(Patho)physiological function of the Multidrug Resistance protein MRP1
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Chapter 6

Effect of p53 hotspot mutants on the expression of multidrug resistance proteins MRP1 and MRP2

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Submitted
Abstract

Loss of functionality of the *p53* tumour suppressor gene has been associated with multidrug resistance. In this study we used A2780 ovarian tumour cells stable transfected with *p53*, containing hotspot mutations either at codon 175 (m175), 248 (m248) or 273 (m273). These mutants showed a different degree of resistance against several anticancer drugs. This enabled us to investigate the relation between expression of drug resistance genes and *p53* mutations. Increased expression of MRP1 and MRP2 was found in m273 and m248 cells. MRP1 was detected at the plasma membrane, only at cell-cell contact sites and MRP2 was predominantly localized intracellularly. The transport activity of MRP1 and MRP2 was determined with membrane vesicles using tritium-labelled leukotriene C₄ (LTC₄) as substrate. Compared to CMV control transfectants, membrane vesicles from m273 and m248 showed a 4.5- and 1.5-fold increased uptake of LTC₄ that was inhibited by MK571. Cytotoxicity tests showed that m273 cells, but not m248, cells were resistant to doxorubicin, mitoxantrone and vincristine. The vincristine resistance was significantly modulated by MK571. In conclusion, loss of *p53* functionality can result in increased levels of certain multidrug resistance proteins. This may contribute to the multidrug resistance phenotype of tumour cells.
Introduction

Mutations of the p53 tumour suppressor gene are the most frequent genetic alterations in human malignancies.\(^1\) Loss of p53 function has been associated with multidrug resistance (MDR).\(^2,3\) Fibroblasts from p53 knockout mice are resistant to chemotherapeutic agents such as etoposide, doxorubicin and 5-fluorouracil.\(^4\) In a screen with 60 human cancer cell lines it appeared that those with a p53 mutation were less sensitive to cytotoxic drugs compared to cell lines expressing wild-type (wt) p53.\(^5\) In addition, specific p53 mutations are associated with doxorubicin resistance in breast cancer.\(^6\) Most of the p53 mutations found in tumours are in conserved regions of the protein; among them are the so-called hotspot mutations located at codons Arg175, Arg248 and Arg273. These mutations occur with a frequency of 6.1 % (175), 9.6 % (248) and 8.8% (273) of all p53 mutations in human cancers.\(^1,7\)

The p53 gene encodes for a 393 amino acid protein, which has functional domains for transactivation, DNA binding, nuclear localisation and oligomerisation. p53 is involved in many cellular processes such as transcription, DNA repair, cell cycle control, senescence and apoptosis.\(^8\) p53 can be activated by DNA damaging agents, hypoxia and decreased levels of ribonucleoside triphosphate pools and this activation is associated with cell cycle arrest. This process allows the DNA repair machinery to restore the DNA damage and prevents mutations and genetic alterations, which can ultimately lead to malignancies. The pro-apoptotic function of p53 seems cell type specific.\(^9\) The p53 protein has been identified as a transcription factor inducing expression of several genes that are directly or indirectly involved in the function of p53 in the cell.\(^10\) However, p53 can also suppress genes that apparently lack p53-binding sites such as the drug resistance genes DNA topoisomerase II\(\alpha\).\(^11\) MDR1-P-glycoprotein (MDR1)\(^12\) and the multidrug resistance protein MRP1.\(^13\) The molecular mechanism(s) involved are unclear.

