ABSTRACT
Patients with metastatic testis tumors are generally curable using chemotherapy, whereas those with disseminated bladder carcinomas are not. We have compared levels of the nuclear enzyme topoisomerase II in three testis (SaSa, 833K, and GH) and three bladder (RT4, RT112, and HT1376) cancer cell lines which differ in their sensitivity to chemotherapeutic agents. The testis cell lines were more sensitive than the bladder lines to three drugs whose cytotoxicity is mediated in part by inhibiting topoisomerase II: amsacrine; Adriamycin; and etoposide (VP16). The frequency of DNA strand breaks induced by amsacrine was higher (1.5- to 13-fold) in the testis cells than in the bladder cells. The level of topoisomerase II-mediated DNA strand breakage in vitro, measured by filter trapping of amsacrine-induced protein:DNA cross-links, was similarly higher in nuclear extracts from the testis than the bladder cells. Western blot analysis showed a generally higher level of topoisomerase II protein in testis than in bladder cell nuclear extracts. Topoisomerase II protein expression broadly correlated with drug-induced strand breakage in both protein extracts and whole cells, but not with population doubling time. However, despite a 2- to 20-fold increased sensitivity to the different topoisomerase II inhibitors, the testis line 833K had a less than 2-fold higher level of topoisomerase II protein than that of the bladder line RT4. These results indicate that the level of expression of topoisomerase II is an important determinant of the relative chemosensitivity of testis and bladder tumor cell lines, but that additional factors must contribute to the extreme chemosensitivity of testis cells.

INTRODUCTION
Disseminated testicular germ cell tumors are cured in over 80% of cases with chemotherapy, whereas bladder cancers and most other solid tumors in adults are not (1). Bladder and testis tumor cell lines reflect the clinically observed drug sensitivities of the two tumor types. For example, testis tumor cell lines are more sensitive to killing by cis-platinum and Adriamycin than are bladder tumor cell lines (2).

Adriamycin interacts with the cellular enzyme, topoisomerase II, causing protein-associated DNA strand breaks and hence, via an as yet unknown mechanism, cytotoxicity (3). A range of other drugs, including the epipodophyllotoxin, VP16 (etoposide), and the intercalating agent, m-AMSA, exert at least part of their cytotoxicity via a similar mechanism (3-5). This raises the possibility that the differences between testis and bladder tumor cells in their sensitivity to certain drugs, observed both clinically and in vitro, are due to differences in expression of topoisomerase II. This hypothesis is supported by the fact that VP16 is one of the drugs currently used in the treatment of nonseminomatous, testicular germ cell tumors (6, 7).

In this study we examined whether differences in the level of topoisomerase II protein are likely to contribute to the extreme sensitivity of testis compared with bladder tumor cell lines to topoisomerase II inhibitors.

MATERIALS AND METHODS
Cell Culture and Conditions. All the cell lines were grown routinely under identical conditions in 25-cm² flasks (Nunc) in RPMI 1640 medium (Gibco) with 5% (v/v) heat-inactivated fetal calf serum (Sera-Lab) derived from a single batch and 2 mM L-glutamine (Gibco) at 36.5°C in a humidified atmosphere of 5%CO₂ in air. Each cell line was used over a maximum of 10 passages to minimize changes that might occur during prolonged culture. All cell lines were Mycoplasma free, as judged by staining with Hoechst 33258.

Drug Sensitivity Measurements. Cellular drug sensitivities were measured using the dimethylthiazol-diphenyltetrazolium bromide (MTT) assay. Exponentially growing cells were detached using trypsin and transferred in 150 μl of medium to Columns 2 to 12 of a 96-well, flat-bottomed microtiter plate (Nunc), Column 1 being the medium/solvent-only control. A separate plate was used for each cell line and each drug. Each cell line was plated at an optimum density, such that the cells were still growing exponentially after 7 days in culture, and the absorbance of the untreated controls did not exceed a value of 2.0. The plates were incubated for 24 h under standard conditions before adding ten cytotoxic drug concentrations in 50 μl of medium to Columns 3 to 12, with the weakest concentration next to the controls. The plates were incubated for a further 6 days before adding 50 μl of a 4-mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) in calcium- and magnesium-free phosphate-buffered saline to each well. After 3 h the fluid contents of each well were aspirated carefully, and 100 μl of dimethyl sulfoxide (Sigma) were added. The purple formazan product was solubilized by gently tapping the plate, and absorbances were measured at 540 nm using an automatic microspectrophotometer (Titertek Multiscan MCC/340 automatic plate reader). Background absorbance (Column 1) was subtracted from each row, and the mean reduction in absorbance at each concentration (one column of 8 wells) was expressed as a proportion of the absorbance of the untreated controls (Column 2).

