Advances in Brief

**N-(5,5-Diacetoxypentyl)doxorubicin: A Novel Anthracycline Producing DNA Interstrand Cross-Linking and Rapid Endonucleolytic Cleavage in Human Leukemia Cells**

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**Abstract**

The cytotoxic and DNA-damaging effects of a novel alkylating anthracycline, N-(5,5-diacetoxypentyl)doxorubicin, were quantified in HL-60 human leukemia cells and in an intercalator-resistant daughter line, HL-60/AMSA. The new drug was cytotoxic to both lines at doses as low as 50 nm for 1 h. N-(5,5-Diacetoxypentyl)doxorubicin produced DNA interstrand cross-linking in both lines. The cross-linking appeared to increase in both lines following drug treatment, but the increase was greater in the resistant line. This appeared to be due to an underestimation of cross-linking, particularly in sensitive HL-60, secondary to time-dependent DNA fragmentation that followed drug removal. This time-dependent DNA fragmentation was probably endonucleolytic cleavage (a feature of apoptosis) as characteristic nucleosomal ladders were produced by N-(5,5-diacetoxypentyl)doxorubicin treatment in a cotemporal time-dependent fashion. This novel anthracycline is the first of a family of alkylating anthracyclines designed to be water soluble, easy to formulate, and capable of producing DNA interstrand cross-linking. Because this last characteristic has previously been associated with doxorubicin analogues of great potency and low toxicity, these newer, more readily formulated drugs may have great clinical utility.

**Introduction**

N-(5,5-DAP)DOX (Fig. 1) is representative of a new class of extremely potent doxorubicin analogues designed and synthesized by Farquhar and Cherif (1, 2). The molecule contains a 5,5-bisacetoxypentyl substituent in the 3'-amino position of the daunosamine sugar. This substituent was designed to undergo hydrolysis to the corresponding aldehyde in the presence of carboxylate esterases, enzymes that are ubiquitous in tissue. It was anticipated that the liberated aldehyde would react with a nucleophilic group proximate to the site of drug intercalation or other form of DNA binding and thus form a covalent adduct. Hopefully, this approach would overcome cellular resistance to antitumor anthracyclines.

We now report on the activity of one of the first members of this new class of compounds in a drug-sensitive/drug-resistant cell pair in which the resistant line contains an intercalator-resistant form of topoisomerase II (3).

Received 8/30/91; accepted 10/29/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This study was supported by USPHS Research Grant CA40090 (L. A. Z.).

**Materials and Methods**

The design and synthesis of N-(5,5-DAP)DOX (Fig. 1) has been described previously (2). The cellular pharmacology, biochemistry, and molecular biology of HL-60 and its amanscine-resistant daughter line, HL-60/AMSA, have been described in detail (3, 4). The cells were grown and radiolabeled for alkaline elution as described previously (3). Soft agar colony formation assays and alkaline elution assays using proteinase were performed and quantified as described in the past (3).

To quantify endonucleolytic cleavage, cells with their DNA labeled as for alkaline elution were exposed to various concentrations of N-(5,5-DAP)DOX for 1 h. The cells were harvested by centrifugation and washed in drug-free medium and then lysed during a 20-min incubation with 10 mM Tris, 1 mM EDTA, and 0.02% Triton X-100 (pH 7.5) on ice. This lysate was centrifuged at 13,000 × g for 10 min. The supernatant contained fragment DNA and the pellet contained intact chromatin (5). This was confirmed by comparing the percentage of DNA in the fragmented fraction with the percentage of DNA in the lysis fraction of filter elution assays performed with aliquots of the same cells (Table 1).

To demonstrate that the DNA in the lysis fraction truly represented endonucleolytic cleavage [i.e., apoptosis (6, 7)], we took aliquots of the identical cells used for quantification of endonucleolytic cleavage and lysed them in the same lysis buffer as above. The supernatant fraction (fragmented DNA) was then brought to 0.5 M NaCl (with 5 M NaCl) and to 50% isopropl alcohol (with 100% isopropl alcohol). Samples were stored at −20°C overnight. The following day the samples were centrifuged at 13,000 × g for 10 min and the precipitated pellet was resuspended in 89 mM Tris-borate buffer (pH 8) (3). This was followed by adding one-sixth volume of 15 mM EDTA, 2% SDS, and 50% glycerol loading buffer and heating the mixture to 65°C for 10 min. The samples were loaded on a 1.5% agarose gel and run at 20 V overnight in the presence of 0.5 µg/ml of ethidium bromide to allow the direct visualization of DNA in the gel.

**Results**

The cytotoxic effects of 1-h treatments of HL-60 and HL-60/AMSA cells with N-(5,5-DAP)DOX were quantified using a soft agar colony formation assay (Fig. 2, top). At N-(5,5-DAP)DOX concentrations in excess of 50 nM, survival in both lines was markedly reduced, with the cytotoxicity being slightly greater in effect on HL-60. The drug produced approximately equal frequencies of DNA interstrand cross-linking in both cell lines immediately following a 1-h treatment (Fig. 2, bottom). However, N-(5,5-DAP)DOX did not produce protein-associated DNA cleavage (data not shown). Together these observations indicated that a cell line that contains an intercalator-resistant form of topoisomerase II and that resists the actions of a number of topoisomerase II-reactive DNA intercalators including anthracyclines (3, 8), is not resistant to this new agent. Additionally, the DNA effect produced by this new drug is distinct from that produced by doxorubicin and other topoisomerase II-reactive anthracyclines (3, 9).