Overexpression of MDR1 and/or MRP1 is seen in various drug resistant cell lines where they cause a MDR phenotype by ATP-dependent extrusion of cytotoxic drugs resulting in reduced intracellular levels.\(^14,15\) MDR1 and MRP1 share only 15% amino acid identity,\(^16\) but both proteins confer resistance to similar, although not identical, groups of anticancer drugs. In contrast to MDR1, MRP1-mediated drug resistance is dependent on the presence of glutathione (GSH) as reviewed by Hipfner et al.\(^17\) The importance of GSH in drug resistance has been extensively studied\(^18\) and is further illustrated by a frequently found coordinated upregulation of MRP1 and \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)GCS), the rate limiting enzyme in GSH biosynthesis, in human cancers.\(^19\) Besides MRP1, two other members of the MRP family, MRP2\(^20,21\) and MRP3\(^22\) have the capacity to confer drug resistance. Expression of MDR1 and MRP1 is repressed by wt p53.\(^15,23\) The involvement of p53 in regulation of MDR1 and MRP1 expression suggests that induction of drug resistance genes may play a role in the MDR phenotype of mutant p53 overexpressing cells.

Recently, the A2780 ovarian cancer cell line has been stably transfected with plasmids containing a mutated p53 sequence resulting in overexpression of the p53 protein containing hotspot mutations either at codon Arg175 (m175), Arg248 (m248)
or Arg273 (m273). Depending on the p53-mutations these cells exhibit a different degree of resistance against several chemotherapeutic drugs such as mitoxantrone, doxorubicin, paclitaxel or vinblastine. We hypothesized that overexpression of MDR transporters may contribute to the cellular resistance against these drugs. Therefore, we investigated the expression of MRP1, MRP2, MRP3, MDR1 and γGCS genes and proteins in the mutant p53-transfected A2780 ovarian cancer cells.

Materials and Methods

Materials
Cell culture reagents and TRIzol reagent was obtained from Life Technologies (Paisly, UK) and SV total RNA isolation system, AMV reverse transcriptase (RT) and RNasin ribonuclease inhibitor was from Promega (Madison, WI). Random primers for cDNA synthesis were obtained from Pharmacia (Uppsala, Sweden). Taq polymerase was from Eurogentec (Seraign, Belgium). Complete protease inhibitor was purchased from Roche Diagnostics (Almere, The Netherlands) and Benzonase was from Merck (Darmstadt, Germany). Monoclonal antibodies against MRP1 (MRPm6) and MRP2 (M2 III-6) were kindly provided by Dr. R. Scheper, (Free University, Amsterdam, The Netherlands). [14,15,19,20-3H(N)]-leukotriene C4 ([3H]LTC4) was obtained from New England Nuclear (Boston, MA) and MK571 was purchased from Biomol (Plymouth Meeting, PA). All other chemicals were from Sigma (St. Louis, MO), unless otherwise indicated.

Cell culture
The human A2780 ovarian tumour cell line was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum in a humidified atmosphere at 37°C with 5% CO₂. Generation of stable p53 transfectants was performed as described. In brief, plasmids carrying no p53 (pCMV-neo), or p53 mutated at codon 175 (m175, Arg → His), 248 (m248, Arg → Trp) or 273 (m273, Arg → His) were introduced in A2780 cells by electroporation and further selected by 1 mg/ml gentamicin (Life Technologies). Overexpression of p53 protein was routinely checked by immunohistochemistry.

RNA isolation and RT-PCR
Total RNA was isolated from the cells with TRIzol Reagent according to the manufacturer’s instructions and further purified using SV total RNA isolation system. Single stranded cDNA was synthesized from 10 μg RNA using 0.5 nmol random primers and 46 U AMV reverse transcriptase in a buffer containing 10 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.0 mM of each dNTP and 60 U RNasin ribonuclease inhibitor in a total volume of 75 μl. Reverse transcription (RT) was performed for 10 min at 25°C and 1 h at 55°C followed by heating at 95°C for 5 min to terminate the reaction. With the cDNA obtained, PCR reactions were performed.
using 3 µl RT reaction mix, 0.5 U Taq polymerase, 50 pmol sense and 50 pmol antisense primer (Table 1) in a final reaction volume of 50 µl.

