Pergamon

Biochemical Pharmacology, Vol. 50, No. 1, pp. 131–135, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00104-2

Short Communication

POTENTIATION OF CHLORAMBUCIL TOXICITY IN B-CLL LYMPHOCYTES USING THE DNA SYNTHESIS INHIBITORS APHIDICOLIN AND 1-β-D-ARABINOFURANOSYLCYTOSINE

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(*Received* 14 *October* 1994; *accepted* 17 *January* 1995)

Abstract—Previous studies in our laboratory have identified enhanced cross-link repair as a primary mechanism of resistance to nitrogen mustards in B-cell chronic lymphocytic leukemia (B-CLL). To evaluate the therapeutic potential for modulation of DNA repair by aphidicolin and 1-β-D-arabinofuranosylcytosine (ara-C), we examined the interaction between these two agents and chlorambucil in lymphocytes from untreated and treated-resistant B-CLL patients. We found that both aphidicolin and ara-C displayed synergy with chlorambucil over a range of inhibitor concentrations. This synergy was not restricted to the resistant samples. Our results indicate that these combinations can enhance the potency of chlorambucil in a clinically relevant model and should be considered for further preclinical and, eventually, clinical trials.

Key words: nitrogen mustard; aphidicolin; 1-β-D-arabinofuranosylcytosine; DNA repair

Drug resistance is one of the major limiting factors in the effective treatment and cure of malignancies. As a result, a great deal of effort has been invested, by both basic and clinical researchers, in trying to understand the mechanisms underlying this phenomenon. We have been studying nitrogen mustard resistance in B-CLL† as a means of developing a clinically relevant model of drug resistance. Following an *in vitro* incubation with melphalan, we found that the percentage of DNA interstrand cross-links in lymphocytes from treated-resistant patients is decreased significantly compared with that in lymphocytes from untreated B-CLL patients [1]. Furthermore, the kinetics of this process indicated that the lymphocytes from treated-resistant patients have an enhanced capacity to remove cross-links compared with those of untreated patients [2]. We recently demonstrated that lymphocytes from patients with nitrogen mustard resistant B-CLL display cross-resistance to mitomycin C and cisplatin but not to UV light or methyl methanesulfonate [3]. Thus, enhanced cross-link specific DNA repair appears to be one of the primary mechanisms of resistance in this model.

A major reason for studying drug resistance is to develop strategies to circumvent this resistance. As a result of poor understanding of the DNA repair systems, few inhibitors exist to modulate their activity. However, the final step of DNA repair is DNA resynthesis, and inhibitors do exist that block this cellular function. Studies involving such inhibitors (aphidicolin, ara-C, hydroxyurea) indicate that they can be used to potentiate the toxicity of cross-linking agents, such as nitrogen mustards and cisplatin [4–7]. It has also been shown that high concentrations of these inhibitors can actually prevent cross-link removal. Such drugs may be useful clinically in combination with nitrogen mustards [8]. In the present report, we examined the abilities of aphidicolin and ara-C to modulate chlorambucil toxicity in B-CLL lymphocytes.

Materials and Methods

Drugs. Chlorambucil, aphidicolin, and ara-C were obtained as powder from the Sigma Chemical Co. Chlorambucil and aphidicolin were dissolved in DMSO at a concentration of 18 and 29.5 mM, respectively. Ara-C was prepared fresh in phosphate-buffered saline at a concentration of 50 mM. Immediately before addition to the cells, the drugs were diluted in phosphate-buffered saline such that the concentration of DMSO in the culture medium never exceeded 0.5%.

B-CLL lymphocyte cell culture and MTT cytotoxicity assay. All patients were diagnosed with B-CLL and separated into two groups (sensitive and resistant) using both clinical criteria as described by Panasci *et al.* [1] and the results from the *in vitro* cytotoxicity assay. Lymphocytes were isolated from the peripheral blood of CLL patients by centrifugation on Ficoll-Hypaque according to Panasci *et al.* [1] and resuspended in lymphocyte culture medium (RPMI 1640, 10% fetal bovine serum, 20 mM HEPES, 10 μg/mL gentamycin). The purified samples were generally greater than 90% B lymphocytes; the mean (±SEM) T cell contamination in the sensitive and resistant populations was 9.7 ± 2.0 and 5.3 ± 2.0%, respectively (no significant difference by an unpaired *t*-test). The MTT assay was performed as described by Plumb *et al.* [9]. B-CLL lymphocytes were diluted to a final concentration of 1.5 × 10^6 cells/mL. The lymphocytes were then exposed to drug, as required, and seeded into 96-well microculture dishes in aliquots of 200 μL (8 replicates of each drug concentration; routinely the standard error was less than 4% of the mean). Following 72 hr of incubation at 37° and 5% CO_2, 50 μL aliquots of 3 mg/mL MTT were added to each well and incubated for an additional 4 hr. The cells

