Drug resistance in malignant rhabdoid tumor cell lines

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Abstract Purpose: We evaluated the in vitro sensitivity of four malignant rhabdoid tumor (MRT) cell lines to six chemotherapeutic agents: 5-fluorouracil, vincristine, carboplatin, doxorubicin, etoposide, and paclitaxel. We also sought to determine whether a defect in the p53 signaling pathway may contribute to the pronounced drug resistance of MRT. Methods: MRT cells were treated with various concentrations of each drug and the effects on DNA synthesis were quantified using a thymidine incorporation assay. In addition, the effect of various concentrations of doxorubicin on cell growth was evaluated in all four cell lines. Functionality of the p53 pathway was evaluated by incubating cells with carboplatin or doxorubicin and monitoring the effects on the levels of the p53, p21WAF1/CIP1, and MDM 2 proteins by Western blot analyses. Results: Vincristine (EC50 0.5–2.9 nM) and doxorubicin (EC50 1.9–5.7 nM) were found to be most effective in inhibiting proliferation and were within clinically relevant concentrations. However, only doxorubicin exhibited cytotoxicity (EC50 2.4–13.1 nM), whereas vincristine and the other drugs tested were cytostatic. Interestingly, all four cell lines had remarkably similar dose response curves to all drugs tested, despite the fact that they were derived from different patients and arose in different tissues. When challenged with DNA-damaging drugs, p53 and the downstream effectors, p21WAF1/CIP1 and MDM 2 were upregulated. Conclusions: These studies indicate that the p53 pathway is functional and responsive to DNA-damaging drugs, and does not likely contribute to the drug resistance of MRT. The in vitro sensitivity of MRT cells to doxorubicin suggests that it may be a clinically important agent for the treatment of MRT.

Keywords Malignant rhabdoid tumor · Doxorubicin · p53 · In vitro screens

Introduction

Malignant rhabdoid tumor (MRT) is a rare and aggressive pediatric solid tumor originally thought to be a variant of Wilms’ tumor, the most common pediatric renal neoplasm [1]. However, ultrastructural, immunohistochemical, and molecular dissimilarities have led to the conclusion that MRT is a separate entity from Wilms’ tumor. In addition to renal MRTs, numerous extrarenal primary tumors composed partly or entirely of rhabdoid tumor cells have been described in the literature, including an entity in the central nervous system, atypical teratoid tumor/rhabdoid tumor (ATT/Rh) [24]. Despite aggressive therapies and irrespective of the location of the tumor, most patients quickly succumb to disease, usually within 1 year of diagnosis [31].

The rarity of MRT has hampered studies to design an effective treatment. Therefore, we employed a collection of MRT cell lines as a model system to test the in vitro sensitivity of these cells to six chemotherapeutic agents. They included carboplatin (a DNA interstrand cross-linking agent), etoposide (VP-16, an epipodophyllotoxin that acts primarily as a topoisomerase II inhibitor), doxorubicin (Adriamycin, a DNA-intercalating anthracycline also known to inhibit the actions of topoisomerase II), the antimitotic agents paclitaxel and vincristine, and the antimetabolite, 5-fluorouracil. Carboplatin and etoposide are two of the compounds of the so-called ICE chemotherapy (ifosfamide, carboplatin, and etoposide) which is one of the clinical therapies often employed against MRT.

We also followed-up on a previous observation that MRT cells demonstrate heterogeneous positive staining...
for p53 [23]. The p53 gene is altered at a high frequency in human tumors and numerous studies have demonstrated that mutations in this gene can alter sensitivity to chemotherapeutic agents (reference 18 and references therein; [3]). In cells in which p53 is functional, it becomes stabilized under conditions of stress or cellular DNA damage. One effect of this can be upregulation of the p21<sub>WAF1/CIP1</sub> cyclin-dependent kinase inhibitor, leading to a cell cycle arrest and presumably allowing cells time to repair damaged DNA before resuming cell division. Therefore, we sought to determine whether p53 was functional in MRT cells by determining whether p53 and its known downstream effectors were upregulated in response to DNA-damaging agents.

Our results indicate that doxorubicin was the most promising chemotherapeutic agent tested since it exhibited cytotoxicity against all MRT cell lines within clinically achievable dosages. The p53 pathway was found to be functional and responsive to DNA-damaging drugs suggesting that alternative mechanisms are responsible for MRT drug resistance.

## Materials and methods

### Cell cultures

Four malignant rhabdoid tumor cell lines designated RT4E, RT5E, STM91-01, and TTC549 were employed in this work. These cell lines were established and maintained as described previously [20, 23] from malignant rhabdoid tumors originating in various locations in the body (Table 1). One of the cell lines, STM91-01, was established from MRT metastatic tumor tissue after treatment with chemotherapy [20]. In addition to those cell lines described previously, TTC549 was similarly established and maintained in RPMI-1640 supplemented with 10% bovine calf serum. A normal kidney (NoK) cell line, a non-immortal primary cell culture derived from proximal tubule kidney epithelial cells, was also employed in this work as a control.

