

Relationship between Topoisomerase II Level and Chemosensitivity in Human Tumor Cell Lines¹

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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 (SuSa, 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, while 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,³ 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).

Received 6/25/91; accepted 10/2/91.

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¹Supported by the Imperial Cancer Research Fund, the Cancer Research Campaign, and the N. E. Thames Regional Health Authority.

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³The abbreviations used are: m-AMSA, amsacrine; IC₅₀, concentration reducing survival by 50%.

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 distilled water, 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 IC₅₀s 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 phase growth (1 to 2 × 10⁵ cells/ml) were labeled for 30 h with 0.015 μCi of [¹⁴C]thymidine per ml (specific activity, 56 mCi/mmol; Amersham, United Kingdom). Following labeling, 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 (DTLKRKSPDLWKE) representing residues 1155–1168 of the topoisomerase II α 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 over-express 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 then transferred to nitrocellulose by electroblotting. The membrane was blocked with MARVEL dried-milk powder and then exposed for 16 h to polyclonal serum T2K2 (diluted 1 in 500). The filter was washed, reacted with ¹²⁵I-labeled Protein A, and autoradiographed. Gels were scanned using the Bio-Image analyzer (MillGen/Biosearch).

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 IC₅₀s between the two cell types. Comparing mean IC₅₀s, the testis cancer cell 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 1 Drug sensitivity of cell lines

Cell line	m-AMSA IC ₅₀ values (ng/ml)	Adriamycin IC ₅₀ values (ng/ml)	VP16 IC ₅₀ values (ng/ml)
Bladder			
HT1376	190.2 ± 27.4 ^a	15.7 ± 1.5	300.8 ± 10.7
RT112	46.1 ± 3.9	11.0 ± 1.3	211.6 ± 20.9
RT4	22.6 ± 3.1	19.7 ± 0.6	317.1 ± 34.5
Testis			
833K	11.8 ± 2.0	1.5 ± 0.1	15.8 ± 1.5
Susa	5.0 ± 0.4	1.2 ± 0.3	10.6 ± 1.3
GH	11.7 ± 1.5	1.0 ± 0.2	15.9 ± 0.7

^a Mean ± SE.

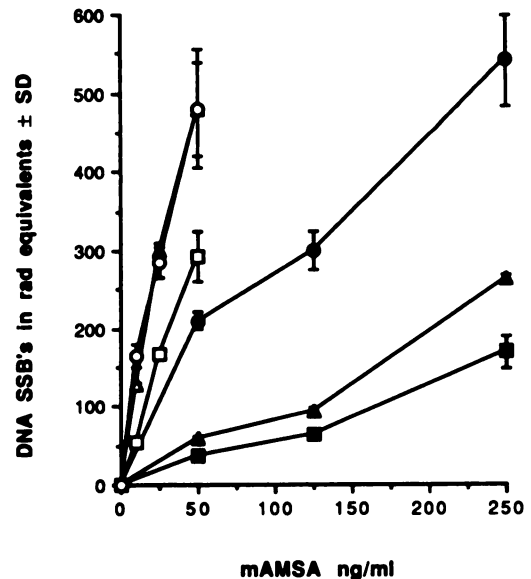


Fig. 1. Frequency of DNA single-strand breaks (in rad-equivalents) produced by a range of m-AMSA concentrations in testis (open symbols) and bladder (closed symbols) cell lines. ○, GH; △, SuSa; □, 833K; ●, RT4; ▲, RT112; ■, HT1376. Points, mean of at least two independent experiments; bars, SE.

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

Cell line	Population doubling time (h)	Relative Topoisomerase II protein level ^a	m-AMSA-specific DNA: protein cross-linking ^b	Single-strand breaks (rad-equivalents) at 50 ng/ml of m-AMSA	Relative m-AMSA sensitivity (IC ₅₀)
Bladder					
HT1376	31	1.0 ^c	0.50	37.5 ± 1.2 ^d (1.0 ^e)	1.0 ^e
RT112	24	1.2	0.75	60.0 ± 2.2 (1.6)	4.1
RT4	37	2.2	2.5	210 ± 10.9 (5.6)	8.4
Testis					
833K	22	3.0	3.5	293 ± 32.0 (7.8)	16
Susa	20	6.6	12	480 ± 59.0 (13)	38
GH	25	7.2	14	480 ± 75.0 (13)	16

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

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

^c Arbitrarily given a value of 1.0.

