Cross-Sensitivity to Topoisomerase II Inhibitors in Cytotoxic Drug-hypersensitive Chinese Hamster Ovary Cell Lines

Craig N. Robson, Paul R. Hoban, Adrian L. Harris, and Ian D. Hickson

Department of Clinical Oncology, University of Newcastle upon Tyne, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, United Kingdom

ABSTRACT

We have isolated a Chinese hamster ovary cell line, designated ADR-1, which exhibits hypersensitivity to a range of drugs which are thought to inhibit the action of the enzyme topoisomerase II. These include anthracyclines, other classes of intercalating agents, and the epipodophyllotoxins, etoposide. No significant sensitivity to radiation, or to mono- and bifunctional alkylating agents was seen, although mild cross-sensitivity to the radiomimetic agent bleomycin was observed.

We have monitored the level of DNA strand breaks induced by topoisomerase II inhibitors in ADR-1 cells using alkaline elution. At equimolar Adriamycin (doxorubicin) doses, more protein-associated DNA strand breaks are induced in ADR-1 cells than in wild-type cells. This enhanced level of drug-induced strand breaks does not appear to be a function of increased drug uptake as both lines accumulate similar levels of radiolabeled daunomycin. Both the rate of repair of strand breaks and the final percentage of strand breaks rejoined was equivalent in the 2 cell lines. These results are consistent with an enhancement in the level of topoisomerase II-dependent DNA breakage in ADR-1 cells following exposure to topoisomerase II inhibitors.

We have previously reported the isolation of 2 bleomycin-sensitive Chinese hamster ovary cell lines, BLM-1 and BLM-2 (C. N. Robson et al., Cancer Res. 45: 5304–5309, 1985). While BLM-1 exhibited cross-sensitivity only to Adriamycin, BLM-2 was shown to be hypersensitive not only to Adriamycin but also to certain alkylating agents and to ionizing radiation. In this paper, we show that both BLM-1 and BLM-2 also exhibit mild cross-sensitivity to a range of topoisomerase II inhibitors.

These results indicate that intercalating agents and epipodophyllotoxins exert their cytotoxicity via common mechanisms and suggest that the maintenance of normal levels of cellular resistance to these agents requires the products of several different genes.

INTRODUCTION

A number of effective antitumor agents, such as Adriamycin, mAMSA, and ellipticine have been shown to interact with cellular DNA by intercalation (for review, see Ref. 1). These drugs, along with the epipodophyllotoxins such as VP16, appear to effect damage to DNA by a similar if not identical mechanism. A common step in their action has been identified as the production of protein-bound single and double strand breaks in DNA (2–4). In vitro studies identified a requirement for a non-histone nuclear protein for the DNA cleavage activity of these drugs (5), which has recently been shown to be a type II topoisomerase (5–8). Confirmation of the involvement of topoisomerase II in intercalator-induced DNA breakage came from the finding that protein-associated strand breaks reacted with an antibody raised against type II topoisomerasers (9).

Despite interacting with a common target enzyme, there are clearly differences in the clinical spectrum of these drugs, with Adriamycin having the widest range of activity in different tumor types; also, the shape of cell survival curves is not identical with the different intercalators, showing that the interaction with topoisomerase II may differ for each drug.

The ability of type II topoisomerases to decatenate DNA has been implicated in the final segregation step of DNA replication (10, 11). A possible role in transcription has also been suggested (12).

Because the enhanced resistance of some cell lines isolated by continuous exposure to anthracyclines, ellipticine, or VP16 has been shown to be mediated via alternative mechanisms to that of an abnormality in topoisomerase II (e.g., drug transport) (13–17), we sought to isolate mutants, using a point mutagen, that were abnormally sensitive to anthracyclines. This was to define targets critical to the normal degree of cellular resistance to these agents and also to try to identify enzymes which interact with topoisomerase II, or which catalyze the biochemical steps occurring before or after the action of topoisomerase II. Mutants hypersensitive to topoisomerase II inhibitors would also be useful for studying the function of the enzyme in a way similar to that of temperature-sensitive mutants.