Drugs. Stock solutions of 1 mg/ml of etoposide (VP16-213; Bristol-Myers) were dissolved directly in medium, Adriamycin (doxorubicin-HCl; Farmitalia Carlo-Erba) in sterile dimethyl sulfoxide, and m-AMSA [4′-(9-acridylamino)methane sulfon-m-amiside; Bristol-Myers] in dimethyl sulfoxide. These stocks were prepared immediately before use and diluted in medium, adding appropriate solvent controls at the highest concentration used.

Statistics. To determine the IC₅₀, linear regressions were plotted using the linear region of the curve, and IC₅₀ values were calculated. The mean ± standard error was calculated from a minimum of 3 experiments for each drug and cell line.

Alkaline Elution. Drug-induced single-strand breaks were measured by DNA alkaline elution (pH 12.1) as described by Kohn et al. (8). Cells in early logarithmic growth were detached using trypsin, washed in phosphate-buffered saline, and seeded onto 100-mm Petri dishes. After allowing the cells to attach for 2 h, the dishes were rinsed and the medium changed to phosphate-buffered saline. Radiolabeling, cells were washed and reincubated for at least 1 h before treatment. Cells were exposed to m-AMSA for 1 h and then prepared immediately for elution. Cells were detached as rapidly as possible by
scraping at 0°C to reduce the possibility of repair. Assays of total single-strand breaks, in the presence of proteinase K, were conducted using 2-μm polycarbonate filters (Nucleopore). Duplicate lanes of each treatment were carried out in all experiments and each experiment was performed independently at least twice. The frequency of single-strand breaks was converted to rad equivalents using a calibration graph derived from elution of DNA from cells treated with a range of irradiation doses. The means ± standard deviation was calculated from the rad-equivalent data.

Filter Binding Assay. Measurement of the extent of covalent binding of topoisomerase II to DNA induced by m-AMSA was carried out as described by Minford et al. (9). Nuclear extracts, equalized for protein content, were incubated with linearized plasmid DNA, labeled at the 3′ end, and various concentrations of m-AMSA at 37°C for 20 min. The reactions were stopped by the addition of 20 mM EDTA, pH 10, and the mixture was applied to a polyvinyl chloride filter (Millipore, 2 μm pore). Filters were processed as described (9).

Antibody Production. A 14mer peptide (DTLKRKPSDLWKE) representing residues 1155–1168 of the topoisomerase IIα amino acid sequence was synthesized, conjugated to bovine thyroglobulin (Sigma), combined with Freund’s adjuvant, and used to immunize rabbits. Following 2 injections, rabbits were immunized with the same peptide conjugated to keyhole limpet hemocyanin (2 further injections). Polyclonal sera were screened by Western blotting of nuclear extracts from parental CHO-K1 and mutant ADR-1 cells previously shown to overexpress topoisomerase II protein (10). One batch of serum (designated T2K2) produced identical Western blot results to those seen with a polyclonal serum raised against purified protein (10).

Western Blotting. Nuclear extracts were prepared from cell pellets by the method of Glisson et al. (11), and their protein content was determined by the method of Bradford (12). Nuclear extracts, equalized for protein content (confirmed by Coomassie blue staining of sodium dodecyl sulfate gels), were electrophoresed on a 7.5% polyacrylamide gel and the mixture was applied to a polyvinyl chloride filter (Millipore, 2 μm pore). Filters were processed as described (9).

**RESULTS**

**Drug Sensitivities.** The sensitivities of the three bladder and three testis cancer cell lines to m-AMSA, Adriamycin, and etoposide are shown in Table 1. The testis tumor cell lines were significantly more sensitive to all three agents than the bladder cancer cell lines, with no overlap in IC50 values between the two cell types. Comparing mean IC50 values, the testis cell cancer lines were 9.1-fold more sensitive to m-AMSA, 12.6-fold to Adriamycin, and 19.6-fold to VP16. Similar relative levels of sensitivity were seen with acute exposure to drugs (data not shown). The relative sensitivities were not related to population doubling times (see summary in Table 2).

**Comparison of DNA Damage Produced by m-AMSA in Bladder and Testis Cell Lines.** DNA damage was measured in the three bladder and three testis cell lines by alkaline elution (8).

