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High dose methylprednisolone can induce remissions in CLL patients with p53 abnormalities

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Abstract Abnormalities of the p53 gene are known to confer detrimental effects in chronic lymphocytic leukaemia (CLL) and are associated with short survival. We have used high dose methylprednisolone (HDMP) to treat 25 patients with advanced refractory CLL of whom 45% had p53 abnormalities shown by one or more methods: flow cytometry, fluorescent in situ hybridisation and direct DNA sequencing. Fifteen were resistant to fludarabine and 16 were non-responders to their most recent therapy. Methylprednisolone had a cytotoxic effect on lymphocytes from 95% of cases assessed by an ex vivo

apoptotic drug sensitivity index (DSI). HDMP was given alone or in combination with other drugs: vincristine, CCNU, Ara-C, doxorubicin, mitoxantrone and chlorambucil, according to the results of DSI. Three patients were treated twice and each treatment was analysed separately. The overall response rate was 77% with a median duration of 12 months (range 7–23+). Responders included 5/10 with abnormal p53, of which two achieved nodular PR. Patients with p53 abnormalities fared worse than those with normal p53. There were no differences in response according to whether HDMP was used alone or in combination. Nine of the 22 evaluable patients (3 NR and 6 PR) have died from progressive disease or transformation. Main toxicity was infection in 7/25 patients. Event free and overall survival were significantly better in responders vs non-responders ($P>0.0001$ and $P=0.04$ respectively). Patients with a DSI of 100% to steroids had a better overall and event free survival, but this was not statistically significant. This study demonstrates that HDMP alone or in combination with other agents is a useful treatment strategy in refractory CLL including patients with p53 abnormalities.

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Introduction

Chronic lymphocytic leukaemia (CLL) usually follows a relatively benign course with affected individuals surviving in excess of ten years, while in others, the disease follows an aggressive course with a shorter survival. Initial management of symptomatic or progressive CLL frequently involves alkylating agents, however resistance frequently develops. An important improvement in therapy during the 1980's was the introduction of the nucleoside analogue fludarabine. Overall response rates of 78% have been reported with fludarabine as first line

therapy, yet the response rate in previously treated patients is variable (12–94%) [1].

Moreover, fludarabine is not curative and most patients will eventually relapse after therapy. There are a number of salvage regimens for relapsed or resistant CLL, but few of these studies were aimed specifically at patients resistant to purine analogues. Furthermore, the myelotoxicity of anthracycline containing regimens and the increased susceptibility to opportunistic infections and autoimmune problems following purine analogues [2], may cause morbidity without the benefit of disease response in resistant patients.

The ex-vivo apoptotic drug sensitivity assay, previously known as differential staining cytotoxicity (DiSC) assay, has been shown to have a 92% predictive accuracy in identifying drug resistance with a sensitivity of 77% [3]. Knowledge of ex-vivo resistance may enable us to detect patients who are unlikely to respond to certain cytotoxics thus avoiding unnecessary treatment and morbidity. Cells from previously treated patients have been found to be more sensitive to methylprednisolone than cells from untreated patients, and a good correlation with ex-vivo responses and response to steroid treatment as well as purine analogues has been reported [3, 4, 5].

There are a number of chromosomal and immunophenotypic markers of worse prognosis and treatment resistance in CLL [6] and it has recently been shown that the feature conferring worse prognosis involves the tumour suppressor gene P53 [7, 8, 9, 10]. Döhner et al. [8] found that patients with p53 deletion failed to respond to either fludarabine or pentostatin, compared with a 36% response rate in patients without deletion. Similarly, Wattel et al. [7] documented a 12.5% vs 80% response rate to chlorambucil in cases with p53 deletion vs cases with no deletion.

The mechanism of action of fludarabine involves the incorporation of its metabolite F-Ara-A (9- β -D arabinofuranosyl-2-fluoadenine) triphosphate into elongating nucleic acid chains resulting in termination of DNA and RNA synthesis [1]. Chlorambucil and other nitrogen mustards produce DNA cross-links and monoadducts inhibiting transcription [11]. All of these agents induce apoptosis, which is most likely dependant on normally functioning or wild-type p53. This was demonstrated by Johnston et al. [12] who measured increased levels of the p53 protein and mdm2 following treatment of CLL cells with fludarabine, cladribine and chlorambucil; and Gartenhaus et al. [13] who measured increased expression of p53 and its downstream target WAF1/CIP1 following treatment with cladribine. P53 independent mechanisms for purine analogue induced apoptosis are described including NAD⁺/ATP depletion by poly ADP-ribose polymerase. However, this mechanism appears to accelerate the late stages of apoptosis rather than initiate the process [14].

