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ORIGINAL ARTICLE

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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-fluororuacil, 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, $p21^{WAF1/CIP1}$, and MDM 2 proteins by Western blot analyses. *Results:* Vincristine (EC_{50} 0.5–2.9 n*M*) and doxorubicin (EC_{50} 1.9–5.7 n*M*) were found to be most effective in inhibiting proliferation and were within clinically relevant concentrations. However, only doxorubicin exhibited cytotoxicity (EC_{50} 2.4–13.1 n*M*), 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, $p21^{WAF1/CIP1}$ 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

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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 $p21^{WAF1/CIP1}$ 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 5×10^4 cells/well (1 cm^2) 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-^{3}H]Thymidine (25 Ci/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 [^{3}H]thymidine incorporated into DNA over the next 18 h was determined as described previously [27].

Cell growth analysis

Cells ($1–2\times10^4$) 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_2. The following day the growth medium was replaced in duplicate wells with medium containing doxorubicin at 0 to 0.1 μ*M*. 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 2×10^4 to 1×10^3 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 μ*M* 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 $p21^{WAF1/CIP1}$ (American Type Culture Collection, Manassas, Va., no. 79928) and *GAPD* (ATCC no. 78463) were labeled with ^{32}P using a Multiprime 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 μ*M* carboplatin or various concentrations of doxorubicin for various times. After treatment, cells were removed from the flasks with trypsin-EDTA, washed three times with PBS, and resuspended in cold cell lysis buffer comprising 150 m*M* NaCl, 10 m*M* Tris, pH 7.2, 5 m*M* EDTA, 0.1% Triton X-100, 5% glycerol, 1 μg/ml leupeptin, 500 μ*M* 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, electroblotted 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. Jiayuh 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-fluororuracil, carboplatin, and

etoposide on MRT cell proliferation using a thymidine incorporation assay. All MRT cell lines tested showed dose-dependent responses to all drugs (Fig. 1). Remarkably, all cell lines behaved in a similar manner qualitatively and quantitatively to the respective drug being tested. This result is illustrated in Table 2 in which the individual EC_{50} values and their means ($\pm$SE) for all the drugs and all the cell lines are shown. In general, doxorubicin, vincristine, and paclitaxel were effective at inhibiting DNA synthesis in the low nanomolar range, whereas carboplatin, etoposide, and 5-fluorouracil required higher concentrations of 0.1–2.5 μM.

Table 1 Malignant rhabdoid tumor cell lines

Identifier	Patient age	Patient sex	Primary location	Metastatic sites
RT4E	10 months	F	Neck[a]	Brain
RT5E	13 years	M	Leg[a]	Lungs, mediastinum, bone
STM91–01	1 year	M	Kidney	Lung[a]
TTC549	6 months	F	Liver[a]	Lung

