Novel Ex Vivo Analysis of Nonclassical, Pleiotropic Drug Resistance and Collateral Sensitivity Induced by Therapy Provides a Rationale for Treatment Strategies in Chronic Lymphocytic Leukemia

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Extensive research into mechanisms of cytotoxic drug resistance and subsequent clinical trials of drug resistance modifiers have produced few encouraging results. In this report, we analyze 4,400+ ex vivo Differential Staining Cytotoxicity (DiSC) assay drug response results from patients with chronic lymphocytic leukemia (CLL) to investigate the development of drug resistance during treatment. Patients were untreated (n = 216) or previously treated with various cytotoxic agents (n = 188). Data was processed to identify ex vivo resistance (or sensitivity) induced by treating patients with prednisolone, chlorambucil, cyclophosphamide, anthracycline, or fludarabine. Induced resistance was apparently not associated with any one known mechanism. Treatment with chlorambucil induced a 10-fold sensitivity to steroids; cyclophosphamide induced greater resistance to anthracyclines than to alkylating agents; anthracyclines induced greatest resistance to chlorambucil, cisplatin, carboplatin, and cladribine. Patients previously treated with at least two regimens were only 2.16-fold more resistant to CLL drugs than untreated patients, but had significantly reduced survival (median survival, 7.9 months compared with 61.1 months for untreated patients). These results suggest that chlorambucil and/or an antimetabolite should be administered before cyclophosphamide or anthracyclines to delay the onset of extensive pleiotropic drug resistance. Because individual differences in drug sensitivity are considerable, specific guidance could be obtained from ex vivo assay results. Furthermore, as a model for investigating drug resistance mechanisms, fresh CLL lymphocytes represent a useful alternative to drug-resistant cell lines.

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MATERIALS AND METHODS

DiSC assay. A detailed description of improved DiSC assay methodology, including details of sample acquisition and the drugs used, has been published. Briefly, blood specimens from patients with CLL in UK hospitals were collected into EDTA and sent to our laboratories within 24 hours of phlebotomy. Patients predominantly fell into two categories: (1) those being entered into the Third UK Medical Research Council (MRC) CLL trial (chlorambucil + chlorambucil + epirubicin), and (2) patients whose specimens were sent to Bath Cancer Research Unit (Bath, UK) for ex vivo chemosensitivity testing to aid in choice of subsequent chemotherapy. After isolation over ficoll-hypaque, lymphocytes were washed and suspended in RPMI 1640 culture medium supplemented with fetal bovine serum (FBS).

Up to 38 drugs were tested at five concentrations in duplicate; the actual number of drugs was dependent on cell yield and the patient’s clinical trial status or their clinical drug resistance. The principal CLL drugs tested were chlorambucil, cyclophosphamide, prednisolone, vincristine, doxorubicin, epirubicin, fludarabine, cladribine, pentostatin, and methylprednisolone. Drug (80 µL) was added to 80,000 cells in 20 µL medium; phosphate-buffered saline (PBS) in medium served as a control.

After 94 hours of incubation (37°C, humidified 5% CO2), 50,000 fixed dead erythrocytes (DRBCs) were added to each tube in 10 µL PBS containing 2% fast-green and 1% nigrosin. The cells were
transferred to microscope slides by cytocentrifugation, air-dried, methanol-fixed, and counterstained with a Romanowsky stain. Subsequent evaluation of slides by light microscopy facilitated the determination of drug efficacy at each concentration compared with controls. DiSC assay results are calculated as the lowest concentration of drug needed to produce a 90% reduction in tumor cell survival (LC_{90}) compared with the control.

**Prior therapy.** Details of prior therapy supplied with the specimen were confirmed with the MRC CLL trial office or by review of the patient's treatment history.

**Data analysis.** All manipulations of these data have been undertaken with log_{10} (LC_{90}) values, giving means and SDs that are used for statistical calculations in equations 2 and 3. The means and SDs in columns 2 and 3 of Table 3 are transformed back to LC_{90}, giving geometric means (GMean, pg/mL) and geometric SDs (GSD). Equation 1 is dependent on the variance of the particular drug's results. For instance, pentostatin gives large resistance factors (up to 66.0; Table 3) so the denominator SD, also distorting the standardized induced resistance. Where many values were obtained (eg, with chlorambucil), found to be similar to the corresponding SD.