Resistance to these treatments, both in vitro and in vivo, correlates with abnormal p53 [12, 15]. Wild-type p53 has been shown to repress the activity of the multi-drug resistance gene, MDR1, however treatment resis-

tance conferred by mutant p53 has been shown to be entirely independent of MDR1 or MDR3 [16, 17].

We describe the response rate and outcome of refractory/advanced CLL patients (half of them refractory to fludarabine) in which a high percentage had abnormalities of p53 determined by a variety of techniques. We also ascertained by the drug sensitivity assay as a predictor of response and showed that steroids, and frequently vincristine, killed CLL cells with abnormal p53 function. This is most likely due to the fact that steroids and vincristine can cause apoptosis independent of the p53 pathway [11, 12]. We conclude that in vitro testing and treatments with cellular killing mechanisms independent of a normally functioning p53 is a rational and effective approach to the management of this poor prognostic group.

Materials and methods

This study included 25 patients with advanced CLL. Diagnosis was confirmed by morphology and immunophenotyping of peripheral blood lymphocytes using a CLL scoring system as previously described [18]. All the patients had received a median of three previous treatments (range 1–6) and 16 were non-responders to their last therapy (Table 1) Out of 25, 24 patients had fludarabine alone, or in combination with other drugs, and 15 had no response to fludarabine, when last used. Fludarabine was not repeated due to autoimmune haemolytic anaemia following a previous course in one patient.

HDMP was given intravenously at a dose of 1gm/m² daily for 5 days repeated at four weekly intervals. All patients were given H2 antagonists and low dose oral antifungal prophylaxis. Patients who were neutropenic were given prophylactic antibiotics, and acyclovir was used in those with a previous history of herpes infection.

Blood pressure was monitored prior to starting treatment and half-hourly during treatment. Urine sugar was monitored daily while having the first course of HDMP and oral hypoglycaemic agents were given if the blood sugar was sustained above 12 mmols/l. Patients received a median of 4.5 courses of HDMP (range 1–8); three patients (cases 10, 12 and 15) were treated on two different occasions and each treatment has been analysed separately.

High dose methylprednisolone (HDMP) was used, alone in 13 of the treatment courses or in combination with other agents in 15, according to the results of the ex vivo apoptotic drug sensitivity assay: vincristine in seven patients and other drugs in eight others (Table 1). The drug sensitivity assay was performed on 20/25 of patients studied as described elsewhere [3].

Where peripheral blood or bone marrow was available, patients' cells were tested for abnormalities of the p53 gene. Fluorescent in situ hybridization (FISH) was performed in 22/25 to detect deletions and; in 21 of these, flow cytometry was carried out for detection of an abnormal p53 protein expression. Flow cytometry was also performed in one patient's sample where FISH data is unavailable. In total, 23/25 were tested for p53 abnormalities by either method. Cells from patients with abnormal p53 by the above methods, and available DNA, were sequenced for mutations.

FISH analysis was performed using standard methods [19] with a p53 locus specific probe (LSI p53 Spectrum Orange, Vysis, Richmond, UK) in combination with a probe specific for the chromosome 17 centromere (CEP17 Spectrum Green, Vysis) to exclude the possibility of monosomy 17 causing loss of hybridization signal for the p53 specific probe. Flow cytometry was carried out on fixed mononuclear cells with the monoclonal antibody DO1 (Novocastra, Newcastle upon Tyne, UK) recognising amino acids 11–25 of both wild-type and mutant p53. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Oxford, UK) [19].

Direct sequencing was conducted in seven samples with either deletion by FISH and/or protein expression by flow cytometry, obtained at the same time as those used for FISH experiments. Sequencing of exons 4–9 of p53 was performed by polymerase chain reaction (PCR) amplification of exonic sequences from genomic DNA followed by fluorescent automated cycle sequencing of both DNA strands. All mutations were re-sequenced from a different PCR product. Obtained DNA sequences were analysed using Sequence Analysis software (version 3.0) (ABI) and aligned and compared to published p53 sequence using Sequence Navigator software (ABI).

The *ex vivo* drug sensitivity assay was carried out on peripheral blood mononuclear cells incubated with drugs for 94 h as described by Bosanquet [20]. Cells from untreated patients were incubated with 7–10 standard CLL drugs: chlorambucil, cyclophosphamide (mafosfamide *in vitro*), prednisolone, vincristine, doxorubicin, epirubicin, fludarabine, cladribine, pentostatin and methylprednisolone. Previously treated patients' cells were incubated with up to 25 other agents. At the end of incubation, fixed duck erythrocytes (as an internal standard) and fast green/nigrosin (to stain dead cells black) were added and the cells cytocentrifuged. Counterstaining with a Romanowsky stain allowed identification of remaining live cells which were evaluated morphologically to determine LC90 s — the lowest concentration of drug to produce a 90% reduction in tumour cell survival compared with control cells [3]. The drug DSI was determined by comparison with all previous assay results with the drug so that 0% is the most resistant and 100% the most sensitive tested.