In this paper, we describe the characterization of 3 CHO cell lines which exhibit hypersensitivity to the cytotoxic effects of a range of topoisomerase II inhibitors. All 3 lines show sensitivity to several different intercalating agents and to VP16, although a number of phenotypic differences between the 3 lines is apparent. The results support the contention that these drugs exert their cytotoxicity via one or more common intracellular targets.

MATERIALS AND METHODS

Cell Culture and Media. Cells were routinely maintained in Ham's F10 medium (Northumbria Biologicals) supplemented with glutamine (3 mmoles/5% fetal calf serum, 5% newborn calf serum, and antibiotics (streptomycin, 100 µg/ml; penicillin, 100 units/ml; and nystatin, 50 units/ml). Cells were grown in Nunc Petri dishes or tissue culture flasks at 37°C under 5% CO2.

Mutagenesis. Cells were mutagenized in growth medium for 24 h with ethyl methane sulfonate (300 µg/ml) as previously described (18).

Mutant Isolation. The isolation of mutants BLM-1 and BLM-2 has been described previously (18). Mutant ADR-1 was isolated using a similar selection protocol, although following a separate mutagenic treatment. Briefly, mutagenized cells were plated to yield single colonies which were overlayed with growth medium containing 0.4% noble agar. After 48 h, when the cells had started to grow into the agar, sterile toothpicks were used to transfer individual colonies onto the surface of gridded agar plates (0.5% agar in medium) which contained either the selective drug in the agar or no drug. Cells growing on the control but not on the drug-containing plate were retested on agar and then transferred to liquid culture for accurate survival testing. All mutants were cloned twice before survival determinations were carried out.

Survival Curves. Exponentially growing cells were trypsinized and seeded in 100-mm Petri dishes to yield 500–10,000 cells. These were allowed to adhere for 4 h before treatment with a DNA-damaging agent, as outlined below.

Radiation. Treatment with UV or X-rays was as described previously (18).

Drug Treatments. Stock solutions of drugs were prepared as described previously (18), with the following additions. Daunomycin was dis...
solved in H₂O and stored in aliquots at −20°C. Ellipticine was freshly prepared in 0.01 M HCl. mAMSA was dissolved in 0.035 M lactic acid and stored at 4°C. Bisantrene was dissolved in dimethyl sulfoxide and stored at 4°C. Mitoxantrone was dissolved in H₂O and stored at 4°C. Vinblastine was dissolved in 0.9% NaCl solution and stored at 4°C.

In general, cells were exposed to a drug for 24 h before being washed twice with phosphate-buffered saline and returned to fresh growth medium. The exceptions were cis-platinum diamine dichloride for which a 2-h exposure was used, and methyl methane sulfonate and VP16, for which 1-h exposures were used. The Petri dishes were then incubated for 10–12 days until visible colonies appeared. These were fixed in methanol:acetic acid (3:1), stained with crystal violet (400 µg/ml) and counted. Colonies containing approximately 50 cells or more were considered as survivors.

Each point on a survival curve represents the average of at least 3 independent experiments. For every experiment, the parental CHO-K1 line was similarly treated to act as a control. The D₃₇ dose is that which reduces cell survival to 37% of control values and represents the average dose required to kill a cell.

The population doubling time for ADR-1 was determined using exponentially growing cells by counting cell numbers over a 48-h period.

Cell Labeling and Alkaline Elution. Cellular DNA was labeled by incubating cells with 0.02 µCi/ml [2,4-C]thymidine (Amersham) for 36–48 h. Following this, cells were incubated for 2 h in label-free medium prior to drug exposure. Cells were then treated with Adriamycin for 1 h and where indicated incubated at 37°C in growth medium to follow the time course of DNA repair.

