*Anti-Cancer Drugs 2001,* **12,** pp. 769–779

## Preclinical report

# Establishment and characterization of adriamycin-resistant human colorectal adenocarcinoma HCT-15 cell lines with multidrug resistance

**Noriko Uchiyama-Kokubu[1] and Toru Watanabe[1]**
[1]Tsukuba Research Institute, Novartis Pharma KK, Ohkubo 8, Tsukuba-shi, Ibaraki 300-2611, Japan.

**The multidrug resistance (MDR) phenotype, either intrinsic and/or acquired, is discussed in relation to several MDR-associated markers such as P-glycoprotein (P-gp) encoded by *mdr1*, multidrug-resistance-associated protein (MRP) encoded by *MRP* and lung-resistance-associated protein (LRP) encoded by *LRP*. Well-characterized *in vitro* models are required to elucidate the mechanisms of MDR. The aim of the present study is the establishment of a drug-resistant subline from human colorectal adenocarcinoma HCT-15 that intrinsically expresses moderate levels of P-gp, MRP and LRP. Three adriamycin-resistant sublines (HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2) were established by stepwise exposure in growth medium that was supplemented with 25–200 ng/ml adriamycin—resulting in a 2.2- to 7.8-fold increase in $IC_{50}$ values by using the XTT assay. They were cross-resistant to MDR-related drugs, epirubicin, mitoxantrone, vincristine, etoposide and taxol, but not the MDR-unrelated drug, mytomycin C. The resistance to adriamycin was confirmed *in vivo* by a lack of sensitivity in athymic nude mice. Gene expression data for *mdr1*/P-gp, *MRP*/MRP and *LRP*/LRP on both mRNA and protein levels demonstrated that the molecules contributing to MDR in resistant sublines are mainly P-gp and partially MRP. The newly established adriamycin-resistant sublines of HCT-15 will provide clinically relevant tools to investigate how to overcome drug resistance and elucidate possible mechanisms of acquired MDR in human colon cancer. [© 2001 Lippincott Williams & Wilkins.]**

*Key words*: Adriamycin, human colorectal adenocarcinoma HCT-15, multidrug resistance, multidrug-resistance-associated protein, lung-resistance-associated protein, P-glycoprotein.

Correspondence to N Uchiyama-Kokubu, Tsukuba Research Institute, Novartis Pharma KK, Ohkubo 8, Tsukuba-shi, Ibaraki 300-2611, Japan.
Tel: (+81) 298 65 2216; Fax: (+81) 298 65 2385;
E-mail: Noriko.Uchiyama@Pharma.Novartis.com

## Introduction

Colorectal cancer is one of the most frequent tumors and is a leading cause of death. Although surgery is successful in a large percentage of cases, the survival rate decreases if the tumor has metastasized to regional lymph nodes or liver.[1] Chemotherapy is the first line of treatment for disseminated disease; however, colorectal cancer is refractory to most chemotherapeutic agents.[2] Unfortunately, very little is known about the mechanisms responsible for the intrinsic drug resistance of the cancer.

For successful cancer chemotherapy, it is necessary to understand the multifunctional mechanisms in the development of resistance to cancer chemotherapy. When tumor cells acquire resistance to antitumor drugs, the resistant cells often show cross-resistance to other antitumor drugs with different chemical structures and cellular targets, including anthracyclines (adriamycin and daunorubicin), vinca alkaloids (vincristine and vinblastine), podophyllotoxines (etoposide) and taxanes (paclitaxel).[3] This cellular resistance is known as multidrug resistance (MDR). The best characterized mechanism responsible for MDR is the overexpression of the P-glycoprotein (P-gp).[4,5] P-gp, encoded by the *mdr1* gene, is an ATP-dependent transmembrane protein that transports antitumor drugs out of cells. In various studies, a correlation of *mdr1* mRNA expression and drug resistance has been demonstrated using human cancer cell lines.[6] Like P-gp, multidrug-resistance-associated protein (MRP) is also an ATP-dependent transmembrane protein, which is expressed in an anthracycline-resistant but P-gp-negative lung cancer line.[7] Recently, the gene encoding the lung-resistance-related protein (LRP) was cloned and expressed in a MDR cell line that does not exhibit P-gp overexpression[8,9]. Numerous models

of an acquired resistant carcinoma were established to elucidate the mechanisms of MDR. For such studies, the resistant sublines have been established from P-gp-negative cell lines through the exposure of MDR-related drugs such as adriamycin and their acquired resistance was mainly dependent on P-gp expression levels.[10] On the other hand, it is difficult to investigate the relationship between intrinsic clinical resistance and expression of resistance-associated markers after chemotherapy, since *mdr1*, *MRP* as well as *LRP* mRNA were often present in the primary tumor specimens before patients received chemotherapy. In addition to the resistance markers found in specimens, samples are often contaminated with *mdr1* mRNA expression in normal human colon tissues. When the intrinsic-resistance tumors acquired additional resistance after chemotherapy, their characteristics become more complex, since the mechanisms of the emergence of drug-resistant phenotypes during chemotherapy are also unknown.