**Table 1.** Primers used for the analysis of expression of MDR genes by RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sense and antisense</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>5’-AATGCGCCAAGACTAGGAAG-3’</td>
<td>990</td>
</tr>
<tr>
<td></td>
<td>5’-ACCGGAGGATGTTGAACAAG-3’</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>5’-CTGGTTGATGAAGGCTCTGT-3’</td>
<td>1067</td>
</tr>
<tr>
<td></td>
<td>5’-CTGCCATAATGTCCAGGGTTC-3’</td>
<td></td>
</tr>
<tr>
<td>MRP3</td>
<td>5’-GCAGGTGACATTTGCTCTGA-3’</td>
<td>564</td>
</tr>
<tr>
<td></td>
<td>5’-CTGAAGGTCACGAGTCTCCC-3’</td>
<td></td>
</tr>
<tr>
<td>γGCS</td>
<td>5’-TGAGATTTAAGCCCCCTCCTCCT-3’</td>
<td>521</td>
</tr>
<tr>
<td></td>
<td>5’-CTACTCCCTCAATAGCGT-3’</td>
<td></td>
</tr>
<tr>
<td>MDR1</td>
<td>5’-AAAAAGATCAACTCGTAGGAGTG-3’</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>5’-GCACAAAAATACACCAACAA-3’</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-AACACCCAGCCATGTACG-3’</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>5’-ATGTCACGCACGATTTCCG-3’</td>
<td></td>
</tr>
</tbody>
</table>

**Isolation of membrane fractions**
Crude membrane fractions were isolated as follows. About 50 x 10⁶ cells were harvested and centrifuged at 1,000 x g for 15 min at 4°C. The pellet was resuspended in 1 mM Tris-HCl, pH 7.4, supplemented with Complete™ protease inhibitor cocktail tablets, with one tablet per 50 ml. Cells were lyzed by 3 cycles of snap-freezing in liquid nitrogen and quick defrosting at 37°C. The resulting suspension was further homogenized by passing 20 times through a 21 gauge needle. The homogenate was incubated for 30 min on ice in the presence of 100 U Benzonase and centrifuged for 15 min at 15,000 x g at 4°C. The pellet was resuspended in 200 µl 1.0 mM Tris-HCl, pH 7.4, supplemented with Complete™.

Membrane vesicles used for transport studies were isolated essentially as described.²⁵ Protein contents were measured by a Bradford-based Biorad protein assay (Biorad laboratories, Hercules, CA).

**Glycosylation assay**
 Thirty µg of crude membrane fractions were incubated overnight at 37°C in 50 mM NaH₂PO₄ buffer, pH 7.5, with 3 mM phenylmethylsulfonyl fluoride and either in the absence or in the presence of 5 U of peptide N-glycosidase F (PNGase F), (New England Biolabs, Beverly, MA).
Immunoblot analysis
Membrane fractions were analyzed for MRP1, MRP2 and MDR1 protein expression by Western Blot as described. Monoclonal antibodies used for protein detection were MRP1 (MRP1), M2 III-6 (MRP2) and C219 (MDR1). Positive control for MRP1 was 2.5 µg membrane vesicles from GLC4/Adr cells, for MRP2 2.5 µg rat liver homogenate and for MDR1 2.5 µg membrane vesicles from A2780AD cells, the MDR counterpart of A2780 cells.

Confocal scanning laser microscopy
Localisation of MRP1 and MRP2 was examined by confocal scanning laser microscopy. Single cell suspensions of 0.5 x 10⁶ cells/ml were grown overnight on coverslips. Cells were washed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and fixed in acetone (-20°C) for 5 min. Coverslips were air dried and cells were rehydrated by placing the coverslips in PBS for 20 min at room temperature. Cells were incubated with monoclonal antibodies MRPm6 (MRP1) and M2 III-6 (MRP2), both diluted 1:50 in PBS/1% bovine serum albumin (BSA), in a humidified chamber overnight at 10°C. Coverslips were then washed three times for 10 min with PBS and incubated for 1 h at room temperature with a FITC-labelled goat anti-mouse IgG (Sigma) diluted 1:400 (MRPm6) and 1:200 (M2 III-6) in PBS/1%BSA. Coverslips were then washed three times for 10 min in PBS, rinsed with water and mounted with Prolong Antifade mounting medium (Molecular Probes, Leiden, The Netherlands). Images were taken with a confocal scanning laser microscope, (TCS 4D, Leica, Heidelberg, Germany) equipped with an argon/krypton laser coupled to a Leitz DM IRB inverted microscope (Leica).