Previously, we and others had demonstrated that another
DNA DAMAGE BY \(N-(5,5\text{-DAP})\)DOX

Table 1  Comparison of methods by which to quantify endonucleolytic cleavage of DNA from drug-treated HL-60 cells

<table>
<thead>
<tr>
<th>Treatment [drug and time following drug removal (min)]</th>
<th>% of cellular DNA in lysis solution with following technique used</th>
<th>Filter-SDS(^a)</th>
<th>Filter-SDS + Proteinase(^b)</th>
<th>Filter-Triton(^c)</th>
<th>Pellet-Triton(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N-(5,5\text{-DAP}))DOX (500 nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>23</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>36</td>
<td>38</td>
<td>28</td>
<td>30</td>
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</tr>
<tr>
<td>120</td>
<td>66</td>
<td>71</td>
<td>61</td>
<td>62</td>
<td></td>
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<tr>
<td>180</td>
<td>89</td>
<td>89</td>
<td>87</td>
<td>89</td>
<td></td>
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<tr>
<td>240</td>
<td>96</td>
<td>94</td>
<td>92</td>
<td>90</td>
<td></td>
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<tr>
<td>Etoposide (100 (\mu)M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>94</td>
<td>95</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>90</td>
<td>91</td>
<td>87</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16</td>
<td>17</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>24</td>
<td>23</td>
<td>20</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Cells were deposited on a polyvinyl chloride filter and lysed with 5 ml of an SDS-containing lysis solution used in filter elution (2% SDS, 0.025 M disodium EDTA, pH 10). Values are percentages of the total DNA that eluted from the filter.

\(^b\) As in Footnote \(a\) with the addition of 0.5 \(\mu\)g/ml of porroteinase.

\(^c\) As in Footnote \(a\) except the lysis solution was the 0.2% Triton X-100 lysis solution described in “Materials and Methods.”

\(^d\) Quantification of endonucleolytic cleavage using Triton X-100 lysis and no filter as described in “Materials and Methods.”

A variety of anthracycline analogues can produce interstrand cross-links. The best known of these analogues is MRA-CN (10–13). Because formulation and solubility problems may have delayed the progression of this agent to the clinic, alternative anthracycline analogues that retain the ability of MRA-CN to cross-link DNA have been designed. \(N-(5,5\text{-DAP})\)DOX is one of these. It behaves similarly to MRA-CN in the cell culture systems used in this work. In particular, \(N-(5,5\text{-DAP})\)DOX produced DNA fragmentation like MRA-CN (12, 13). The new data presented here suggest that this fragmentation is secondary to endonucleolytic cleavage [i.e., apoptosis, (6, 7) [Fig. 3]. This cleavage explains the excess DNA detected in the lysis fraction of elution assays and the apparent decline in cross-linking following drug removal.

The association between the cleavage and the decreased cross-
linking is best appreciated in Fig. 3. Although cross-linking is higher in resistant cells than in wild-type cells, there is an accompanying lower amount of DNA detected in the lysis fraction from resistant cells. Because the latter are intercalator resistant on the basis of a drug-resistant form of topoisomerase II (3, 8), it is possible that topoisomerase II, a target for other intercalating anthracyclines (3, 9, 15), may be involved in the manner in which N-(5,5-DAP)DOX kills cells. On the other hand, a large production of protein-associated DNA cleavage was not detected in cells treated with the new drug which suggests that its primary target is not topoisomerase II.

N-(5,5-DAP)DOX also allowed us to demonstrate that the fragmented DNA we detect in the pH 10 elution lysis is probably which constitutes the bulk of the DNA of the nucleosomal ladder detected in gel assays of endonucleolytic cleavage, a feature of apoptosis (6, 7) (Table 1). These same changes were produced by a topoisomerase II-reactive drug that does not produce interstrand cross-links (etoposide) (Table 1). Thus, apoptosis can be triggered by cytotoxic agents with different initial effects on cellular DNA (6, 7, 16–21). Note, however, that the DNA fragmentation following N-(5,5-DAP)DOX was clearly not topoisomerase II-mediated, because such DNA cleavage (e.g., etoposide-induced, protein-associated DNA cleavage) required SDS to detect (Table 1), while DNA fragmentation following N-(5,5-DAP)DOX did not.

The chemical nature of the interstrand cross-link produced by N-(5,5-DAP)DOX is not known. However, the facts that both this agent and MRA-CN are modified in the 3′-amino position of doxorubicin (Fig. 1) and that both agents cross-link DNA suggest that this site may be uniquely positioned to produce cross-linking once the drug has intercalated. In this regard, Gao et al. (22) have demonstrated that in the presence of formaldehyde a daunorubicin analogue modified with a sugar at the 4′-oxygen position of daunomycin formed a covalent adduct with a DNA hexamer through a 3′-amino methylene bridge. These authors suggested that an anthracycline with “a reactive (e.g., alkylating) functional group at the 3′-nitrogen position” might produce a good anticancer drug. In fact, Farquhar et al. (23) previously reported the synthesis and biological evaluation of a series of doxorubicin analogues bearing chemically reactive substituents at this position. N-(5,5-DAP)DOX, the new analogue described here, is a further extension of this approach. Studies are planned to define the size and composition of the 3′-amino substituent for maximal cytotoxic activity and to determine whether this new class of compounds is active in vivo while retaining low cardiotoxicity.

Acknowledgments

We wish to thank Dr. Mike Story of the Department of Experimental Radiotherapy, M. D. Anderson Cancer Center, for teaching E. Altschuler the method of assessing endonucleolytic cleavage and Dr. Raymond Meyn for critical reading of the manuscript. We also thank Diane Rivera for editing the manuscript.

References

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