<table>
<thead>
<tr>
<th>Drug sensitivity of cell lines</th>
<th>m-AMSA IC50 values (ng/ml)</th>
<th>Adriamycin IC50 values (ng/ml)</th>
<th>VP16 IC50 values (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bladder</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT1376</td>
<td>190.2 ± 27.4</td>
<td>15.7 ± 1.5</td>
<td>300.8 ± 10.7</td>
</tr>
<tr>
<td>RT112</td>
<td>46.1 ± 3.9</td>
<td>23.0 ± 1.3</td>
<td>216.6 ± 20.9</td>
</tr>
<tr>
<td>RT4</td>
<td>22.6 ± 3.1</td>
<td>19.7 ± 0.6</td>
<td>317.1 ± 34.5</td>
</tr>
<tr>
<td><strong>Testis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>833K</td>
<td>11.8 ± 2.0</td>
<td>1.5 ± 0.1</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>SuSa</td>
<td>5.0 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>16.6 ± 1.3</td>
</tr>
<tr>
<td>GH</td>
<td>11.7 ± 1.3</td>
<td>1.0 ± 0.2</td>
<td>15.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Mean ± SE.

Initially dose-response studies were carried out on all lines to determine the degree of damage induced by m-AMSA. The testis lines showed a much steeper dose response to m-AMSA-induced single-strand breaks than the bladder cell lines (Fig. 1). A dose of m-AMSA (50 ng/ml) was selected which produced a frequency of single-strand breaks that could be measured in all lines by the high-sensitivity alkaline elution technique. There was no overlap in sensitivity to single-strand break formation between the two cell types (Table 2).

The frequency of single-strand breaks in rad-equivalents was 8-fold greater in the testis lines GH and SuSa than in the bladder cell line RT112, and 13-fold greater than in HT1376 (Fig. 1; Table 2). These results are in agreement with the sensitivity of the testis lines to the cytotoxic effects of this drug. However, the level of DNA damage in the testis cell line 833K was only 1.4-fold greater than that in the bladder cell line RT4.

**Comparison of in Vitro DNA:Protein Cross-Linking Produced by m-AMSA.** The level of DNA:protein cross-linking induced by m-AMSA in vitro was measured by the filter binding assay of Minford et al. (9). Fig. 2 shows that the testis cell lines SuSa and GH showed both a higher baseline (absence of m-AMSA) level of DNA:protein cross-linking and of m-AMSA-induced cross-linking than either 833K (testis) or the three bladder lines. The summary in Table 2 shows the calculated level of DNA:protein cross-linking that can be assumed to be m-AMSA specific (cross-linking in the presence of 6 μg/ml of m-AMSA minus that in the absence of drug). The testis cell extracts showed higher levels of m-AMSA-specific DNA cross-linking activity than the bladder cell extracts.

**Topoisomerase II Protein Level.** Fig. 3 shows a representative Western blot of nuclear proteins from the bladder and testis cell lines using anti-topoisomerase II peptide antiserum. A high level of topoisomerase II protein as found in both SuSa and GH (testis) cells, a level 2- to 3-fold above that seen in the third testis line, 833K. RT112 and HT1376 cells, both bladder tumor cell lines, expressed very much lower levels of protein than SuSa or GH cells (6- to 7-fold). However, 833K (testis) and RT4 (bladder) showed similar topoisomerase II protein levels. These data are summarized in Table 2. A similar relative level...
Table 2 Summary of properties of bladder and testis cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population doubling time (h)</th>
<th>Relative Topoisomerase II protein level</th>
<th>m-AMSA-specific DNA: protein cross-linking</th>
<th>Single-strand breaks (rad-equivalents) at 50 ng/ml of m-AMSA</th>
<th>Relative m-AMSA sensitivity (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT1376</td>
<td>31</td>
<td>1.0†</td>
<td>0.50</td>
<td>37.5 ± 1.2** (1.0*)†</td>
<td>1.0†</td>
</tr>
<tr>
<td>RT112</td>
<td>24</td>
<td>1.2</td>
<td>0.75</td>
<td>60.0 ± 2.2 (1.6)†</td>
<td>4.1</td>
</tr>
<tr>
<td>RT4</td>
<td>37</td>
<td>2.2</td>
<td>2.5</td>
<td>210 ± 10.9 (5.6)†</td>
<td>8.4</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>833K</td>
<td>22</td>
<td>3.0</td>
<td>3.5</td>
<td>293 ± 32.0 (7.8)</td>
<td>16</td>
</tr>
<tr>
<td>SuSa</td>
<td>20</td>
<td>6.0</td>
<td>12</td>
<td>480 ± 59.0 (13)</td>
<td>38</td>
</tr>
<tr>
<td>GH</td>
<td>25</td>
<td>7.2</td>
<td>14</td>
<td>480 ± 75.0 (13)</td>
<td>16</td>
</tr>
</tbody>
</table>

† Based upon densitometric scanning of Western blots. Values represent the mean of 2 independent determinations.

* Percentage of DNA bound at 6 μg/ml of m-AMSA minus control in the absence of m-AMSA.

 Arbitrary given a value of 1.0.