The significance attached to treatment-induced resistance factors is dependent on the variance of the particular drug's results. For instance, pentostatin gives large resistance factors (up to 66.0; Table 3) but also has a large variance (GSD, 58.4; Table 3); an induced resistance of fivefold would therefore, be insignificant. For drugs such as epirubicin that yielded small variances (GSD, <2; Table 3), an induced resistance of fivefold would be very significant. To allow for this, induced resistance results were standardized as follows. In Figs 2, 4, and 8:

\[
\text{Resistance Factor} = \frac{\text{GMean}_t}{\text{GMean}_u} \tag{1}
\]

where subscript \(t\) indicates the treated group and subscript \(u\), the untreated group.

Equations 2 and 3 give a linear scale, whereby positive and negative values indicate induced resistance or sensitivity, respectively (Figs 2 through 8). The SEMs on these graphs are similarly transformed:

\[
\text{Standardized induced resistance} = \frac{\bar{t} - \bar{u}}{\text{SD}_u} \tag{2}
\]

where \(\bar{t}\) indicates the mean of the log LC_{90} values for the group of treated patients; \(\bar{u}\), the mean of untreated patients; and \(\text{SD}_u\), the SD for untreated patients, of the relevant drug. In Figs 3 and 5 through 7:

\[
\text{Standardized induced resistance} = \frac{\bar{t} - \bar{c}}{\text{SD}_t} \tag{3}
\]

where \(\bar{c}\) indicates the mean of the log LC_{90} values for the control group of patients. Ideally, the denominator for this equation should be \(\text{SD}_u\), but occasional small numbers in these groups may distort the SD, also distorting the standardized induced resistance. Where many values were obtained (eg, with chlorambucil), SD, values were found to be similar to the corresponding SD; so the denominator of SD, was used throughout.

Equations 2 and 3 give a linear scale, whereby positive and negative values indicate induced resistance or sensitivity, respectively (Figs 2 through 8). The SEMs on these graphs are similarly transformed:

\[
\text{Standardized SEM} = \frac{\text{SEM}}{\text{SD}_c} \tag{4}
\]

Statistical analysis. It should be noted that these results are sensitive to sample size; SEMs for different drugs are variable and are shown on the graphs (Figs 2 through 8). Statistical significance was calculated by Student's \(t\) test, and these values are indicated in the figures. A total of 177 comparisons of control versus treated results are presented in Figs 2 through 8. Table 1 gives the level of significance expected by chance versus that observed. It is seen from this that 9 of 11 results where \(0.01 < P < 0.1\) are expected to be actually significant, as are 17 of 24 results where \(0.01 < P < 0.05\). In many cases, the significance of one result is reinforced by a similar result for drugs in the same biochemical class, suggesting true significance rather than chance result. Thus, it is likely that daunorubicin and epirubicin in Fig 5 are truly significant, because all other anthracyclines behave in the same way, while the vincristine result may well be due to chance, as the vincristine result goes in the opposite direction.

**RESULTS**

This report is based on 4,491 drug LC_{90} values using 34 different drugs and representing the results of 404 DiSC assays performed on 345 patient specimens\(^9\); 216 assays on 112 patients were confirmed with the MRC CLL trial office or by review of the patient's treatment history.