Transport studies
Transport of [³H]LTC₄ (1.5 nM) into isolated membrane vesicles was measured as described. Briefly, membrane vesicles (40 µg protein) were rapidly thawed and incubated in a buffer containing 4 mM ATP or AMP-PCP, the non-hydrolyzable ATP-analogue, 10 mM MgCl₂, 10 mM creatine phosphate (Roche Diagnostics, Almere, The Netherlands), 100 µg/ml creatine kinase (Roche Diagnostics), 10 mM Tris, pH 7.4 and 250 mM sucrose. After 1 min prewarming, [³H]LTC₄ (1.5 nM) was added and samples were taken after 3 min and diluted into ice-cold stopsolution (10 mM Tris/250 mM sucrose, pH 7.4). These dilutions were filtered and radioactivity was counted. The MRP1 inhibitor MK571 (3 µM final concentration) was added together with the membrane vesicles. ATP-dependent transport was calculated by subtracting AMP-PCP values from the ATP values.

γ-GCS activity and GSH levels
To determine the γGCS activity, cell pellets were resuspended in 150 mM Tris-HCl, pH 7.4, briefly sonicated and the lysates were centrifuged at 15,000 x g for 15 min at 4°C. The supernatants were used further for determination of γGCS activity according
Mutant p53-mediated expression of MRP1 and MRP2

to the method of Seelig and Meister using the coupled enzyme procedure. The reaction was followed by measuring the decrease in NADH absorbance at 340 nm at 37°C. Specific activity of the \( \gamma \text{GCS} \) enzyme was defined as \( \mu \text{mol NADH oxidized/min/mg protein} \) with 1 \( \mu \text{mol/min/mg} \) set to 1 U. The specificity of the method was confirmed by using buthionine sulfoximine (BSO), an inhibitor of \( \gamma \text{GCS} \). The GSH levels were determined according to the method of Tietze.

**Drug sensitivity assay**

Cell survival after drug exposure was tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microculture assay. For CMV and m273 1,250 cells, for m175 2,000 cells and for m248 2,500 cells were seeded in a total volume of 200 µl medium in a 96-well culture plate. Drug incubations were performed for 96 hrs with each drug concentration tested in quadruplicate. For modulation studies, MK571 (50 µM final concentration) was added 1 h before the drug incubations started.

**Results**

**mRNA analysis of drug resistance genes in mutant p53 overexpressing cells**

To investigate the relation between specific p53 mutations and drug resistance genes, we analyzed the expression of \( \text{MRP1}, \text{MRP2}, \text{MRP3}, \text{MDR1} \) and \( \gamma \text{GCS} \) genes by RT-PCR in A2780 ovarian tumour cells, the CMV control transfectants and the mutant p53 overexpressing cells. (Figure 1). The \( \beta \)-actin gene was used as control to compare expression levels, while the human liver hepatoma cell line HepG2 and human liver (HL) tissue served as positive controls for all genes analyzed. \( \text{MRP1} \) mRNA was detected in all cells but showed particularly increased levels in m273 cells. Expression of the \( \text{MRP2} \) gene was demonstrated in the m248 cells and at a lower level in the CMV transfectants. No expression of this gene was found in the other mutants or in the A2780 cell line, while a relatively strong expression was observed in the HepG2 cells and in HL tissue. \( \text{MRP3} \) gene expression was detected only in HepG2 cells and HL tissue. A relatively high expression of the \( \text{MDR1} \) gene was seen in HepG2 cells and HL tissue, while \( \text{MDR1} \) expression was very low in m273 cells and not detectable in the other mutants nor in the CMV transfectants or the A2780 cells. All cell lines showed expression of the \( \gamma \text{GCS} \) gene. Compared to CMV transfectants, the expression of \( \gamma \text{GCS} \) in m273 cells was slightly decreased.

