A B S T R A C T

Background: The development of chemoresistance represents a major obstacle in the successful treatment of cancers such as Non-Hodgkin’s Lymphoma (NHL). With the recognition of important roles for both p53 and its more recently described paralog p73 in mediating the activity of anti-cancer drugs, there has been increasing recognition that cellular resistance to anthracyclines could and does arise through failure of p53 family member signalling. Despite these advances in understanding how cells respond to DNA damage in vitro, and how this is affected by molecular genetic changes which affect p53 family member signalling, the contribution of these to in vivo chemoresistance has not been definitively established. Our major task now is to determine how these changes operate individually and collectively in vivo to produce the phenotype of clinical chemoresistance, and how we can translate this knowledge into clinically useful strategies to improve the outcome of chemotherapy.

Materials and Methods: In this study we used two cell lines derived from Nigerian patients with Burkitt’s lymphoma in a suspension type cell culture. The reduction of the tetrazolium salt to a blue-black formazan product by living not by dead cells can be used to measure growth inhibitory effects (cell proliferation inhibitory) of tumor cells.

Results: An alternative option for p53+ (resistant) cells is to use a PGP reversal agent in combination with DOX, but reducing the dose of DOX when combined with chemosensitizer.

Conclusion: The altered cellular dose in chemoresistant cell lines may provide a rational basis for the use of modified anthracycline based regimens in chemosensitizers, preferably non-genotoxic, in the treatment of tumors expressing chemoresistance phenotype with p53 over-expression.

Keywords: chemoresistance, chemosensitizers, NHL cell line model.
Chemoresistance is the major limitation to the success of CHOP in non-Hodgkin’s lymphoma (NHL) patients. The well-studied form of drug resistance involving altered membrane transport is multi drug resistance (MDR) cells. MDR is a phenomenon in which a cellular phenotype develops resistance in vitro to a single cytotoxic drug, such as a vinca alkaloid, an anthracycline or a podophyllotoxin, and also becomes resistant to other structurally and/or mechanistically dissimilar drugs.1-2 Many forms of MDR result from the expression of ATP-dependent efflux pumps with broad substrate specificity. These pumps belong to a family of ATP-binding cassette (ABC) transporters that share sequence and structural homology, and so far 48 human ABC genes have been identified. Alteration of the subcellular distribution of the drug by “trapping” in intracellular organelles is another way in which the cell can inactivate the drug. This type of resistance has been described for doxorubicin.3 The altered cellular dose, low dose, in MDR cell lines may provide a rational basis for the use of modified anthracycline based regimens in combination with PGP reversal agents, chemosensitizers, preferably non-genotoxic, in the treatment of tumours expressing chemoresistant phenotype with p53 over-expression.4 Table 1 show some gain-of-function activities of mutant p53, which may contribute to chemoresistance.

**Table 1. Some gain-of-function activities of mutant p53**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Increase in expression of multidrug resistance-associated protein (MRP1)</td>
<td>Tsang et al., 2003 4</td>
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<tr>
<td>Activation of DuTPase expression</td>
<td>Pugachava et al., 2002 5</td>
</tr>
<tr>
<td>Down-regulation of CD95(Fas/APO-1)</td>
<td>Zalcenstein et al., 2003 6</td>
</tr>
<tr>
<td>Induction of gene amplification</td>
<td>El-Hizawi et al., 2002 7</td>
</tr>
<tr>
<td>Complex formation with TAp73</td>
<td>Marin et al., 2000 8</td>
</tr>
<tr>
<td>Complex formation with TAp63</td>
<td>Strano et al., 2000 9</td>
</tr>
<tr>
<td></td>
<td>Bergamaschi et al., 2003 10</td>
</tr>
<tr>
<td></td>
<td>Monti et al., 2003 11</td>
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</tbody>
</table>

*Chemicals:*

Doxorubicin, verapamil, fluphenazine, quercetin, indomethacin, Tween-20 and Triton X-100 were obtained from Sigma (Pool, Dorset, UK).

*MTT assay:*

The reduction of the tetrazolium salt, MTT, (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to a blue-black formazan product by living (but not by dead cells) can be used to measure growth inhibitory effects (cell proliferation inhibitory) of tumour cells. The advantage of the MTT assay is that the results are read automatically with a microplate spectrophotometer. The methodology of the in vitro growth/growth inhibition
MTT assay described below represents a modification of the original MTT colorimetric assay described by Mosmann.19