DNA single-strand breaks were assayed by the procedure of alkaline elution, essentially as described by Kohn et al. (19). Briefly, approximately 5 x 10⁵ cells were impinged onto polycarbonate filters (Nuclepore), lysed with 20 mM EDTA-2% sodium dodecyl sulfate, pH 9.7, and exposed to 0.5 mg/ml proteinase K (Sigma) for 1 h. DNA was eluted from the filters using a solution of 0.1 M tetrapropylammonium hydroxide (Aldrich)-0.02 M EDTA (free acid), pH 12.1, containing 0.1% sodium dodecyl sulfate, at a flow rate of 0.035 ml/min. Fractions were collected over 15 h at 90-min intervals.

The use of a Watson-Marlow 202U/AA 16-channel constant speed peristaltic pump gave highly reproducible flow rates in each channel.

Estimate of Repair of DNA Single-strand Breaks. The percentage of repair of single-strand breaks was calculated from the percentage of DNA retained on the filter after 9 h of elution, according to the equation

\[
\frac{(DNA_t^A - (DNA_0^A)}{(DNA_0^A) - (DNA_0^A)}
\]

where (DNA₁^A) is the percentage of DNA retained after recovery time \(t\), with Adriamycin; (DNA₀^A) is the percentage of DNA retained at time \(t = 0\), with Adriamycin; and (DNA₀) is the percentage of DNA retained from untreated cells.

Drug Uptake. Accumulation of [³H]daunomycin was measured essentially by the method of Bates et al. (20).

RESULTS

Sensitivity to DNA-damaging Agents and Anticancer Drugs. The cell lines ADR-1, BLM-1, and BLM-2 were tested for exhibiting hypersensitivity to a range of topoisomerase II inhibitors as well as to several other DNA-damaging agents and anticancer drugs.

Fig. 1 shows survival curves for the 3 lines following a 24-h exposure to Adriamycin. BLM-1 and BLM-2 exhibit around 2-fold sensitivity to Adriamycin, as judged by D₃₇ values (Table 1). ADR-1 cells, however, show a significantly greater level of sensitivity to Adriamycin (Table 1). Equivalent degrees of hypersensitivity to Adriamycin were also observed with the 3 lines following 1-h drug treatments (data not shown).

To determine whether this general feature of BLM-1 and BLM-2 showing mild sensitivity to Adriamycin and ADR-1 showing more extreme sensitivity was seen with other topoisomerase II inhibitors, we studied the survival response of the 3 lines to VP16 and to several different classes of intercalating agents. Figs. 2, 3, and 4 show survival curves for these lines following exposure to VP16, ellipticine, and bisantrene, respectively. As with Adriamycin, BLM-1 and BLM-2 exhibit 1.5 to 3-fold sensitivity to these agents, while ADR-1 cells show greater degrees of sensitivity (Table 1). Similar data were obtained for daunomycin, mAMSA, and mitoxantrone, and are given in Table 1 as D₃₇ values for killing by these agents.

Because BLM-1 and BLM-2 were isolated on the basis of sensitivity to bleomycin and have been shown here to exhibit cross-sensitivity to topoisomerase II inhibitors, we wished to determine whether ADR-1 cells show any cross-sensitivity to bleomycin. The result (Fig. 5) shows that ADR-1 cells exhibit mild sensitivity to bleomycin (around 2-fold, as judged by D₃₇ values, Table 1).

We have also tested the 3 cell lines for exhibiting hypersensitivity to a range of other DNA damaging agents and anticancer drugs. While BLM-2 cells show hypersensitivity to X-rays, UV, mitomycin C, cis-platinum diamine dichloride, ethyl methane sulfonate, melphalan, and chlorambucil (Table 1), both BLM-1 and ADR-1 cells show near wild-type levels of resistance to all of these agents (Table 1). In addition, all 3 lines show no sensitivity to vinblastine or vincristine (Table 1).