**Protein expression of MRP1, MRP2 and MDR1 in mutant p53 overexpressing cells**

Crude membrane fractions were isolated to examine the expression of MRP1, MRP2 and MDR1 proteins using immunoblot analysis (Figure 2). MRP1 was overexpressed in m273 cells, while in the other mutants no significant difference in MRP1 expression was detected in comparison with the CMV transfectants. Increased levels of MRP2
were observed in m248 cells and to a lower extent also in the CMV transfectants. Expression of MDR1 was only detected in membrane fractions from A2780AD cells, which served as positive control for MDR1 expression (data not shown). We also investigated expression levels of MRP1 and MRP2 (Figure 3) in enriched membrane subfractions used for kinetic analysis of MRP1 and MRP2 function. Again an increased MRP1 expression was seen in membrane vesicles from m273 cells. Expression of MRP2 was detected only in membrane vesicles from m248 cells, in contrast to the results obtained with the crude membrane fractions where MRP2 staining was also detected in CMV membranes. This may be due to further purification of the membrane preparation used for transport studies.

MRP1 protein in the mutant p53 overexpressing cells seems to have a slightly lower molecular weight compared to A2780 and CMV control cells (Figure 2). Treatment of the crude membrane fractions with PNGase F that cleaves the N-linked glycosylation resulted for all cell lines in a deglycosylated protein with identical mass (data not shown). Thus, in the mutant p53 transfected cells, MRP1 is less glycosylated than MRP1 in A2780 cells and CMV transfectants.

**Subcellular localisation of MRP1 and MRP2**

Confocal scanning laser microscopy was used to study the subcellular localisation of MRP1 and MRP2 in m273 and m248 cells, respectively. Increased MRP1-staining was observed at the plasma membrane of m273 cells but only at cell-cell contact sites (Figure 4). Much lower MRP1-staining was found in CMV transfectants. No increased MRP1-signal was seen in the other mutants (data not shown). MRP2 was
predominantly localized at intracellular vesicular structures in m248 cells, whereas MRP2 was not detected in CMV transfectants (Figure 4) or other mutants (data not shown).

**Figure 2.** Immunoblot analysis of MRP1 and MRP2 proteins in crude membrane fractions. Twenty µg of protein was separated on 7.5% SDS-PAGE and transferred to nitrocellulose by electroblotting. Positive control for MRP1 was 2.5 µg membrane vesicles from GLC4/Adr cells and for MRP2, 2.5 µg rat liver homogenate. Protein levels were analyzed with monoclonal antibodies against MRP1 (MRPr1) and MRP2 (M2 III-6). Primary antibodies were visualized by enhanced chemiluminescence. Sizes of molecular weight markers are indicated in kDa.

**Figure 3.** Immunoblot analysis of MRP1 and MRP2 protein levels in enriched membrane subfractions. Analysis of protein expression was performed precisely as described in the legend of figure 2.

**ATP-dependent [³H]LTC₄ transport activity from mutant p53 overexpressing cells**

To investigate MRP1 and MRP2 function, transport studies were performed using [³H]LTC₄ as substrate. ATP-dependent uptake of [³H]LTC₄ into membrane vesicles from m175 cells was slightly decreased compared to CMV. In both CMV and m175 membrane vesicles, MK571 inhibited ATP-dependent [³H]LTC₄ uptake, indicating that this transport is MRP1-mediated. Compared to CMV, ATP-dependent [³H]LTC₄ uptake into m248 and m273 membrane vesicles was increased 1.7- and 4.9-fold, respectively. This transport activity was reduced by MK571, an inhibitor for MRP1 and MRP2.¹¹ These results demonstrate that MRP1 is functionally
overexpressed in m273. The transport activity measured with m248 membrane vesicles is most likely due to the transport function of both MRP1 and MRP2.