[a]Sites from which cell lines were established

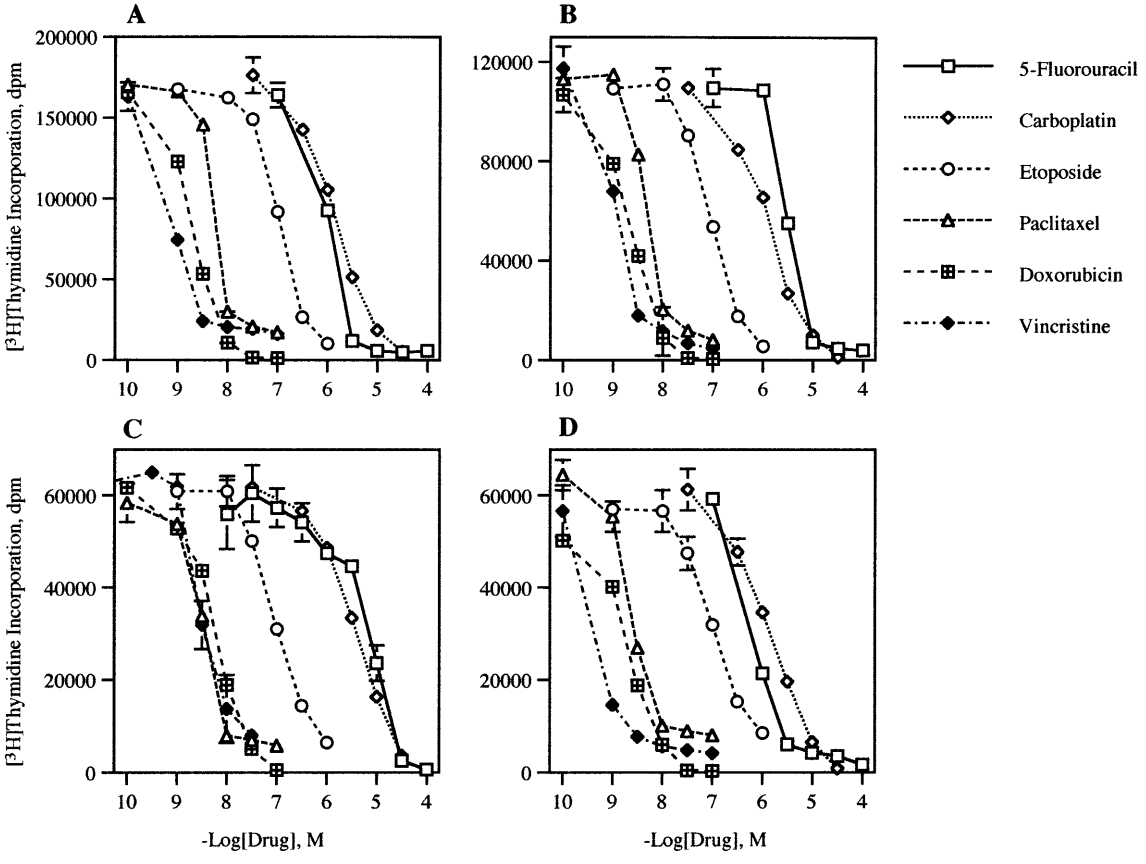

Fig. 1A–D Effect of chemotherapeutic drugs on [^{3}H]thymidine incorporation in MRT cell lines. The MRT cell lines, STM91-01 (**A**), RT4E (**B**), RT5E (**C**) and TTC549 (**D**), were grown in the presence of the indicated concentrations of drug for 24 h and the amount of [^{3}H]thymidine incorporated over the next 18 h was determined. For clarity, thymidine incorporation in the absence of drug was graphed as the lowest dose tested in a particular series. Data represent assays performed in triplicate with error bars indicating standard deviation

Cell growth assays

Cells treated with micromolar concentrations of doxorubicin began rounding up and detaching from the plates, and this cytotoxic effect was absent at low concentrations of any of the other drugs. To study this further, cells were plated on multiwell plates, treated with doxorubicin for 72 h, quantified by staining with crystal violet, and compared with cells that had been allowed to grow for the same time in the absence of doxorubicin. As can be seen in Fig. 2, the cell growth curves correlated nicely with the DNA synthesis curves with EC_{50} values of doxorubicin in the nanomolar range (2.9–13.1 nM). To examine the cytotoxic effect more directly, STM91-01, RT4E, and TTC549 cells were treated with 1 μM doxorubicin for 24 h which caused approximately 50% of the cells to detach from the plates. A correlation between cytotoxicity and cell detachment from the substrate was verified by trypan blue staining. During the next 24 h most remaining cells detached from the flasks. Despite reports of apoptosis induction by doxorubicin in other cell lines [13, 19, 25, 32], we were unable to verify morphological changes characteristic of apoptosis in the majority of the doxorubicin-treated MRT cells using time-lapse videomicroscopy, nuclear staining with 4′,6-

Table 2 EC_{50} values (nanomolar) for inhibition of [^{3}H]thymidine incorporation