DNA Damage and Repair. Using alkaline elution, we have studied the level of DNA strand breaks induced in ADR-1 cells following exposure to different concentrations of Adriamycin. Fig. 6a shows representative elution profiles for ADR-1 cells following a 1-h treatment with up to 2 µg/ml Adriamycin. The comparable data for wild-type cells is shown in Fig. 6b. Two differences can be observed upon comparison of the elution profiles for the 2 lines: (a) ADR-1 cells have a higher basal level of strand breaks than do CHO-K1 cells; and (b) at equimolar doses of Adriamycin, significantly more strand breaks are induced in ADR-1 cells than in wild-type cells.
CHO CELLS HYPERSENSITIVE TO TOPOISOMERASE II INHIBITORS

TaWe 037 values for wild-type and mutant strains following treatment with DNA-damaging agents and anticancer drugs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Adriamycin (ng/ml)</th>
<th>VP16 (μg/ml)</th>
<th>Ellipticine (ng/ml)</th>
<th>Bisantrene (ng/ml)</th>
<th>Daunomycin (ng/ml)</th>
<th>mAMSA (ng/ml)</th>
<th>Mitoxantrone (ng/ml)</th>
<th>Melphalan (μg/ml)</th>
<th>Vinblastine (ng/ml)</th>
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<tr>
<td>CHO-K1</td>
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<td>1.82</td>
<td>232</td>
<td>786</td>
<td>115</td>
<td>18</td>
<td>7.4</td>
<td>0.66</td>
<td>900</td>
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<tr>
<td>ADR-1</td>
<td>44*</td>
<td>0.41*</td>
<td>60*</td>
<td>248*</td>
<td>30*</td>
<td>5.5*</td>
<td>2.3*</td>
<td>0.68*</td>
<td>810*</td>
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<tr>
<td>BLM-1</td>
<td>110*</td>
<td>1.29*</td>
<td>142*</td>
<td>520*</td>
<td>85*</td>
<td>11*</td>
<td>4.8*</td>
<td>0.63*</td>
<td>380*</td>
</tr>
<tr>
<td>BLM-2</td>
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<td>1.12*</td>
<td>95*</td>
<td>540*</td>
<td>81*</td>
<td>8.5*</td>
<td>4.8*</td>
<td>0.61*</td>
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<table>
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<tr>
<th>Vinblastine (ng/ml)</th>
<th>Chlorambucil (μg/ml)</th>
<th>Ethyl methane sulfonate (μg/ml)</th>
<th>cis-Platinum diamine dichloride (μg/ml)</th>
<th>Mitomycin C (ng/ml)</th>
<th>UV (J/m²)</th>
<th>X-rays (rads)</th>
<th>Bleomycin (μg/ml)</th>
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<td>(3.9)*</td>
<td>(365)</td>
<td>(1.37)</td>
<td>(110)</td>
<td>(10.8)</td>
<td>(137)</td>
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<tr>
<td>ADR-1</td>
<td>145*</td>
<td>3.9*</td>
<td>330*</td>
<td>1.31*</td>
<td>85*</td>
<td>10.9*</td>
<td>132*</td>
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<tr>
<td>BLM-1</td>
<td>150*</td>
<td>(3.9)</td>
<td>(360)</td>
<td>(1.35)</td>
<td>(90)</td>
<td>(10.8)</td>
<td>(140)</td>
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<tr>
<td>BLM-2</td>
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<td>(120)</td>
<td>(0.62)</td>
<td>(59)</td>
<td>(6.9)</td>
<td>(74)</td>
</tr>
</tbody>
</table>

* Significant difference with wild type, at P < 0.01.
* No significant difference (P > 0.05).
* Numbers in parentheses have been reported previously (18).
* Significant difference with wild type, at P < 0.05.

**Fig. 2.** Survival of wild-type CHO-K1 (•), ADR-1 (■), BLM-1 (○), and BLM-2 (□) cells following exposure to VP16. Points, mean of 3 independent experiments.

In experiments where proteinase K treatment was removed from the alkaline elution protocol, not only were the Adriamycin-induced strand breaks not revealed in either cell line, but also the difference in basal strand break levels in ADR-1 cells was no longer evident.