^d Mean ± SE.

^e Numbers in parentheses, relative values.

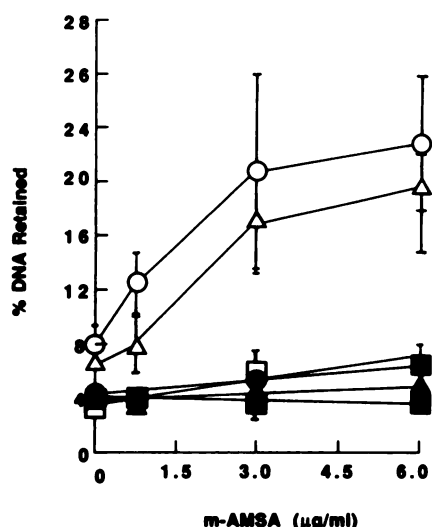


Fig. 2. DNA binding activity induced by m-AMSA measured by retention of protein:DNA complexes on filters and in nuclear extracts from testis (open symbols) and bladder (closed symbols) cell lines. ○, GH; △, SuSa; □, 833K; ●, RT4; ▲, RT112; ■, HT1376. Points, mean of 3 independent experiments; bars, SE.

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 cis-platinum and Adriamycin than the bladder tumor cells (2). This suggests that sensitivity to these drugs is probably an intrinsic property of testis tumor cells.

In this study we have measured expression of a nuclear enzyme, topoisomerase II, which may be important in determining intrinsic sensitivity to certain drugs in human cells. Low levels of topoisomerase II protein have been demonstrated in leukemic cells from patients with chronic lymphocytic leukemia. This cell type is resistant to killing by Adriamycin, both

clinically and *in vitro* (13). Conversely, increased expression of topoisomerase II protein was correlated with sensitivity to intercalating agents and epipodophyllotoxins in a mutant rodent cell line (10).

We have shown that the level of topoisomerase II-mediated DNA strand breakage following m-AMSA treatment was higher in three testis tumor cell lines than in three bladder cell lines. This is in agreement with the finding that the testis tumor cell lines were 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.

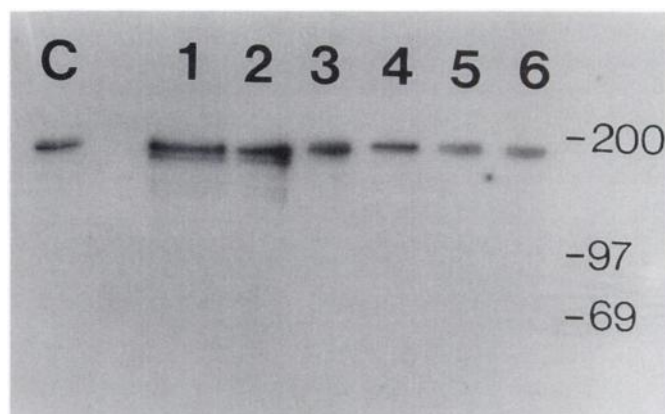


Fig. 3. Western blot of nuclear extracts of testis and bladder cell lines using topoisomerase II antibodies. Lane 1, SuSa; Lane 2, GH; Lane 3, 833K; Lane 4, RT4; Lane 5, RT112; Lane 6, HT 1376. Molecular weights (kDa) are shown on the right. Lane C shows an extract from a control cell line (HeLa).

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 tumor cells fell into two narrow ranges and did not directly correlate with topoisomerase II protein expression. 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 there was a continuum of cell sensitivity that broadly reflected drug-induced DNA damage and topoisomerase II levels. These results taken together suggest that topoisomerase II levels are an important component but not the sole mechanism through which clinical topoisomerase II inhibitors exert their cytotoxic effects.

Topoisomerase II protein exists in two structurally similar forms in human cells, designated α (*M*, 170,000) and β (*M*, 180,000) (16). It is probable that both forms of the enzyme were detected on our Western blots. Fig. 3 shows 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 *cis*-platinum 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-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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⁴ Unpublished results.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 1991;51:6592-6595.

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