![Image](image.png)

**Figure 4.** Subcellular localisation of MRP1 and MRP2 in CMV transfectants (A and C), m273 cells (B) and m248 cells (D). MRP1 was stained with the monoclonal antibody MRPm6 (1:50), (A and B). MRP2 was stained with the monoclonal antibody M2 III-6 (1:50), (C and D). The primary antibodies were visualized by a secondary FITC-labelled antibody with a 1:400 dilution for MRPm6 and a 1:200 dilution for M2 III-6. Images were taken with a confocal scanning laser microscope. Bar = 10 µm.

**γGCS activity and GSH levels**

GSH is a crucial factor in MRP1 and MRP2 function as MDR proteins. Figure 1 shows that expression of the γGCS gene is not significantly changed in m175 and m248 cells and is slightly decreased in m273 cells. To determine whether the activity of the enzyme is altered, we measured γGCS activity and GSH levels in the p53 mutants and CMV transfectants. HepG2 cells were used as positive control for the γGCS assay. The γGCS activity determined in the p53 mutants was not different from the CMV transfectants. The HepG2 cells showed a γGCS activity of 4.5 ± 0.4 U which is about 3-fold higher compared to the CMV transfectants (Table 2).
The GSH levels from m248 and m273 cells were not different from CMV transfectants. However, m175 cells showed increased GSH levels compared to CMV transfectants and the other mutants (Table 2).

Table 2. γGCS enzyme activity and GSH levels in mutant p53 overexpressing cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>γGCS activity (U)</th>
<th>GSH (nmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>1.7 ± 0.2</td>
<td>11.1 ± 2.5</td>
</tr>
<tr>
<td>m175</td>
<td>1.8 ± 0.3ns</td>
<td>22.5 ± 2.9a</td>
</tr>
<tr>
<td>m248</td>
<td>1.9 ± 0.2ns</td>
<td>8.5 ± 1.9b</td>
</tr>
<tr>
<td>m273</td>
<td>1.5 ± 0.3ns</td>
<td>10.7 ± 2.4b</td>
</tr>
<tr>
<td>HepG2</td>
<td>4.5 ± 0.5a</td>
<td></td>
</tr>
</tbody>
</table>

One U γGCS enzyme activity is defined as 1 µmol NADH oxidized per min/mg protein. Data represents mean ± S.D. of at least three independent experiments. Statistical significance was tested using a one-way ANOVA test. HepG2 cells were used as positive control for the γGCS assay. *ns* not significant compared to CMV transfectants or the other mutants; *a* P < 0.05 compared to CMV; *b* P < 0.05 compared to m175.

**Figure 5.** ATP-dependent uptake of ^[3]H^LTC_4 into membrane vesicles from CMV transfectants, m175, m248 and m273 cells. Uptake of ^[3]H^LTC_4 was measured for 3 min in the presence of ATP or the non-hydrolyzable ATP analogue AMP-PCP. ATP-dependent uptake was calculated by subtraction of AMP-PCP levels from ATP levels. MK571 was used in a final concentration of 3 µM. Data shown are means ± SD from two experiments with quadruplicate determinations.

**Drug sensitivity of mutant p53 overexpressing cells**

Previously we have demonstrated that our p53 mutant cells showed a different degree of resistance against several anticancer drugs. In this study we found overexpression of MRP1 and MRP2 in m273 and m248 cells. In order to test whether MRP1 and MRP2 play a role in the drug resistance phenotype of the p53 mutants, we measured the cytotoxicity of three different anticancer drugs in the presence or the absence of MK571 using the MTT assay.
The m175 cells only showed significant resistance to doxorubicin (1.8 fold) and this was modulated by MK571 (Table 3). Resistance levels found in m248 cells were not different from CMV transfectants. However, compared to CMV transfectants, the m273 cells showed resistance to doxorubicin (2.8 fold), mitoxantrone (5.9-fold) and to vincristine (1.7-fold). In these cells, only resistance to vincristine was significantly modulated by MK571 (Table 3).