We have also compared the rate of repair of the strand breaks in the 2 lines. The result (Fig. 7) shows that for around 1 h after the removal of Adriamycin, strand breaks are still being generated in both cell lines. However, following this period, both the rate of repair and the percentage of strand breaks rejoined after 5 h are approximately the same in both lines.

To determine whether the increased level of DNA damage induced in ADR-1 cells was due to enhanced drug uptake, we compared the level of daunomycin accumulation in CHO-K1 and ADR-1 cells. No significant difference could be detected following exposure to radiolabeled drug for either 10, 60, or 90 min (Table 2).

**Fig. 3.** Survival of wild-type CHO-K1 (•), ADR-1 (■), BLM-1 (○), and BLM-2 (□) cells following exposure to ellipticine. Points, mean of 3 independent experiments.

Stability of Phenotypes, Population Doubling Times, and Plating Efficiencies. ADR-1 cells have been grown in continuous culture for 9 months without showing any evidence of phenotypic reversion. The doubling time for these cells was found to be 15 h compared to 13 h for wild-type cells. The plating efficiency was 50–60% for ADR-1 cells and 70–80% for the wild type.

These data for BLM-1 and BLM-2 have been reported previously (18).

**DISCUSSION**

We have described the isolation of a new CHO cell line, ADR-1, which exhibits hypersensitivity to the cytotoxic effects of a range of topoisomerase II inhibitors. This is the first report of the isolation of any mammalian cell line showing such a profile of drug sensitivities. We have also shown that 2 mutants, previously shown to exhibit hypersensitivity to bleomycin, are also mildly cross-sensitive to topoisomerase II inhibitors.

It is of interest that ADR-1 cells are specifically hypersensitive to the cytotoxic effects of a strictly limited range of DNA-damaging agents. Because topoisomerase II has been confirmed...
as the intracellular target for both the intercalating drugs and the epipodophyllotoxins (for review, see Ref. 21), it seems likely that an alteration in a topoisomerase II-dependent reaction is the underlying cause of drug hypersensitivity in ADR-1 cells. Consistent with this view is the finding that, at equimolar doses, Adriamycin induced more DNA strand breaks in ADR-1 cells than in wild-type cells. This is also unlikely to be the result of increased intracellular drug accumulation since transport stud

ies showed no significant difference between the cell lines.

A number of drug-resistant cell lines have been isolated by continued exposure to anthracyclines. These cells generally exhibit cross-resistance to other intercalating agents, to VP16, and to *Vinca* alkaloids (22–27). This so-called MDR phenotype is associated with increased expression of a family of high molecular weight membrane glycoproteins and appears to be mediated at least in part via decreased intracellular drug accumulation resulting from increased drug efflux (for review, see Ref. 28). It is unlikely that a “reversal” of this MDR phenotype has occurred in ADR-1 cells because we have been unable to demonstrate any alteration in drug uptake and also because the
normal level of resistance to Vinca alkaloids has been maintained.

Recently, cell lines have been isolated which are specifically resistant to intercalators and epipodophyllotoxins (29, 30). These lines have been shown to induce fewer protein-associated DNA strand breaks following exposure to topoisomerase II inhibitors. ADR-1 cells would appear to possess the reciprocal phenotype to these cells, being hypersensitive and not resistant to inhibitors. Like ADR-1 cells, these resistant mutants were also isolated in a one-step procedure, in contrast to many other multidrug-resistant lines (31). It thus seems that MDR cells may have many alterations if produced by continued drug exposure, including transport and topoisomerase II activity, which will make analysis of critical individual resistance pathways more difficult.

The strand breaks induced by intercalators are protein associated and are therefore not revealed without deproteinization. The higher background level of protein-concealed breaks in ADR-1 cells suggests that these lesions are not intrinsically lethal and that processes occurring after strand scission may be important in producing the final killing effect. The phenotype exhibited by ADR-1 cells may reflect a mutation involving the site of interaction between topoisomerase II and intercalators, and hence purification of the mutant protein and isolation of the gene will be of major interest.