The modification of the original in vitro assay procedures has been described (Scudiero et al., 1988). In the present study, 2 x 103cells/100µl Raji cells sub-lines (TK+ and TK-) were harvested from exponential-phase maintenance cultures (75-90% confluence in T-25 cm2 flasks, Corning, NY, USA). The cells were counted by trypan blue exclusion dye using a haemocytometer and a light microscope (Nikon, Japan) and dispensed as replicated cultures in multi-well cell culture microplates (Corning, NY, USA) for 24 hours. Following incubation in 5% CO2 at 37°C, 100µl of cell culture medium alone, cell culture medium containing chemosensitizers, cell culture medium containing doxorubicin and cell culture medium containing chemosensitizers in combination with doxorubicin were dispensed within the appropriate wells and incubated for 48 hours. A stock solution of MTT was prepared as follows: 5mg MTT/ml in PBS was prepared under sterile conditions and stored at 4°C for a maximum of one month and at -20°C for more than one month. A working solution of MTT was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in pre-warmed standard culture medium. Fifty microlitres of this solution were added to each well (resulting in 50µgMTT/250µl total medium volume), and microplates were incubated at 37°C for 4 hours. At the end of this period, the microplates were centrifuged (500g) for 10 minutes and then the upper media removed by aspiration from each well by pipetting out (just 10-20µl left in the bottom of each well). Then 150µl DMSO was added to each well. The dark blue crystals, which were the products of living cells, were dissolved overnight at 37°C, and then the microplates were read on a microplate reader, using a wavelength of 570nm. From the beginning, the microplates were normally read within 45-60 minutes of adding the DMSO. Optical densities (OD) were obtained through the computer programme and the results printed out and kept for data analysis after being transferred to Excel 2003 spreadsheet. The value for the blank (DMSO) was subtracted from the raw absorbance values. All samples were analysed in quadruplicate and all procedures were carried out in the Type 2 cabinet hood (except during centrifugation and reading step of microplates) to prevent and avoid any possible contamination.

Stock solutions of the drugs:

Doxorubicin, verapamil and fluphenazine (always freshly prepared) were dissolved in dH2O at a final concentration of 1mM, was aliquoted and stored at -20°C. Indomethacin dissolved in ethanol and quercetin dissolved in DMSO at a final concentration of 1mM aliquoted and stored at -20°C until further usage.

Doubling time of the Raji cells sub-lines:

The number of cells aliquoted to each well was determined as a function of the population doubling time of each sub-line using the linear relationship between absorbance and the number of cells in the growth medium during the incubation time. The basis of this test is that any drugs, regardless of site or mechanism of action will interfere with the growth of each cell sub-line and there would be a reduction of the growth rate reflected by cell number. The growth characteristics of Raji sub-lines were studied by counting the number of the cells over a 5-day period from an initial plating density 2 x 103 cells/well. This ensures that each chemosensitivity study was carried out during the logarithmic growth phase of each Raji cells sub-line. Table 1 shows the results.

Treatment of Raji cells:

Raji cells were seeded in 96 well microplates at 2 x 103cells/well in 100µl of the complete growth medium. This cell concentration ensured logarithmic cell growth during the assay. The two far left and right columns were filled with cell culture medium alone to reduce evaporation and avoid any possible contamination from the remaining inner columns. Quadruplicate wells were used for the control, control plus chemosensitiser, doxorubicin alone, doxorubicin in combination with the chemosensitiser. The mean concentration was determined in three independent experiments, each performed in quadruplicate, as mentioned above.

Results

The results showed that DOX was able to inhibit proliferation of the Raji cell sub-lines alone and in combination with different sub-groups of chemosensitizers. How-
ever, Tween-20 and Triton X-100 significantly enhanced the inhibitory effects of DOX in combination indicating the disruption of cell membranes. Both cell sub-lines behaved in a similar manner.

Figure 1 Treatment of Raji (TK+) cells with different subgroups of p-glycoprotein reversal drugs after 48 hours incubation at 37°C and 5% CO2. Black columns show the cells alone, white columns show the cells which were treated with a chemosensitiser, black columns with white dots show the cells treated with 25nM DOX and white columns with black dots show the cells which were treated with 25nM DOX with chemosensitiser. Means of optical densities were determined and analysed by a two tailed Student t-test and bars indicate mean ± SE for two independent experiments.

Figure 2 Treatment of Raji (TK-) cells with different subgroups of p-glycoprotein reversal drugs after 48 hours incubation at 37°C and 5% CO2. Black columns show the cells alone, white columns show the cells which were treated with a chemosensitiser, black columns with white dots show the cells treated with 25nM DOX and white columns with black dots show the cells which were treated with 25nM DOX with a chemosensitiser. Means of optical densities were determined and analysed by a two tailed Student t-test and bars indicate mean ± SE for two independent experiments.