Drug	STM91-01	RT4E	RT5E	TTC549	Mean ± SE
Vincristine	0.47	2.9	1.0	0.84	1.3 ± 0.5
Doxorubicin	1.9	5.7	1.9	2.2	2.9 ± 0.8
Paclitaxel	5.0	3.4	4.3	2.6	3.8 ± 0.5
Etoposide	108	96	92	106	100 ± 3
Carboplatin	1370	3747	635	1181	1733 ± 597
5-Fluorouracil	1070	5045	3092	856	2515 ± 850

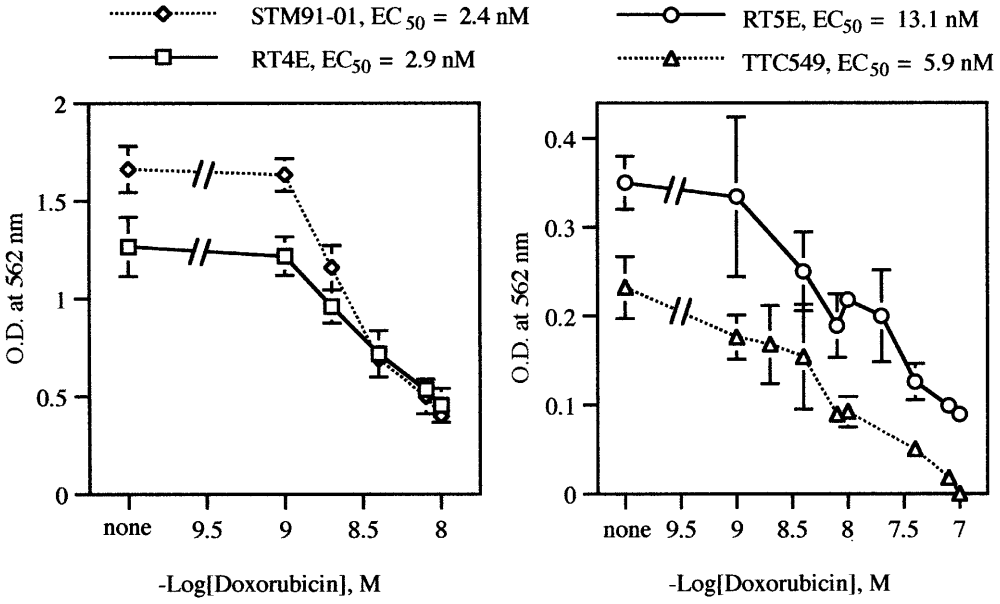

Fig. 2 Viability assay of four representative MRT cell lines exposed to doxorubicin in vitro. Four representative MRT cell lines, TTC549, RT4E, RT5E, and STM91-01 were treated in triplicate with concentrations of doxorubicin ranging from 10^{-9} to 10^{-7} *M* for 72 h and then stained with 0.2% crystal violet in 20% methanol. The amount of staining was quantified by solubilization in ethanol and determination of optical density (OD) at 562 nm. Error bars represent the standard deviations. The EC_{50} values for growth suppression are shown beside the cell line designations

diamidino-2-phenylindole (DAPI), and internucleosomal cleavage assays (data not shown).

Analysis of p53

The resistance of the MRT cells to several of the drugs tested, coupled with our previous demonstration of p53 accumulation in several MRT cell lines [23], prompted an additional investigation to determine whether the p53 pathway was responsive to DNA damage inflicted by chemotherapeutic agents. We measured the accumulation of the p53 protein in MRT cells exposed for various times to carboplatin and doxorubicin. Similar determinations of gene expression and protein accumulation of $p21^{WAF1/CIP1}$ were also made. Figure 3 shows that for two of the MRT cell lines, RT4E and STM91-01, carboplatin treatment induced p53 protein accumulation through the 24-h test period. Subsequent induction of the $p21^{WAF1/CIP1}$ protein was also apparent, although the kinetics were different from those seen with p53 induction. Of the time-points evaluated, $p21^{WAF1/CIP1}$ accumulation was greatest at 24 h in RT4E cells but peaked at 4 h in STM91-01 cells. These results suggest a functional p53 pathway, since both the p53 and $p21^{WAF1/CIP1}$ proteins accumulated in response to the DNA damage induced by carboplatin.