**Discussion**

Mutations of the p53 tumour suppressor gene are common in diverse types of human malignancies and are frequently associated with a drug resistant phenotype.5,28,6 Our data provide evidence for an association between mutated p53 and increased expression of MRP1 and MRP2 and support the idea that mutation of p53 leads to a decreased sensitivity for anticancer drugs.29

**Table 3.** Drug resistance profiles of CMV transfectants and different p53 mutants.

<table>
<thead>
<tr>
<th>cells</th>
<th>doxorubicin</th>
<th>mitoxantrone</th>
<th>vincristine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MK571</td>
<td>MK571</td>
<td>MK571</td>
</tr>
<tr>
<td>CMV</td>
<td>11.1 ± 1.7</td>
<td>8.5 ± 1.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>m175</td>
<td>20.4 ± 0.1$^a$</td>
<td>15.9 ± 0.2$^b$</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>m248</td>
<td>15.0 ± 2.3</td>
<td>11.7 ± 1.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>m273</td>
<td>30.8 ± 1.7$^a$</td>
<td>21.9 ± 1.7</td>
<td>4.1 ± 0.6$^a$</td>
</tr>
</tbody>
</table>

Sensitivity to doxorubicin, mitoxantrone and vincristine was determined using the MTT assay in the absence or the presence of MK571. Data represent IC$_{50}$ values (means ± SD) of at least two experiments with quadruplicate determinations. $^a$ different from CMV transfectants (one way ANOVA, P < 0.05); $^b$ different from the IC$_{50}$ value measured without MK571 (unpaired two-tailed Student’s t-test, P < 0.01).

Most of the mutations found in p53 are located in the conserved hydrophobic region (amino acids 100-293) which binds to specific DNA sequences.8 In this study we used p53 constructs with mutations at the highly conserved codons 175 (Arg → His), 248 (Arg → Trp) and 273 (Arg → His). These are frequently occurring, so-called hotspot, mutations. Residues Arg248 and Arg273 seem to be involved in direct contact with the DNA, while residue Arg175 is supposed to play a role in stabilizing the structure of the DNA binding surface of p53.7 Since codons 175, 248 and 273 are located in the
Mutant p53-mediated expression of Mrp1 and Mrp2

DNA-binding domain of the protein, research has been focussed on the transcriptional activity of the protein mutated specifically at these codons.

In the current study we found an increased expression of Mrp1 and Mrp2 after stable transfection of A2780 ovarian carcinoma cells with mutant p53 constructs. The mechanism behind this is unknown. Apparently, the promoter regions of the Mrp1 and Mrp2 genes lack consensus p53 binding sequences. Thus it is unlikely that p53 has a direct role in regulation of these drug resistance genes. It has been demonstrated that wt p53 negatively regulates genes that apparently lack p53 binding elements including DNA topoisomerase IIα,11 Mdr1 12 and Mrp1.13 This suggests that the p53-dependent regulation of Mrdr1, Mrp1 and Mrp2 does not occur at the level of gene transcription.