![Fig 1. Relationship between LC_{90} values and induced resistance values presented in Figs 2 through 8; chlorambucil results for Figs 2 and 6 shown as an example. (A) All the LC_{90} values from 215 chlorambucil tests on untreated patients. To the left is the mean (\(\bar{u}\)), indicated by an asterisk; \(\pm \text{SD}_u\), of these data, which is defined as an induced resistance of zero (right-hand y axis). This SD becomes the unit by which induced resistance is measured (right-hand y axis; also, y axes in Figs 2 through 8; see equations 2 and 3). To the right of these data are \(\bar{u} \pm 1\text{SEM}\), used as the control in Figs 2, 4, and 8. (B) Chlorambucil LC_{90} values for all treated patients; the bar to the right is the mean (\(\bar{t}\)) \(\pm \text{SEM}\) of these data. On average, treated patients are seen to be 2.47 times (0.92 SDs) more resistant to chlorambucil. This result is indicated along with other drugs tested in Fig 2. (C) The LC_{90} values on the left (chlorambucil results of patients who have had chlorambucil + prednisolone + cyclophosphamide + vincristine) act as a control for the effect of anthracycline. The mean (\(\bar{b}\)) \(\pm \text{SEM}\) of these data is indicated by the \(\square\) and forms the baseline SEM for the chlorambucil bar in Fig 6 (indicated by the shorter horizontal dotted line). To the right of these data are results from patients who have also received anthracycline; they are more resistant by a factor of 0.95 SDs. The data points for each group show a distribution close to normal with little skewness or kurtosis. Results for other drugs shown in Figs 2 through 8 were calculated in the same manner.\]
untreated patients and 188 assays where the patients had received cytotoxic therapy.

While the treatment of CLL in the United Kingdom has commonly followed the pattern of chlorambucil + prednisolone, followed by CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone), followed by fludarabine, we found a considerable diversity in patient pretreatment status: 49 different combinations of drugs (between one and four different regimens) had been given to the 188 patients in the pretreated group. Treatment other than that mentioned above included dexamethasone, high-dose methylprednisolone, vinblastine, etoposide, epirubicin, mitoxantrone, daunorubicin, amnacrine, α-interferon, and irradiation.

Within this diversity of therapy administered to CLL patients, we were able to establish pretreatment subgroups to isolate the effect of treatment with single drugs on ex vivo drug sensitivity. Details of how the comparisons were undertaken are shown in Fig 1 (with chlorambucil as an example), and the results are presented in Figs 2 through 8. Table 2 shows details of age, sex, and disease duration (date of diagnosis to DiSC assay date) for each of these subgroups. The median age of all subgroups is close to the typical value for CLL. The median disease duration for the pretreatment groups ranges from 3.0 to 5.0 years and does not obviously relate to prior treatment; thus, the medians for chlorambucil only, additional anthracycline (± other drugs), or additional fludarabine (± other drugs) are 3.3, 3.0, and 3.1, respectively. The ratio of males to females is more variable. However, in separate analyses of both the untreated and all previously treated data, we found no significant difference in mean drug sensitivity between males and females for any of the 34 drugs tested ($P > .1$).

In addition, when the results for Fig 7 (showing effect of fludarabine) were generated using males only (for control and treatment groups; Table 2), the pattern of induced resistance (including statistical significance) was confirmed. Binet stage at assay was similar in 104 untreated patients (24, A; 32, B; 48, C) and 74 previously treated patients (23, A; 10, B; 41, C) where data were available. Several other confounding influences could have potentially skewed the data. However, it was beyond the scope of this analysis to allow for how much drug a patient had received, how long they were treated, the schedule used, or whether or not they became clinically resistant to the drug.

Overall results (comparing 188 pretreated with 216 untreated CLL assays) are given in Fig 2. With the exception of the corticosteroids (prednisolone, methylprednisolone, and dexamethasone) and vincristine, it is apparent that the administration of cytotoxic therapy has induced resistance to most drugs. Subdivision of the data into pretreatment subgroups (Figs 3 through 7) reveals intriguing drug response patterns within the general picture of acquired resistance shown in Fig 2. Indeed, in certain cases, in addition to the observed pleiotropic drug resistance, results point to collateral sensitivity to a small number of drugs.