 Mean ± SE.

 Numbers in parentheses, relative values.

of topoisomerase II protein was seen by Western blotting with a second antipeptide antibody and with polyclonal antiserum raised against purified topoisomerase II protein (data not shown). Similar differences in protein levels were also seen on Western blots of whole cell extracts (data not shown).

DISCUSSION

Clinical response to chemotherapy is influenced by many factors, including individual patient pharmacokinetics, and tumor bulk and vascularization. Despite this, when testis and bladder tumor cells were established in tissue culture, where all external factors relating to drug delivery are removed, the testis tumor cells remained more sensitive to killing by m-AMSA than the bladder cell lines (2). The level of topoisomerase II-mediated strand breakage varied among the testis tumor cell lines, with SuSa and GH cells having a comparably high level, but 833K a lower level which was similar to that in the bladder line RT4. The bladder lines RT112 and HT1376 showed a lower level of strand breakage than any of the other cell lines. These variations in m-AMSA-induced DNA strand breakage were apparently dependent upon a nuclear factor (presumably topoisomerase II), and largely independent, therefore, of factors such as drug uptake, as the DNA:protein cross-linking in nuclear extracts was also elevated in the testis compared with the bladder cell lines. Indeed, there was a good correlation between m-AMSA-induced strand breakage in cellular DNA and m-AMSA-specific DNA protein cross-linking in nuclear extracts. An explanation for this variation in topoisomerase II strand breakage activity in the cell lines was the relative level of expression of topoisomerase II protein, as seen by Western blotting. A summary of these data is shown in Table 2.
Although 833K cells expressed a relatively low level of topoisomerase II protein compared with that of the other two testis cell lines, and only slightly higher than that of the highest expressing bladder cancer cell line, RT4, they were markedly more sensitive to VP16 and Adriamycin than any of the bladder cell lines. IC₅₀ values for these two drugs in the testis and bladder cancer expressing bladder cancer cell line, RT4, they were markedly lower than the IC₅₀ values found with m-AMSA an apparently more specific topoisomerase II inhibitor, that there was a continuum of cell sensitivity that broadly reflected drug-induced DNA damage and topoisomerase II levels. These results taken together suggest that measurement of topoisomerase II levels in individual tumours might predict response to chemotherapy. However, the extreme and uniform hypersensitivity of the testis cell lines to certain topoisomerase II inhibitors is unlikely to result solely from the degree of expression of topoisomerase II protein.

This indicates that a component of the drug sensitivity in these cell lines must be an intrinsic property not explained by variation in topoisomerase II activity. Although VP16 and Adriamycin in part exert their cytotoxic effects via topoisomerase II, it is likely that other mechanisms, such as free radical generation, are also important (14, 15).

In contrast to the findings with VP16 and Adriamycin, we found with m-AMSA an apparently more specific topoisomerase II inhibitor, that the predominant form of topoisomerase II in the bladder cell extracts is of a slightly higher molecular weight than that in the testis extracts. Whether this represents a difference in relative expression of the α and β forms is unknown. It is possible that a change in expression from the β to the α form in the testis cells could sensitize them to topoisomerase II inhibitors, as the α form has been shown to be preferentially sensitive to these drugs (17). Further work is needed to confirm this suggestion.

Testis tumor cell lines are sensitive to a wide range of drugs, including cisplatinum and vincristine (Ref. 2; Footnote 4) which appear to act via mechanisms independent of topoisomerase II. We demonstrated previously reduced repair of cis-platinum-induced DNA intrastrand cross-links in SuSa cells, relative to 833K and RT112 cells (18), and this probably contributes to the extreme sensitivity to cis-platinum seen in this line.

Many of the topoisomerase II-inhibitory drugs are transported by the multidrug resistance transporter (mdr protein or P-glycoprotein). However, we were unable to detect any P-glycoprotein in any of the cell lines by immunocytochemical analysis using C219 antibody (data not shown).

It seems likely that the high level of topoisomerase II protein expressed in these testis tumor cell lines contributes to their sensitivity to m-AMSA, Adriamycin, and VP16. Conversely, the low level of topoisomerase II protein seen in bladder cancer cell lines probably contributes to their relative resistance to chemotherapeutic agents. This may be of clinical relevance in that measurement of topoisomerase II levels in individual tumors might predict response to chemotherapy. However, the extreme and uniform hypersensitivity of the testis cell lines to certain topoisomerase II inhibitors is unlikely to result solely from the degree of expression of topoisomerase II protein.

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* Unpublished results.
Relationship between Topoisomerase II Level and Chemosensitivity in Human Tumor Cell Lines

Andrew M. Fry, Christine M. Chresta, Stella M. Davies, et al.


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