Doxorubicin, which was cytotoxic to the MRT cells, could also induce the p53 pathway. As shown in Fig. 4A, Northern blot analysis demonstrated a marked increase in $p21^{WAF1/CIP1}$ mRNA expression in the 4 h after treatment of the cells with 1 μ*M* doxorubicin. Western blot analysis (Fig. 4B) demonstrated an increase in p53 protein accumulation in both cell lines after only 2 h of treatment. While p53 accumulation in STM91-01 cells peaked 2 h after exposure, the maximal accumulation in RT4E cells occurred during the 4- to 8-h period. Similarly, an increase in the $p21^{WAF1/CIP1}$ protein was detectable in both cell lines 2 h after treatment with the drug with accumulation continuing through the 4- to 8-h period. Since $p21^{WAF1,CIP1}$ can be induced by p53-independent mechanisms, we also determined whether the MDM 2 protein, another gene target for p53, was induced by doxorubicin treatment. Figure 4B demonstrates that an increase in MDM 2 protein levels occurred in both cell lines. Therefore, two proteins known to be responsive to p53 were upregulated in response to doxorubicin treatment.

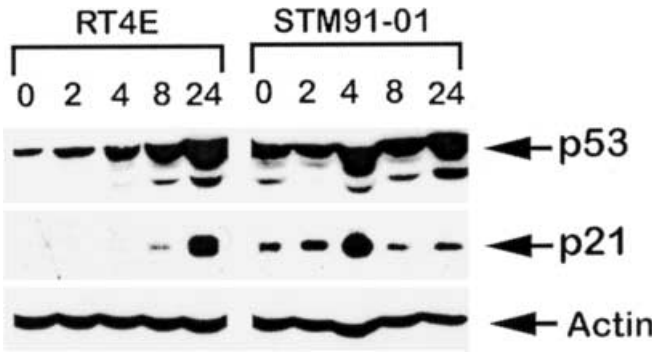

Fig. 3 Western blot analysis of carboplatin-induced p53 accumulation and induction of p21 in MRT cells. RT4E and STM91-01 cells were treated with 100 μ*M* carboplatin for the times (hours) noted above the lanes, then harvested, and lysates prepared. Protein (20 μg) was subjected to SDS-PAGE, blotted, and incubated with a monoclonal antibody to p53 (*top*) or p21 (*center*). The prominent lower band in the p53 blot is uncharacterized, but has been noted previously in other cell lines [18]. *β*-Actin is shown as a control for loading (*bottom*). The data are representative of two independent experiments