The Mdr1 gene expression in m273 cells is specific but was very low. It has been proposed that mutant p53 can induce Mdr1 expression by a dominant negative effect on the suppressor activity of wt p53.12 This may also be true for the increased Mdr1 gene expression in m273 cells. The precise mechanism of Mrp1 and Mrp2 expression in relation to p53 is not known. In analogy to Mdr1, overexpression of mutant p53 might interfere with the suppressor activity of wt p53. It has been shown that induction of Mrp1 promoter activity mediated by the transcription factor Sp1 is strongly reduced by wt p53.13 Sp1 forms a heterocomplex with wt p53 and this suggests that wt p53-mediated repression of Mrp1 expression may be a result of p53-Sp1 complex formation, which prevents transactivation of the Mrp1 promoter. Mutated p53 also binds to Sp1 but this might result in a complex with reduced repression activity, causing an increased Mrp1 expression. Further research is required to elucidate the precise mechanism.

The expression of Mrp2 is induced in m248 cells and to a much lower extent in the CMV transfectants. We speculate that overexpression of p53 mutant m248 might interfere with repression of Mrp2 promoter activity in A2780 cells, or may result in increased levels of transcription factors that induce Mrp2 expression. Alternatively, m248 and CMV cells differ from the other mutants in that they have a constitutive overexpression of p21 (Waf1/Cip1), which is involved in cell cycle arrest.9 Furthermore, cell cycle analysis showed an increased percentage of m248 cells in the G1 phase and a decreased percentage of cells in the S phase (S de Jong et al., submitted). Thus, m248 cells seem to be in a less-proliferative state. Recently, it has been demonstrated that Mrp2 expression in freshly isolated hepatocytes is related to the absence of cyclin D1, a protein expressed in late G1 and mediating entry into the S-phase of the cell cycle.31 The increased Mrp2 expression in CMV and m248 cells may be related to alterations in the cell cycle status of these cells.

The localisation of Mrp1 in m273 cells at cell-cell contact sites is consistent to observations with other cell lines.32,31 In contrast, the localisation of Mrp2 in m248 cells was predominantly intracellular. In polarized cells, Mrp2 is found at the apical membrane 33,34 and also in transfected polarized epithelial cells, Mrp2 is targeted to the apical membrane.35 However, in non-polarized cells such as kidney carcinoma cells, and in non-polarized transfected cells, Mrp2 is localized mainly at intracellular membranes.36,20 A2780 cells have a strictly non-polarized phenotype. This may explain the intracellular localisation of Mrp2 in m248 cells.
Although m248 cells have an increased expression of functional MRP2, they do not show significantly increased resistance to any of the tested drugs. This may be due to the relative low expression levels that can not significantly contribute to drug resistance. In contrast, m273 cells, with highest MRP1 expression, show increased resistance against doxorubicin, mitoxantrone and vincristine. However, the MRP1 inhibitor MK571 only significantly modulates vincristine resistance. This indicates that doxorubicin and mitoxantrone resistance is more complex than vincristine-related resistance and appears to be partly dependent on mechanism(s) other than MRP1.\textsuperscript{37} This is further illustrated by the fact that MRP1 per se confers higher resistance towards vincristine than to doxorubicin and no resistance to mitoxantrone.\textsuperscript{38}

Based on the MK571 modulation, the doxorubicin resistance in our p53 mutants seems at least partial dependent on MRP1. The MRP1-mediated doxorubicin resistance in m175 cells is probably related to the increased GSH levels in these cells. MRP1-mediated drug transport is dependent on the GSH concentration.\textsuperscript{39,25} Alternatively, GSH is important for the detoxification of xenobiotics and increased levels are associated with drug resistance. Doxorubicin is known to generate toxic metabolites, such as 4-hydroxyalkenals. These metabolites are easily conjugated to GSH and are potential MRP1 substrates.\textsuperscript{40} Blocking of MRP1 might thus cause an inhibition of a cellular defence pathway against the toxic effects of doxorubicin resulting in increased sensitivity.

In conclusion, we demonstrate that overexpression of p53 mutated at codons 273 or 248 results in increased expression of functional MRP1 and MRP2. In addition, increased MRP1 expression is associated with vincristine resistance. Thus, loss of wt p53 functionality may result in increased levels of certain multidrug resistance proteins and this may contribute to the drug resistant phenotype of tumour cells.

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References