There is little evidence that treating patients with prednisolone (Fig 3) induces ex vivo resistance, with the exceptions being prednisolone, methylprednisolone, and pentostatin. Administration of chlorambucil as a single agent (Fig 4) induces
no significant increase in resistance to the principal CLL drugs. In fact, the most interesting and statistically significant change is the strongly enhanced sensitivity induced to prednisolone and methylprednisolone. Treatment with cyclophosphamide, on the other hand, has induced a widespread pleiotropic drug resistance (Fig 5) with relatively large increases in resistance to the antimetabolites and anthracyclines, as well as to chlorambucil and etoposide. For most drugs, the degree of anthracycline resistance (Fig 6) is small compared with that caused by cyclophosphamide (Fig 5), and the pattern of resistance is quite contrary to that induced by anthracyclines in cell lines. While we see increased resistance to the synthetic compounds (chlorambucil, platinums, cladribine) and dexamethasone, there has been little change in resistance to the naturally derived MDR drugs commonly associated with anthracycline resistance (anthracyclines, vincas, etoposide, ansamycin). Treatment with fludarabine (Fig 7) significantly increased resistance to cladribine and probably to fludarabine ($P > .06$), as well as to chlorambucil.

Figure 8 shows the degree of induced drug resistance to 29 drugs determined in 32 samples from patients pretreated with multiple drugs, many of whom had end-stage disease. All had received a minimum of five principal CLL drugs: both chlorambucil and cyclophosphamide, prednisolone, vincristine, and an anthracycline. In addition, 18 of 32 had been treated with at least one other regimen including fludarabine (nine samples), etoposide (five samples), second anthracycline (three samples), and radiotherapy (three sam-
The interesting observation from these data is that drug resistance is not universally induced despite the multiplicity of chemotherapy that these patients have received. Thus, while significant resistance to chlorambucil, cyclophosphamide, and the antimetabolites is observed, only small changes are observed for the other principal CLL drugs, and residual sensitivity to the steroids.

The resistance factors (equation 1) arising from varied drug treatments are presented in Table 3. Although the resistance induced by multiple prior chemotherapy (Table 3, last column, and Fig 8) is for most drugs in the order of 0.5- to 5-fold (average, 2.16-fold), this small increase in cellular resistance has conferred almost total resistance to subsequent therapy in vivo, as the median survival of these patients was only 7.9 months from assay date compared with 61.1 months for untreated patients. The largest absolute increases in sensitivity are approximately 10-fold for the steroids, after treatment with chlorambucil. The large resistance factors for pentostatin parallel the large SD in untreated specimens and demonstrate the need for results to be normalized.
Fig 6. Effect of pretreatment with an anthracycline on ex vivo drug sensitivity. Control group, 80 specimens pretreated with chlorambucil ± prednisolone ± cyclophosphamide ± vincristine (13 to 72 LC₅₀ values for each drug; mean, 37); bars, 31 specimens pretreated with chlorambucil ± prednisolone ± cyclophosphamide ± vincristine plus epirubicin (n = 15) or doxorubicin (n = 10) or mitoxantrone (n = 6) (12 to 28 values; mean, 21). *P < .05; **P < .01; ***P < .001.

Fig 7. Effect of pretreatment with fludarabine on ex vivo drug sensitivity. Control group, 58 specimens pretreated with chlorambucil ± prednisolone (12 to 51 LC₅₀ values for each drug; mean, 31); bars, 11 specimens pretreated with chlorambucil ± prednisolone plus fludarabine (4 to 11 values; mean, 7). *P < .05; ***P < .001.

The following is a summary of the changes in drug resistance shown in Figs 2 through 8: (1) ex vivo chlorambucil, fludarabine, cladribine, and pentostatin results are most adversely affected by treatment; (2) increased ex vivo resistance is only occasionally induced to the anthracyclines; (3) prior treatment has variable effects on the ex vivo response to the steroids, vincas, and thiopurines, such that ex vivo resistance induced by treating patients with one drug was sometimes counteracted by ex vivo sensitization induced by treating with another.