Results

P53 abnormalities

Ten out of 22 patients (45%) had hemizygous p53 deletion by FISH, 7/9 with deletion had also p53

expression by flow cytometry and 4/7 had mutations by direct sequencing. The remaining three patients had normal sequencing of exons 4–9. A summary of the p53 results is shown in Table 2.

Response to treatment

Twenty-five patients have been treated, three patients have been treated twice and are analysed separately giving a total of 28 treatment courses. Twenty-two patients (25 treatments) were evaluable and responses are summarised in Table 1. Three patients were unevaluable for response as they developed serious infection following the first course of treatment.

Seventeen of the 22 (77%) evaluable patients responded to treatment, two of these had a nodular PR documented by BM trephine biopsy. Even of the five non-responders (NR), four had a significant reduction of the lymphocyte count and decrease in lymph node size but short of PR; the other NR progressed while on treatment. Five of the ten patients with p53 abnormalities responded (2 nodular PR, 3 PR). However patients with p53 abnormalities still fared worse than those with normal p53 (Table 3).

Two out of three patients treated twice (cases 10, 12 and 15) responded a second time to HDMP giving a total of 19/25 (76%) responses. Of these 19, the median event free survival (EFS) from start of treatment was significantly longer than in the non-responders (Table 3).

Table 2 Results of p53 studies

Patient	Sex	P53 by FISH	Flow cytometry	Direct sequencing				
				Mutation	Exon	Codon	Amino acid substitution	
1	AB	M	Hemizygous deletion	Not available	No DNA available			
2	MD	M	Hemizygous deletion	Normal	No mutation			
3	GH	M	Hemizygous deletion	Over expression	No mutation			
4	BJ	M	Hemizygous deletion	Over expression	No DNA available			
5	PM	M	Hemizygous deletion	Over expression	CGT to CAT	8	273	Arg to His
6	FN	M	Hemizygous deletion	Over expression	CGT to CAT	8	273	Arg to His
7	FS	F	Hemizygous deletion	Over expression	CGC to CAC	5	175	Arg to His
8	DT	M	Hemizygous deletion	Normal	No DNA available			
9	AW	M	Hemizygous deletion	Over expression	No mutation			
10	DB	M	Diploid	Normal				
11	MB	F	Diploid	Normal				
12	GC	M	Diploid	Normal				
13	RG	M	Diploid	Normal				
14	TH	M	Diploid	Normal				
15	JI	M	Diploid	Normal				
16	MJ	M	Not available	Not available				
17	KK	M	Diploid	Normal				
18	RM	M	Diploid	Normal				
19	CM	M	Diploid	Normal				
20	DP	F	Diploid	Normal				
21	BS	F	Diploid	Normal				
22	SN	M	Diploid	Normal				
23	GP	M	Not available	Not available				
24	ABE	M	Hemizygous deletion	Over expression	TGT to TTT	8	277	Cys to Phe
25	JPMG	M	Not available	Normal				

Table 3 Survival and EFS by response and p53 status

Patient group (number of patients)	Median event free survival (months) (Kaplan-Meier)	<i>P</i> value (log rank)	Patient group (number of patients) ^a	Median overall survival (months) (Kaplan-Meier)	<i>P</i> value (log rank)
Responders to HDMP (19)	12.9	<0.0001	Responders to HDMP (17)	57	0.04
Non responders to HDMP (6)	5.5		Non responders to HDMP (5)	24	
Normal p53 (16)	12.9	0.05	Normal p53 (13)	57	0.0002
Abnormal p53 (9)	7.6		Abnormal p53 (9)	20	

^a Three patients were treated twice: for overall survival only the earlier of the two treatments was analysed

The remission duration from the end of treatment ranged from 2.2 to 18.9+ months with median of 6.6 months. Five patients remain in remission with a range of 4.7+–18.9+ months (median 9.4+ months). Nine of the 22 evaluable patients have died; three were non-responders who died of progressive CLL. The six others had responded to treatment, but four died of progressive disease and two of them with large cell lymphoma.

A summary of patients' responses, previous treatments and p53 status are displayed in Table 1. Responders to HDMP had a better overall survival and event free survival from start of treatment as determined by log rank statistics. (Table 3). Other chemotherapy agents were added in 15 cases where they showed a good ex vivo sensitivity. Twelve achieved PR including one nodular PR (case 17). Comparison of HDMP alone vs in combination with other therapies shows no advantage in the addition of other drugs in event free survival. It is of interest that of the three patients treated on two separate occasions, two (cases 10 and 12) had a longer duration of response when HDMP was given with another drug.