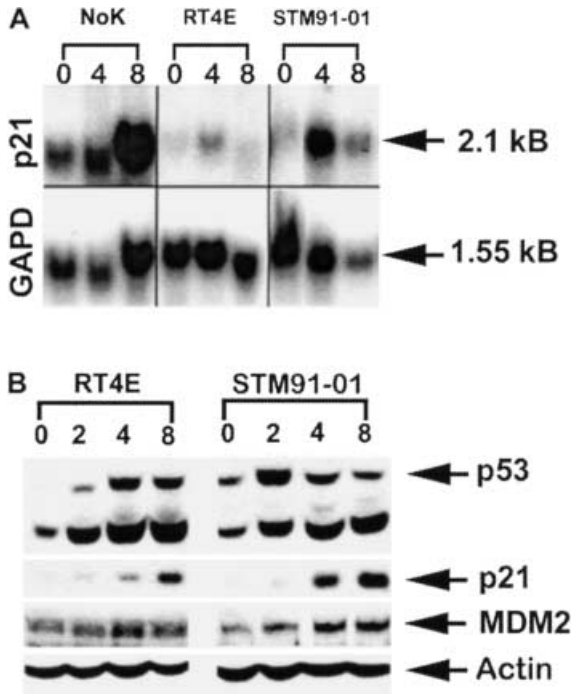

Fig. 4A, B Doxorubicin-induced p53 accumulation and induction of genes in MRT cells. **A** Northern blot analysis of NoK, RT4E, and STM91-01 cells treated with 1 μ*M* doxorubicin for the times noted above the lanes (hours). Cells were harvested and RNA extracted. Total RNA (6 μg) was size-fractionated on a formaldehyde-agarose gel, transferred to a nitrocellulose membrane, probed for p21 or the GAPD control, and exposed to X-ray film. **B** Western blot analysis of RT4E and STM91-01 cells treated with 1 μ*M* doxorubicin and protein harvested as described for Fig. 3 except that an additional set of lysates was blotted and incubated with a monoclonal antibody to MDM 2. For the MDM 2 antibody, 80 μg protein was loaded on the gel. The p53 induction is representative of two independent experiments

The experiments demonstrated a rapid accumulation of p53 and its downstream effectors when cells were incubated with rather high concentrations of doxorubicin, which simply demonstrated that the cells have a functional p53 pathway. We next determined whether p53 would accumulate in cells at doses of doxorubicin approaching the EC_{50}. To test this, we incubated STM91-01 and RT4E cells with various concentrations of the drug and determined p53 levels by Western blot analysis. In Fig. 5 it can be seen that p53 accumulation occurred in both cell lines at doses down to 10 n*M*. Therefore, p53 levels responded to low concentrations of doxorubicin, potentially tying doxorubicin-mediated growth inhibition to a p53-dependent pathway.

Discussion

Malignant rhabdoid tumor has been described as one of the most lethal tumors of childhood, having the highest relapse rate of any of the pediatric renal tumors [31]. Extrarenal primary locations are numerous, in particular the CNS where MRT cells constitute an often undetected component of some medulloblastomas and primitive neuroectodermal tumors [2]. Regardless of location, the presence of MRT cells carries a grave prognosis. The rarity of the tumor has compromised the development of in vitro and in vivo data that might aid clinicians in their search for effective treatments. We employed our collection of MRT cell lines to examine the in vitro effectiveness of several agents commonly used in MRT treatment as well as to evaluate the functionality of one potential mechanism of drug resistance in MRTs.

Doxorubicin, a commonly used anthracycline antibiotic, was the most effective drug of those tested against the MRT cell lines. Not only did it inhibit DNA synthesis and cell growth at nanomolar concentrations, it was also cytotoxic to the majority of MRT cells. The resistance of MRT cells to many of the drugs tested, as well as a previous discovery of apparent accumulations of p53 protein in a subpopulation of MRT cells [23], prompted additional investigation to determine functionality of the p53 protein, since it has been implicated in drug resistance mechanisms. The p53 pathway participates in at least two potential cellular outcomes as a result of DNA damage, either apoptosis or cell cycle arrest/senescence. The mechanism whereby the choice between these pathways is made is not always certain and is potentially affected by cell type and the extent of DNA damage. Wild-type p53 induces expression of $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor leading to G_1/S arrest, presumably a checkpoint to allow cells to survey DNA damage and to effect repairs, if possible, before proceeding through the G_1 restriction point. Some studies have correlated p53 loss in tumor cells with resistance to anticancer agents [14, 15], and others have found that p53 loss sensitizes cells to some agents (e.g. those that damage DNA) and confers re-