DISCUSSION

The overall impression drawn from this analysis is that chemotherapy has induced unexpected ex vivo changes in cellular drug sensitivity. The changes are unexpected because they are sometimes at variance with the prevailing wisdom derived from cell lines made artificially drug-resistant in vitro. For instance, treating patients with anthracyclines (MDR-associated drugs) induced more ex vivo resistance to steroids and platinums while treating with cyclophosphamide induced resistance to all the MDR drugs except vincristine. This suggests little or no induction of MDR, corroborating other work in which we assessed the expression of MDR in some of these specimens (using C494 and JSB1): no correlation was found between MDR expression and drug sensitivity, irrespective of whether or not the drugs were expected to induce MDR.22

The absence of a conspicuous relationship between our ex vivo findings and the resistance factors induced in cell lines23 suggests that this posttreatment pleiotropic resistance observed in fresh CLL cells is most likely caused by the complex interplay of a number of resistance mechanisms, some of which may as yet be unidentified. This endorses the conclusion of a study of the mechanisms of chlorambucil resistance in CLL: no correlation was found between any of the individual parameters measured (GSH, GST, protein-bound sulfhydryl) and clinical response, pointing to the involvement of multiple mechanisms.24 The investigators reporting a recent drug resistance study using HL60 promyelocytic cell line with clinically relevant (less than 10-fold) induced resistance likewise concluded that the best explanation for their results was the coinduction of multiple resistance mechanisms.18

Indeed, if the limited, induced resistance observed in this study is shown to represent the culmination of effects produced by multifactorial resistance mechanisms, it is not surprising that the numerous clinical trials with drug resistance modifiers have identified only a minority of patients who
show benefit. It could be valuable, however, to choose a drug resistance modifier on the basis of prior individual ex vivo drug sensitivity testing with a panel of modifiers.23

CLL is by nature a chronic disorder, so that patients receive a series of gradually more aggressive treatments as disease control becomes more problematic. No cures have been documented21 although complete phenotypic remissions have recently been described.22 The results presented here may, therefore, have useful implications for current and future treatment strategies in CLL.

First, chlorambucil induced almost no resistance to other CLL drugs (Fig 4), which validates its use as first-line therapy. It is also inexpensive and relatively nontoxic. Second, in view of the sensitization to the steroids induced by chlorambucil, the use of high-dose steroids (such as methylprednisolone) either after or alternating with chlorambucil would be worthy of investigation.30 Third, the purine analogue antimetabolites are seemingly appropriate for second-line therapy, because fludarabine also induced little pleiotropic drug resistance to other drugs (Fig 7). Fourth, treatment involving cyclophosphamide should, perhaps, be delayed until other cytotoxic agents have been administered, because it induced pronounced pleiotropic drug resistance (Fig 5).

Obviously, these conclusions are based on the mean results of many patients. Likewise, clinical trial results, which guide standard treatment practice, are based on the mean results of many patients. However, the interpatient differences in this study are often larger than those between the mean values of different pretreatment groups. Similarly, ex vivo cytotoxicity testing of untreated patients with childhood acute lymphoblastic leukemia subdivided for factors such as age and immunophenotype—while yielding prognostically significant information (as with this present study)—showed larger differences between patients than between the groups.31 Thus, a more accurate indication of the most appropriate treatment options for an individual patient, at any particular juncture of their disease, might be gained by undertaking an ex vivo drug sensitivity test.

The fludarabine-induced resistance to cladribine (and to a lesser extent, pentostatin; Fig 7) confirms the limited response to a second antimetabolite seen in the clinic.32,33 Therefore, the selection of the antimetabolite most likely to produce an optimal clinical response is important; it would be prudent to use ex vivo assay results to guide in the choice of these agents, not only for clinical efficacy but also for cost-effectiveness, bearing in mind both the considerable expense of these drugs and their associated debilitating immunosuppression. Ineffective antimetabolite therapy is a costly treatment option.

Many of the patients who had received multiple prior therapy (Fig 8) had advanced disease. Due to the residual sensitivity to the steroids, these drugs have quite often appeared to be the most active ex vivo. Consequently, high-dose methylprednisolone has been administered in a number of such cases, with 4 of 12 patients achieving excellent partial responses and surviving an unexpected extra 2 to 3 years.30 This ex vivo sensitization to steroids by administration of chlorambucil finally gives an explanation of the known facts, that steroids do little for patients at diagnosis (11% response rate34) but are anecdotally known to be more effective in end-stage disease.