Table 1. Logarithmic growth phase of each Raji cells sub-line

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<thead>
<tr>
<th>Cell sub-line (name)</th>
<th>Doubling time (hour)</th>
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<tr>
<td>Raji TK+</td>
<td>23 ± 1.5</td>
</tr>
<tr>
<td>Raji TK-</td>
<td>20 ± 2</td>
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The NHL in vitro model was able to demonstrate manipulation and modulation of cells in the presence of a chemosensitiser to overcome the chemoresistance obstacle, when using a low dose of DOX. It is clear that the p53 tumour suppressor gene is vital in assuring genome stability. DNA double-strand breaks represent chromosomal lesions that can lead to mutation, tumour transformation, or apoptosis. Double-strand breaks can occur as a result of chemotherapy. For example, the cytotoxic activity of DOX appeared to correlate more closely with the formation of DNA double-strand breaks than with single strand lesions. Mammalian cells possess potent and efficient mechanisms to repair double strand breaks, and thus complete normal development as well as mitigating oncogenic potential and preventing cell death. When the repair process of double-strand breaks fail, chromosom-
The current thinking about double-strand breaks and their lack of repair is that they cause chromosomal instability resulting in a MDR phenotype. Instead of making the genome more unstable (by continuing chemotherapy for resistant patients), it may be better to keep the genome stable in NHL patients. According to the present study, it appears that stability can be maintained by reducing the dose of DOX and combining it with a PGP reversal drug such as Tween-20 or Triton X-100 in resistant patients with p53 over-expression. In such resistant NHL patients at least 50% of their cells are not operating effectively (normally). Our earlier results have shown that p53+ NHL cells with alkylating damage are irreparable (23). Continuing conventional chemotherapy is probably not very beneficial for resistant patients due to their lack of repair, which might also associated with MGMT depletion. This could increase the risk of further mutation, which could cause even more chromosomal lesions as an additive result of the p53 deficient repair machinery.24 Hence it may be more appropriate to modulate the effects of DOX in combination with ideally non-genotoxic chemosensitizers in the presence of the mutant p53 to overcome the phenomenon. Therefore, using some of the PGP reversal agents could be a promising way forward not only for NHL resistant patients, but also for the other human tumours resistant to the standard regimens. With regards to alkylating damage to DNA, a study of cyclophosphamide induced DNA damage and repair occurring in vivo was conducted in the brown Norway rat myeloctic leukemia model by Wang and his colleagues.25 DNA single-strand breaks, DNA-DNA interstrand cross-links, DNA-protein cross-links, and DNA double-strand breaks were measured by alkaline and neutral elution in their study. No SSB or DSB were observed at 4 h after the cyclophosphamide injection over the dose range of 15-250 mg/kg, but a low level of SSB was observed at 18-28 h after cyclophosphamide treatment. These data suggest that the cytotoxic effects of cyclophosphamide were mediated mostly by DNA interstrand cross-links and DNA-protein cross-links. Single strand breaks appearing late after cyclophosphamide injection in vivo may be a reflection of repair of DNA-DNA interstrand cross-links and DNA-protein cross-links and an indication of the optimal timing for administration of DNA-repair inhibitors. This observation is of interest because DSB can potentate the instability of the genome of the NHL patients. Therefore the therapeutic benefit of cyclophosphamide in this model, when it is given during the CHOP regimen might help resistant patients. In contrast the study by Valenzuela and her colleagues revealed that DOX induced DSB. They evaluated their results by using pulsed-field gel electrophoresis and compared with cellular effects, PGP expression and intracellular glutathione levels.26 Nevertheless, the lack of repair capacity which has been demonstrated in p53+ NHL patients makes the cyclophosphamide option of limited benefit.

Based on the present results, an alternative option for p53+ (resistant) cells is to use a PGP reversal agent in combination with DOX, but reducing the dose of DOX when combined with such an agent as suggested by the results which have been achieved in the present study. Tween-20 and Triton-X100 showed highly significant differences in growth arrest in both cells sub-lines alone and a combination with DOX suggesting the use of liposomal doxorubicin delivery as a new administration for p53+ (resistant) NHL patients. This should be an effective strategy for standard regimens based on doxorubicin (doxorubicin-based regimens). The results also suggest a new effective strategy for pharmaceutical industries. Another limitation factor in NHL resistant patients reflects the fact that there is also some evidence that chemotherapy can produce secondary cancers.27 The most serious side effect is the possibility of developing a second tumour known as acute myeloid leukaemia. This complication is quite rare, and the importance of chemotherapy in treating non-Hodgkin’s lymphoma in children is more important than the risk, which means that cyclophosphamide might be useful in young resistant patients and perhaps not elderly patients. Therefore liposomal doxorubicin might help in elderly patients with p53 over-expression. Thus the identification of novel early biomarkers from drug-induced toxicity could aid drug discovery by improving the toxicity prediction process. However, the question is how to minimize the cardiotoxic effect but maintain the antitumor effect of DOX.28-29 It is well known that the lack of a complete and long-lasting response to chemotherapy is a main drawback limiting the clinical potential of DOX in NHL treatment. Proteomic techniques have identified novel biomarkers with the potential to enable prediction of response to anticancer therapy, particularly chemore-
sistance. Before these “potential prognostic markers” are applied clinically, however, further studies are required to validate whether these alterations are the cause or the result of resistance to anticancer therapy.  

**References**