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† Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; B-CLL, B-cell chronic lymphocytic leukemia; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 1. Results from the combination of chlorambucil and aphidicolin in the cytotoxicity assay

(A)		Aphidicolin					
		2 μM		5 μM		12 μM	
	Patient	DMF	Toxicity	DMF	Toxicity	DMF	Toxicity
Sensitive	JGO	1.6	0	2.2	0	3.2	7
	SLA	1.5	2	2.8	11	9.4	34
	RME	1.7	0	2.1	7	3.4	16
	SSK	1.3	0	1.5	0	3.0	6
	NNO	NA	NA	2.0	3	2.4	14
	MLE	1.4	0	1.8	0	2.2	0
Resistant	EST	1.4	3	1.9	9	3.3	15
	PVA	1.1	0	1.7	0	2.2	0
	EHA	1.3	2	1.9	5	2.6	8
	AMO	1.3	0	1.4	0	2.4	3
	FSA	1.2	0	1.7	0	3.2	10
	SKR	1.3	0	1.7	0	2.3	0
	RSL	1.9	10	2.3	10	3.1	16

(B)		Aph	CLB			
	Patient	EC_{50} (μM)	EC_{50} (μM)	I_2	I_5	I_{12}
Sensitive	JGO	55	3.9	0.65	0.55	0.53
	SLA	55	4.7	0.72	0.45	0.32
	RME	91	4.8	0.63	0.53	0.42
	SSK	>100	5.7	0.81	0.73	0.45
	NNO	132	7.4	NA	0.54	0.51
	MLE	251	7.8	0.74	0.58	0.51
Resistant	EST	88	14	0.72	0.57	0.44
	PVA	194	15	0.92	0.61	0.51
	EHA	130	17	0.76	0.56	0.48
	AMO	203	32	0.79	0.76	0.47
	FSA	NA	38	NA	NA	NA
	SKR	>100	50	0.82	0.65	0.56
	RSL	NA	75	NA	NA	NA

B-CLL lymphocytes were exposed to 0, 2, 5, and 12 μM aphidicolin for 30 min at 37° prior to treatment with chlorambucil. Cytotoxicity was measured using the MTT assay. Cytotoxicity profiles were also established for aphidicolin. EC_{50} represents the concentration of drug required to produce a 50% reduction in viable cells following a 3-day incubation. In panel A, the dose modifying factor (DMF) is calculated as the ratio of the EC_{50} chlorambucil to the EC_{50} of chlorambucil in the presence of a fixed concentration of aphidicolin. Toxicity refers to the cytotoxicity of the inhibitor alone (% cell death compared with controls). Panel B presents the analysis of synergy for the data in panel A. I_x is calculated according to the formula in Materials and Methods. $I_x < 1$ indicates synergy between chlorambucil (CLB) and aphidicolin (Aph) at X μM aphidicolin. NA = not available.

and formazan crystals were pelleted in the wells by centrifugation at 350 *g* for 10 min. The supernatant was removed, and the formazan crystals were resuspended in 200 μL DMSO followed by addition of 25 μL of Sorensen's glycine buffer (0.1 M glycine plus 0.1 M NaCl equilibrated to pH 10.5 with 0.1 N NaOH). The $O.D._{570}$ was measured using an ELISA reader.

Analysis of synergy. Synergy was determined by the calculation:

$$I_X = \frac{EC_{50}C/I_X}{EC_{50}\,CLB} + \frac{Tox\,I_X}{EC_{50}\,I}$$

where $EC_{50}C/I_x$ = the concentration of chlorambucil required to produce 50% kill in combination with the inhibitor at concentration X; EC_{50} CLB = the concentration of chlorambucil required to produce 50% kill in the absence of inhibitor; Tox I_x = the concentration of inhibitor in the combination; EC_{50} I = the concentration of inhibitor required to produce 50% kill in the absence of chlorambucil. According to this formula when $I_x < 1$, then the interaction is synergistic. If $I_x = 1$, the interaction is additive, and $I_x > 1$ indicates antagonism [10].