Of the principal CLL drugs, the increase in cellular resistance observed in the more heavily pretreated patients ranged from 0.34 for methylprednisolone to 48.54 for pentostatin, with a geometric mean of just 2.16-fold (Table 3, last column). The predominantly low values of these resistance factors (Table 3) are in agreement both with the twofold to sixfold increase in resistance considered to be clinically most relevant35 and with recently published factors for reduced ex vivo radiolabeled drug accumulation and reduced ex vivo...
intracellular doxorubicin fluorescence in patients with refractory acute nonlymphocytic leukemia.\textsuperscript{35} Despite the small magnitude of this elevated cellular resistance, it is probably sufficient to cause treatment failure,\textsuperscript{37} emphasizing a major drawback of looking exclusively at extremely resistant cell lines: forms of resistance that are relevant in patients may be missed altogether.\textsuperscript{37}

The DiSC assay and other ex vivo assays, for instance the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], FMC (fluorometric microculture cytotoxicity), and ATP (adenosine triphosphate luminescence) assays, have been shown to have an overall predictive accuracy for resistance of 90%.\textsuperscript{38,40} Because intrinsic drug resistance may be sufficient to hinder clinical response and clinical resistance is probably multifactorial, the measurement of just one or two mechanistic markers (PgP, GSH, topoisomerase II, etc) is likely to be of limited predictive use.\textsuperscript{31} Only when all the mechanisms of neoplastic cellular resistance (whether intrinsic, acquired, or induced) have been unravelled will it be feasible to identify them at an early stage with a view to their circumvention.\textsuperscript{37} Ex vivo assays, however, are ideally suited to the task of exploring methods of resistance circumvention, by virtue of their high predictive accuracy and their ability to relate patterns of ex vivo pleiotropic drug resistance (and collateral sensitivity) to the specific prior therapy administered. In addition, these assays are supported by over 4,200 published clinical correlations.\textsuperscript{39,40} The DiSC assay, for example, is currently used routinely in our laboratories to establish a patient’s individual drug-resistance profile.\textsuperscript{30,42} Because it relates to the particular stage of the patient’s disease progression (including specific prior chemotherapy), the DiSC assay identifies drugs to which the patient is likely to be resistant and provides an ideal adjunct to current methods of selecting appropriate therapy for the patient.\textsuperscript{43}

The method of analysis reported here is a paradigm, readily repeated for other disease types. Furthermore, its use as a coadjuvant to traditional methods of clinical trial design could both augment and refine chemotherapeutic approaches.
to the treatment of the common leukemias and tumors. The benefits of incorporating ex vivo methodologies into the identification of new treatment strategies include eliminating less-relevant agents from a number of possibilities, expediting the discovery of hitherto unexplored treatment options, and reducing the number of second-rate or blind alley trials, thereby minimizing both the financial burden on health service budgets and the risks (to individual patients) of inappropriate therapy. In the rarer tumors where clinical trials cannot be undertaken, ex vivo assays could be effectively used to map out patterns of drug resistance ex vivo, thus highlighting as yet unidentified regimens of known cytotoxics that would be worthy of investigation.

The pleiotropic resistance observed in this work strongly suggests that multiple resistance mechanisms are being induced by chemotherapy and that ex vivo drug sensitivity testing of CLL is a valuable model for studying both the mechanisms and the drug resistance modifiers to overcome them. In addition, the results of this analysis provide further rationale for treatment strategies in CLL. However, because "predicting an individual patient’s prognosis is still a challenge" and ex vivo drug response profiles show large variations, more precise guidance would be available if individuals were tested for ex vivo drug sensitivity at each point of treatment choice. The novel method of analysis used here is applicable to a diversity of malignancies and could offer great potential in aiding both the design of clinical trials and the development of new anticancer agents.

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REFERENCES

26. Mughal TI, Goldman JM: Chronic leukemias: Can they be...
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