### Thymidine incorporation assay

All drugs used in this work were obtained from Sigma (St. Louis, Mo.). Stock solutions were prepared in phosphate-buffered saline (PBS) (carboplatin and 5-fluorouracil) or dimethyl sulfoxide (DMSO) (etoposide, paclitaxel, doxorubicin and vincristine) and stored at -20°C. For thymidine incorporation assays, cells were seeded at 5×10<sup>4</sup> cells/well (1 cm<sup>2</sup>) in 48-well clusters and grown for 24 h in growth medium. The culture medium was removed and replaced with medium containing the indicated concentration of drug and grown for an additional 24 h. Vehicle (DMSO) was included, as appropriate, in control wells lacking drug. [methyl-<sup>3</sup>H]thymidine (25 Cl/mmol; Amersham Pharmacia Biotech, Piscataway, N.J.) was added to culture supernatants to a final concentration of 0.5 μCi/ml and the amount of [<sup>3</sup>H]thymidine incorporated into DNA over the next 18 h was determined as described previously [27].

### Cell growth analysis

Cells (1–2×10<sup>4</sup>) were plated in triplicate in a 96-well microtiter plate (Becton Dickinson, Franklin Lakes, N.J.) and allowed to establish overnight in an incubator at 37°C under an atmosphere supplemented with 5% CO<sub>2</sub>. The following day the growth medium was replaced in duplicate wells with medium containing doxorubicin at 0 to 0.1 μM. The plates were returned to the incubator for 72 h after which the cells were washed with PBS and stained with 0.2% crystal violet in 20% methanol for 30 min [10]. The crystal violet was removed and the wells were washed three times with water and allowed to air-dry. The stain was resolubilized in 200 μl ethanol and the optical density (OD) was read with a Molecular Devices Vmax kinetic microplate reader at 562 nm. A preliminary test of this assay done by adding quantified amounts of cells to the wells and staining as above indicated that the assay was linear from 2x10<sup>4</sup> to 1x10<sup>6</sup> cells/well and that the limit of detection was approximately 300–600 cells/well.

### Northern blot analysis

For the induction studies, two MRT cell lines (STM91-01 and RT4E) and NoK cells were treated with 1 μM doxorubicin for 0, 2, 4, and 8 h. Total RNA was extracted at specified times from each of the cell lines as described previously [23]. cDNA fragments isolated from cloned portions of the genes for p21<sub>WAF1/CIP1</sub> (American Type Culture Collection, Manassas, Va., no. 79928) and GAPD (ATCC no. 78463) were labeled with 32P using a Multiscribe DNA labeling system (Amersham Pharmacia Biotech) or a Random Prime DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. All hybridizations were performed as previously described [21].

### Western blot analysis

Whole-cell protein extracts (WCEs) were made from cell cultures of RT4E and STM91-01 cells treated with 100 μM carboplatin or various concentrations of doxorubicin for various times. After treatment, cells were removed from the flasks with trypsin-EDETA, washed three times with PBS, and resuspended in cold cell lysis buffer comprising 150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, 1 μg/ml leupeptin, 500 μM phenylmethylsulfonyl fluoride, and 1 μl/ml (0.0078 TIU/ml) aprotinin. The cells were subjected to a freeze-thaw cycle and the cell debris pelleted in a microfuge at 4°C and 5000 g for 10 min. The protein concentration of the resulting supernatants was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.,) according to the manufacturer’s instructions and using bovine serum albumin (BSA) as a standard. WCEs were stored at -80°C until used. All extractions were performed on ice.

Replicate samples from each of the WCEs were fractionated on 10% or 12.5% SDS-polyacrylamide gels, electrophoresed onto a membrane (Immobilon-P; Millipore, Bedford, Mass.) and then probed with DO-1 monoclonal antibody to human p53 or WAF1 (Ab-1) monoclonal antibody to p21 (both Oncogene Sciences, Cambridge, Mass.) both at 1 μg/ml in 3% BSA/PBS. A hybridoma cell line (PAB4B11) secreting antibodies to MDM 2 was generously provided by Dr. Juiyh Lin (University of Michigan, Comprehensive Cancer Center, Ann Arbor, Mich.). A monoclonal antibody to β-actin (Sigma) at a dilution of 1:5000 was used to evaluate lane loading. In each case an anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) was used and the results visualized by a chemiluminescence method (ECL, Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

## Results

### In vitro chemosensitivity testing using a thymidine incorporation assay

We examined the in vitro effects of doxorubicin, vincristine, paclitaxel, 5-fluorouracil, carboplatin, and