Results and Discussion

The main objective of the study of drug resistance is the development of improved therapies by which this resistance could be overcome. It is now clear from our studies that enhanced cross-link repair is one factor that correlates with resistance in B-CLL [1–3]. However, it is also clear that this repair system belongs to an undefined pathway and, therefore, will be difficult to target with standard chemotherapeutic agents. DNA repair can be divided into two steps: (I) incision and release of the damaged site, and (2) resynthesis of the lost DNA [11]. As we know very little about the repair mechanism in B-CLL, targeting step I would be difficult. We can, however, target step 2 using specific inhibitors of DNA polymerization (resynthesis).

The first inhibitor used was aphidicolin. Aphidicolin

Table 2. Results from the combination of chlorambucil and ara-C in the cytotoxicity assay

(A)		Ara-C					
		0.5 μM		1.0 μM		2.0 μM	
	Patient	DMF	Toxicity	DMF	Toxicity	DMF	Toxicity
Sensitive	SLA	3.0	11	4.9	13	6.9	30
	RME	1.6	5	2.2	13	9.6	45
	SSK	2.7	5	7.7	28	NA	NA
	MLE	1.8	0	2.3	4	NA	NA
Resistant	EST	6.5	17	29	46	NA	NA
	EHA	3.7	1	6.0	14	NA	NA
	AMO	2.4	0	3.2	0	6.1	11
	FSA	2.3	0	NA	0	4.1	6
	SKR	1.6	0	2.3	4	4.2	25

(B)	Patient	Ara-C EC_{50} (μM)	CLB EC_{50} (μM)	$I_{0.5}$	$I_{1.0}$	$I_{2.0}$
Sensitive	SLA	3.6	4.7	0.50	0.48	0.70
	RME	3.2	4.8	0.78	0.77	0.73
	SSK	2.9	5.7	0.54	0.47	NA
	MLE	5.0	7.8	0.64	0.64	NA
Resistant	EST	1.2	14	0.57	0.87	NA
	EHA	2.0	17	0.46	0.67	NA
	AMO	6.6	32	0.48	0.46	0.47
	FSA	7.3	38	0.51	0.55	0.52
	SKR	11.0	50	0.67	0.53	0.43

B-CLL lymphocytes were exposed to 0, 0.5, 1.0, and 2.0 μM ara-C for 30 min at 37° prior to treatment with chlorambucil. Cytotoxicity was measured using the MTT assay. Cytotoxicity profiles were also established for ara-C. EC_{50} represents the concentration of drug required to produce a 50% reduction in viable cells following a 3-day incubation. In panel A, the dose modifying factor (DMF) is calculated as the ratio of the EC_{50} chlorambucil to the EC_{50} of chlorambucil in the presence of a fixed concentration of ara-C. Toxicity refers to the cytotoxicity of the inhibitor alone (% cell death compared with controls). Panel B presents the analysis of synergy for the data in panel A. I_x is calculated according to the formula in Materials and Methods. $I_x < 1$ indicates synergy between chlorambucil (CLB) and ara-C at X μM ara-C. NA = not available.

competes with dCTP for binding in the active site of DNA polymerase α [12]. Freshly isolated lymphocytes were incubated at 37° in the presence of various concentrations of aphidicolin for 30 min prior to the addition of chlorambucil. The aphidicolin was left in the culture throughout the 3-day incubation period. As the concentration of aphidicolin increased, so did the effect on chlorambucil cytotoxicity (Table 1A). Strong synergy was obtained using this combination in samples from resistant patients (Table 1B). It was our expectation that this combined effect of aphidicolin and chlorambucil would be more pronounced in resistant lymphocytes as compared with sensitive lymphocytes since the resistant cells display greater cross-link removal. However, when sensitive lymphocytes were screened under the same conditions, similar observations were made (Table 1, A and B). So, while aphidicolin displays synergy with chlorambucil in our model, this effect was not specific to resistant lymphocytes.

The next DNA synthesis inhibitor used was ara-C. Ara-C was chosen because this cytosine analog can compete with dCTP for binding in all polymerases tested *in vitro* [12]. As polymerases β and δ have also been implicated in the repair reactions [11], it is possible that this inhibitor will provide greater enhancement of toxicity than aphidicolin, which primarily inhibits α. As with aphidicolin, the cells were exposed to increasing concentrations of ara-C prior to chlorambucil treatment. The effect of ara-C in combination with chlorambucil was tested in both sensitive and resistant lymphocytes. While strong synergy was obtained (Table 2, A and B), the steep cytotoxicity curve and varying degrees of resistance made it difficult to obtain concentrations that were non-toxic in all samples tested. In fact, it appears that there was a correlation between resistance to ara-C and chlorambucil ($r = 0.846$, $P = 0.004$, $N = 9$). There was no significant correlation between the toxicities of aphidicolin and chlorambucil ($r = 0.497$, $P = 0.1732$). Thus, while there exists synergy between ara-C and chlorambucil, cross-resistance to ara-C could defeat its usefulness in combinational therapy. It should be noted, though, that the two patients with low-level resistance (EST and EHA) did not display cross-resistance to ara-C. In fact, they exhibited greater sensitivity to ara-C than the sensitive population. It is possible that such patients could benefit from the ara-C/chlorambucil combination.