Just as the Adriamycin-sensitive mutant is mildly bleomycin sensitive, so the bleomycin-sensitive mutants are somewhat anthracycline sensitive. Because the bleomycin-sensitive mutants differ markedly from each other and from ADR-1 in cross-sensitivity pattern, they are unlikely to bear the same mutation. The results suggest that BLM-1 and BLM-2 may have a defective protein that interacts with topoisomerase II. The presence of topoisomerase II inhibitors may thus sensitize the cells because of an interaction between topoisomerase II and the mutant protein.

Cross-resistance between bleomycin and Adriamycin has not been reported in either the MDR phenotype or in topoisomerase II mutants previously. There has been a report of a bleomycin-resistant cell line which exhibited mild cross-resistance to VP16 (32), although normal levels of sensitivity to Adriamycin were maintained in this line. Bleomycin strand breakage is sequence specific, 5'-guanine-pyrimidine sequences being most susceptible, with the pyrimidine sugar being attacked (33). Intercalators are also known to show preference for alternating purine-pyrimidine sequences (34). It has been suggested that the bithiazole moiety of bleomycin intercalates and may be responsible for the double-strand breakage (35, 36). Thus the bithiazole moiety may be important in the cross-sensitivity shown between the different classes of mutant. The cross-sensitivity is however surprising since the double-strand breaks produced by bleomycin and by intercalators differ. Moreover, intercalators readily produce sister chromatid exchanges whereas equitoxic doses of bleomycin do not (37).

Both bleomycin and intercalators show preference for inter- nucleosomal or transcriptionally active DNA (38). It has been suggested that one function of topoisomerase II is to introduce torsional stress into transcriptionally active chromatin. Thus, topoisomerase II is associated with the nuclear matrix and its sites of cleavage may be the anchorage sites for the DNA loops of mitotic chromosomes (39, 40). If topoisomerase II was involved in a repair enzyme complex, defective interactions of the mutant topoisomerase II could produce hypersensitivity to bleomycin-induced strand breaks.

The intercalating drugs that we assessed were chosen for their different propensities to form lipid peroxides or free radicals, and it is clear that these mutants are hypersensitive to Adriamycin (which is a potent lipid peroxidizing agent) and to mitoxantrone (which can even lower basal lipid peroxidation levels and inhibit Adriamycin-induced peroxidation) (41). The marked hypersensitivity of these cells to Adriamycin as well as to VP16, mitoxantrone, ellipticine, and mAMSA suggests that the major mechanism by which cells are killed by Adriamycin is not related to free radicals or membrane toxic effects.

It is of interest that BLM-2 cells show sensitivity to bleomycin, X-rays, and Adriamycin. Cell lines generated from AT patients are characterized by their hypersensitivity, particularly to ionizing radiation and bleomycin (42), but also in milder form to Adriamycin (43).

The phenotype exhibited by BLM-1 cells is unique among previously reported mutants of mammalian cell lines. Extreme sensitivity is only seen with bleomycin, with a less dramatic response to topoisomerase II inhibitors. Because bleomycin generates base damage via a free radical mechanism (44) and intercalators such as Adriamycin generate free radicals during their action (45), it is possible that BLM-1 cells are deficient in the elimination of highly reactive species such as superoxide free radicals. Although the major action of mitoxantrone is not thought to involve the generation of oxygen free radicals (41), it has recently been reported that this drug can itself be converted to a free radical (46), although there is no evidence that this species is cytotoxic.

The cross-sensitivities of these mutants show previously unsuspected interactions between bleomycin, VP16, and anthracyclines that may be relevant clinically. If they act at sequential steps biochemically, there may be synergistic interactions, and elucidation of these targets may help drug design or biochemical modulation. These drugs are used together for high grade lymphomas, teratomas, and Hodgkin's disease, but their scheduling and dosing is empirically current. Further understanding of mechanisms of interaction may allow optimal therapeutic use.

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REFERENCES

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