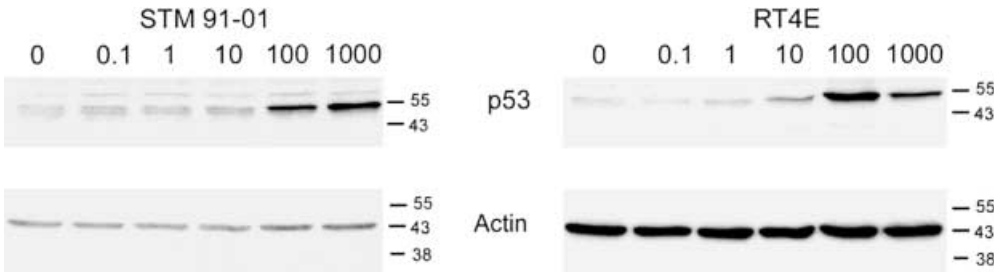

Fig. 5 Concentration-dependence of doxorubicin-induced p53 accumulation. Western blot analysis of STM91-01 and RT4E cells incubated with doxorubicin at the concentrations indicated in nanomolar above the lanes for 2 h (STM91-01) or 8 h (RT4E). Protein was harvested and 20 μg was loaded on the gel and blotted for p53 (*top*) or actin (*bottom*). The migration of molecular mass markers (in kilodaltons) is indicated to the right of each gel. The data are representative of two independent experiments

sistance on others [6, 7, 17, 30]. In the NCI anticancer drug screen, cells with mutant p53 exhibit less growth inhibition to DNA-damaging agents and antimetabolites, but the effect of antimitotic agents is independent of p53 status [18].

We have previously reported detection by immunohistochemistry of a heterogeneous accumulation of p53 protein in several MRT cell lines and primary tissue [23]. This observation suggests a potential dysfunction in this pathway, since the ability to detect p53 by this method is usually associated with aberrant accumulations of the protein, often due to mutations. Since a previous sequence analysis of the p53 mRNA in four of the MRT cell lines had disclosed no mutations [23], we elected in these studies to determine the functionality of the molecule. We found that exposure of MRT cell lines to either carboplatin or doxorubicin induced accumulation of the p53 protein and subsequent transcriptional upregulation of $p21^{WAF1/CIP1}$. We also found that doxorubicin treatment provoked an increase in another direct target of p53, MDM 2 [8]. It was of interest that both drugs activated the p53 pathway, as a 72-h exposure to high concentrations of carboplatin was largely cytostatic (data not shown). However, even low concentrations of doxorubicin triggered a response that ultimately led to the death of the majority of MRT cells. This suggests that, while carboplatin concentrations were sufficient to inflict DNA damage as evidenced by the accumulations of p53 and subsequently $p21^{WAF1/CIP1}$, either this damage was insufficient to trigger a cell death pathway or DNA repair mechanisms were adequate to maintain cell viability, at least through the 72-h test period. Doxorubicin, on the other hand, quickly provoked oncosis [16] in the majority of cells. Thus, we conclude from these studies that p53 is functional in the two MRT cell lines evaluated and is likely functional in the majority of MRT since we were unable to detect mutations in p53 cDNA in four of four MRT cell lines tested [23]. This conclusion is in concordance with the role of *p53* in maintaining normal DNA ploidy [4, 9, 11], as most MRT cell lines and tumors exhibit a relatively normal karyotype.

Another candidate for involvement in drug resistance in MRTs is a recently described gene on chromosome 22 which has been implicated as a tumor suppressor involved in tumorigenesis of MRT [26]. The protein product of this gene, variably designated INI1 [12], BAF47 [29], or SMARCB1 [22], is a member of the mammalian SWI/SNF family of chromatin remodeling complexes. Examination in our laboratory and others of the MRT cell lines employed here using RT-PCR, individual amplification of each of the nine exons from genomic DNA, and selective sequencing have confirmed mutation of the INI1 gene in all of them (Wright et al., manuscript in preparation; [5]). Thus, lack of the INI1 protein product may lead to transcription defects that result in a particular drug resistance profile in MRTs, conferring resistance to some drugs or, conversely, sensitivity to others.

With regard to the etiology of MRTs, there was no significant toxicity correlation in drug resistance or *p53* and $p21^{WAF1/CIP1}$ expression between cell lines established from renal and those from extrarenal tumors or between cell lines established from metastatic tissue and those from primary tumor tissue. Thus, these studies add further support to the contention that MRT is not simply a phenotype but represents a distinct malignant entity. That these findings may be relevant to treatment of MRT patients has been recently demonstrated by a clinical study suggesting that doxorubicin is an important component of a successful treatment regimen [28].

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