Toxicity

The most common side effect observed was insomnia or hyperactivity and the most serious side effect was infection, which was recorded in seven of the 25 patients. These were pneumonia (3), herpes zoster (1), *E.coli* septicaemia (1), oral candida (1), tuberculous osteomyelitis (1) and pyrexia of unknown origin. Treatment was stopped in three patients after one course due to infection and one of these died from intractable pneumonia. Two patients required oral hypoglycaemic agents and one patient required insulin due to reversible steroid induced diabetes. One patient had a spontaneous wedge collapse of his L2 vertebrae, four months following completion of treatment.

Correlation with the drug sensitivity assay

Ex vivo apoptotic drug sensitivity assay results were available in 22/25 evaluable cases and showed 40–100% ex vivo sensitivity of CLL lymphocytes to methylprednisolone. Eleven out of 17 responders had a DSI of 100% whereas only one out of five of the NRs had a DSI

of 100% (Table 1). All responders had values equal to or greater than 50%.

In all 20 cases analysed, methylprednisolone alone or in combination with another drug showed the greatest activity ex vivo.

Discussion

The treatment of refractory CLL remains disappointing and there are several reports of salvage regimens. Keating et al. [21] reported a 56% response rate with fludarabine in 68 previously treated patients, with a mean of two previous treatments, including 13% complete remissions. However Monserrat et al. used fludarabine in 68 heavily treated CLL patients and documented an overall response rate and improved survival in only 28% [22]. De Rossi et al. combined fludarabine with prednisolone in 22 pre-treated, chlorambucil refractory patients and reported 36% responses [23]. Marotta et al. [24] combined low dose fludarabine with cyclophosphamide in 20 patients, refractory to conventional therapy achieving an overall response rate of 85%. Tallman et al. [25] used cladribine in 26 relapsed refractory patients and attained a 31% remission rate. Bowen et al. [26] described a 50% response rate using subcutaneous Campath 1-H in fludarabine resistant CLL with a median survival of 11 months.

In our study, all patients were refractory to alkylating agents and 72% were resistant to fludarabine. Ex vivo drug sensitivity suggested sensitivity to steroids (40–100%) in all cases where it was performed. Resistance to fludarabine with this assay was 20/26 and resistance to all nucleoside analogues was found in 11/26. Treatment with HDMP produced a 76% response rate in these refractory patients, including half of those with p53 abnormalities. This compares favourably with previous responses to fludarabine (0/12) and Chlorambucil (1/8) in patients with p53 abnormalities [7, 8]. Vincristine is also noted to cause apoptosis in a p53 independent fashion and notably is the most commonly used drug with HDMP in this series. Although HDMP used showed no survival advantage over HDMP, in combination therapy with other drugs the 4/5 patients who remain in remission had an additional drug – so this may prove to be an advantage with time. In addition, two of the three patients treated twice with HDMP had a longer response when another agent was used.

Abnormalities of the p53 gene whether detected by protein expression [9], FISH [8] or direct DNA sequencing [27] all confer worse prognosis and drug resistance in CLL and in the hierarchical model described by Döhner et al. [6], p53 deletion by FISH is the abnormality, conveying the worst prognosis. The generally accepted mode of drug resistance is felt to be an impairment of the apoptotic process upon which most of the cytotoxics rely [17]. Other studies have demonstrated that steroid and vincristine cytotoxicity is independent of the p53 pathway [12]. Although the association of drug resistance and poor prognosis with abrogation of normal p53 function is well known, there is little in the literature to suggest a way forward in overcoming this problem. Some experimental work and early clinical studies suggest that proteasome inhibitors may offer an alternative apoptotic pathway to p53 dependent mechanisms [28, 29]; however, these drugs are not readily available in practice. It has been previously shown that in vitro sensitivity to steroids correlates with clinical outcome, and nucleoside resistance ex vivo has a similar in vivo correlation [3]. The role of steroids in lymphocyte induced apoptosis is well known. These agents induce apoptosis by a number of mechanisms including inhibition of cytokine production, alteration of mitochondrial membrane potential causing caspase activation, down regulation of cyclinD3 causing G1 arrest and inhibition of NF- κ B, a transcription factor complex important in cell survival [28, 29, 30, 31, 32, 33].

In summary, CLL patients with abnormal p53 are unlikely to respond to standard therapy. Treatments directed outside p53 dependent apoptotic pathways, such as steroids or vincristine, have a greater chance of success. We have demonstrated that these agents are effective in resistant patients where abnormalities of p53 are identified by current methods. This treatment causes little or no myelosuppression, so it can be used in stage C patients with profound cytopenias. Although almost one third of patients had infective episodes following treatment and the immunosuppressive effects of high dose steroids are well recognised, HDMP may not be the sole factor involved as CLL itself has a higher rate of infection due to its intrinsic immunodeficiency [34]. We conclude that HDMP alone or in combination with other drugs, guided by an ex vivo drug sensitivity assay, is a logical and effective treatment strategy in resistant CLL including patients with p53 abnormalities.

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