In the case of both aphidicolin and ara-C, the dose modifying factor was correlated to the toxicity of the inhibitor. A comparison of aphidicolin toxicity and dose modifying effect revealed a correlation coefficient (r) of 0.891 ($P = 0.0001$). For ara-C, the correlation was slightly weaker ($r = 0.759$), but still highly significant ($P = 0.0004$).

One surprising finding from this study was the correlation between ara-C and chlorambucil resistance. Although we examined a small population (9 patients), the statistics for the correlation indicated that it was highly significant ($P = 0.0004$). This observation is somewhat confusing because the resistant lymphocytes exhibit about two times the DNA

synthesis activity of the sensitive cells and would be expected to be more sensitive [13]. However, ara-C resistance could arise from a number of sources including reduced deoxycytidine kinase activity (this enzyme converts ara-C to its toxic form ara-CTP) or increases in the dCTP pool (ara-C is a competitive inhibitor) [14]. It has been reported recently that chlorambucil treatment induces ribonucleotide reductase activity, which in turn can increase deoxynucleotide pools [15]. Therefore, the observed resistance to ara-C may result from cellular changes required for the DNA polymerization system to adapt to the enhanced repair process.

It is clear from the data that both aphidicolin and ara-C can potentiate chlorambucil toxicity in both sensitive and resistant lymphocytes. Ara-C has been used clinically for several years, and trials are currently underway using aphidicolin, so that dosing schedules and toxicities will soon be known. Aphidicolin provided the most consistent synergism of the two inhibitors, indicating that this combination deserves further investigation. Phase I clinical trials with aphidicolin glycinate (a soluble form of aphidicolin) demonstrated that a concentration of 3 μg/mL (9 μM) is achievable in humans [16]. At 5 μM aphidicolin, we have shown modification of toxicity ranging from 1.5- to 2.8-fold. The addition of 12 μM aphidicolin to the chlorambucil treatment could enhance toxicity 3-fold. Previous trials have shown that a concentration of 0.5 μM ara-C is achievable *in vivo*, and this dose is well tolerated [17]. In some cases, we found that 0.5 μM ara-C provided strong potentiation of chlorambucil toxicity (3- to 6-fold, Table 2A). The drawback of using ara-C is the severe myelosuppression associated with this drug. Moreover, the observation that ara-C resistance correlated with resistance to chlorambucil further hinders the use of this combination.

Aphidicolin is considerably less toxic than ara-C in the lymphocytes and demonstrated no significant correlation with nitrogen mustard resistance. This is enough to bring some resistant lymphocytes into the sensitive range. As clinical trials are currently underway with aphidicolin, this combination should be considered for diseases currently receiving single agent nitrogen mustard therapy (B-CLL, multiple myeloma). Also, ara-C was able to produce > 6-fold enhanced toxicity in one resistant patient (EST, Table 2A). As regards therapy, if resistant cells were tested *in vitro*, the patients that would fare best with either aphidicolin or ara-C could be identified before therapy begins.

The results of these studies clearly establish B-CLL as a quick, effective system for examining drug toxicity in a clinically relevant model of resistance. Using this strategy, a potential therapeutic effect has been established for the aphidicolin/chlorambucil and ara-C/chlorambucil combinations. By combining the *in vitro* cytotoxicity assay using B-CLL cells with a method for measuring toxicity in hematopoeitic progenitor cells (e.g. CFU-C assay), a screening system could be developed to investigate new combinations of alkylating agents and chemosensitizers. This same system could then be used with individual resistant patients to see who would best benefit from the treatment. The MTT assay has already been used to predict clinical response to chlorambucil in untreated patients, with very promising results [18, 19]. In conclusion, nitrogen mustard resistant B-CLL is the first demonstration of a tumor that develops drug resistance *in vivo* and can be studied readily *in vitro*. This model should prove useful in understanding the mechanisms of *in vivo* drug resistance and developing strategies to circumvent resistance in B-CLL, as well as other lymphoid malignancies.

Acknowledgements—This work was supported by a private donation from Evelyn Steinberg Alexander and by Fonds de la Recherche en Santé du Québec, Reseau du Cancer. L.P. is a recipient of the Gertrude-Stanley Vineberg clinical scientist award.

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