, to the drug resistance caused by several previous rounds of chemotherapy and the fact that all the patients had to stop treatment early due to disease progression or neurological toxicity.

Previously Podhorecka *et al* have conducted an *in vitro* study to assess the interactions of thalidomide with fludarabine in terms of induction of apoptosis (25). At early time-points (<24 h) thalidomide induced DNA damage as evidenced by increased phosphorylation of histone H2AX as well as inducing low levels of the apoptotic marker caspase-3, however thalido-

midide did not increase overall CLL-cell death when used as monotherapy. Thalidomide and fludarabine in combination demonstrated an enhancement of fludarabine induced CLL-cell killing suggesting that thalidomide may 'prime' CLL-cells for nucleoside-induced cytotoxicity by co-inducing low-levels of DNA damage. Our study in contrast demonstrated that following prolonged CLL-cell incubation (72 h) with thalidomide a minority of cases of CLL displayed increased cell death but that fludarabine-induced cell death was maximal and not further enhanced by thalidomide. This suggests that *in vitro* thalidomide may initially sensitise CLL-cells to fludarabine-mediated cytotoxicity resulting in more rapid cell killing. In addition thalidomide has been noted to reduce levels of regulatory T-cells *in vivo* possibly facilitating a direct cytotoxic T-cell effect against CLL-cells (26).

In the present study it was found that cyclophosphamide was relatively ineffective both with and without thalidomide. Eleven of the 25 samples showed an increase in cell death when treated with cyclophosphamide and thalidomide in comparison to cyclophosphamide alone. Eight of these samples had a difference between the induced cell death of $\geq 5\%$. As expected, dexamethasone displayed significant cytotoxic activity against cultured CLL cells, and 14 of the 25 samples tested showed an increase in cell death when treated with combined dexamethasone and thalidomide, in comparison with dexamethasone alone. Eleven of these samples showed a difference between the induced cell death of $\geq 5\%$ including some from patients with adverse prognostic factors.

Corticosteroids exert their cytotoxic effect in a p53-independent manner and the observation that two of the patients in whom thalidomide enhanced dexamethasone mediated-cytotoxicity towards cultured CLL cells possessed a p53 deletion suggests that the effect of thalidomide on CLL cell viability may also be independent of p53 activity. CLL patients whose malignant cells possess p53 deletions have a very poor prognosis, with a median survival of less than 18 months, and show a weak response to alkylators and purine analogues. Combinations of agents such as alemtuzumab and methylprednisolone, whose cytotoxic activity is independent of p53, have been investigated therapeutically in CLL with some success although at the expense of considerable toxicity (27). Our results suggest that a thalidomide/corticosteroid-based regimen may be effective in a proportion of CLL patients, including those with poor prognostic features.

The combination of thalidomide with either cyclophosphamide or dexamethasone is associated with a modest enhancement of the *in vitro* cytotoxicity of these latter two agents towards CLL cells. However, as thalidomide has complex anti-angiogenic and anti-inflammatory effects when used *in vivo* for the treatment of malignancy it is likely that the relatively small increase in cytotoxic effect observed *in vitro*, will be further enhanced in a clinical setting. Thalidomide and dexamethasone may therefore be a rational combination on which to base the development of future chemotherapy regimens for the treatment of CLL.

Acknowledgements

We would like to thank the Haematological Malignancy Diagnostic Service, Leeds General Infirmary, Leeds, for

providing IgV_H mutation and CD38 expression data, and Hull York Medical School and Pharmion Corp. for their financial support. These data were presented at the British Society of Haematology conference, Glasgow, April, 2008.

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