Based on this background, the aim of the present study is to establish well-characterized intrinsic-resistance tumor models that acquire additional resistance after chemotherapy. Some MDR cell lines, especially in renal and colon cancer cells, are reported to be intrinsically resistant and co-express three resistance-associated markers (P-gp, MRP and LRP) or express high levels of at least two of them.[11] Among these, a human colorectal adenocarcinoma HCT-15 cell line was originally derived from specimens of human colon adenocarcinomas prior to exposure to chemotherapy and expresses moderate levels of three resistance-associated markers.[11,12] Thus, we felt that HCT-15 will provide the best available resistance model after exposure to adriamycin, one of the well-known antitumor agents. In this report, to investigate which resistant marker significantly contributes to MDR in establishing adriamycin-resistant cells, we analyzed both mRNA and protein expression levels of three resistant markers, *mdr1*/P-gp, *MRP*/MRP and *LRP*/LRP, as well as utilizing the functional rhodamine 123 assay.

## Materials and methods

### Materials

Adriamycin, epirubicin and mytomycin C were purchased from Kyowa Hakkou Kogyo (Tokyo, Japan), and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), 2-[6-amino-3-imino-3H-xanthen-9-yl]-benzoic acid methyl ester (rhodamine 123), paclitaxel (taxol) and verapamil were from Sigma (St Louis, MO). Vincristine was purchased from Shionogi (Osaka, Japan) and mitoxantrone from Lederle (Tokyo, Japan). Etoposide was purchased from Nihon Kayaku (Tokyo, Japan).

The mouse monoclonal antibody MRK16 was purchased from Kyowa Medex (Tokyo, Japan). The rat monoclonal antibody MRPr1 and mouse monoclonal antibody LRP56 were from Nichirei (Tokyo, Japan). Fluorescein isothiocynate (FITC)-labeled goat anti-rat or anti-mouse immunoglobulin G (IgG) were purchased from Tago Immunologicals (Camarillo, CA). Mouse or rat monoclonal IgG was from Chemicon (Temecula, CA).

The Gene Amp RNA PCR kit was purchased from PE Applied Biosystems (Foster City, CA) and polymerase chain reaction (PCR) MIMIC from Clontech (Palo Alto, CA). Isogen was purchased from Nippon Gene (Tokyo, Japan). All other chemicals were of analytical grade.

### Animals and tumor cells

Female 6-week-old BALB/c *nu/nu* mice weighing 19–23 g each were purchased from Clea (Tokyo, Japan). They were maintained under specific pathogen-free conditions at 25°C in an atmosphere with 50% humidity. Lighting was operated automatically on a 12-h light/dark cycle. All animal studies were done under the guidelines of the Novartis Tsukuba Research Institute (Novartis Pharmaceuticals, Tsukubashi, Ibaraki, Japan).

Human colorectal carcinoma HCT-15 (CCL 225) cells were obtained from ATCC (Rockville, MD) through Dainippon Pharmaceutical (Osaka, Japan). Adriamycin-resistant sublines of HCT-15 were established by continuous exposure to adriamycin. Cells were suspended in culture medium in the presence of 25 ng/ml adriamycin (HCT-15/ADM1) and 50 ng/ml adriamycin (HCT-15/ADM2) for 1 year. HCT-15/ADM2-2 cells were selected in 200 ng/ml adriamycin for 1 year. HCT-15 and HCT-15/ADM cells were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 $\mu$g/ml kanamycin in an atmosphere containing 5% $CO_2$ at 37°C. After the development of the resistance phenotype, HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 cells were maintained in the same growth medium supplemented additionally with 25, 50 and 200 ng/ml adriamycin.

### *In vitro* growth inhibition assay

XTT assay was carried out as described previously.[13] In brief, parental and resistant sublines were seeded in 96-well tissue culture plates at a density of

$5 \times 10^3$ cells/well. After 24 h incubation, the cells were exposed to the drugs for 72 h and then relative cell growth was assessed by staining with XTT. Following 4 h incubation with the tetrazolium dye, the absorbance at 450 nm was measured by use of a microplate reader (Molecular Devices, Menlo Park, CA). The $IC_{50}$ values were defined as the concentration producing the cell growth at 50%. Relative resistance of antitumor drugs was determined by dividing $IC_{50}$ values of resistant sublines by that of parental cells.

### *In vivo* solid tumor studies

A suspension of cells ($10^7$ cells in 0.1 ml of Hanks' balanced salt solution) was injected into the right subaxillary region of individual athymic mice (day 0). The control and treatment groups consisted of 10 and five mice, respectively. Adriamycin treatment (8 mg/kg i.v.) was initiated after tumors were established (100–500 $mm^3$, as estimated by caliper measurement). The tumor volume ($v$) was calculated from the equation:

$$v = 1/2 \times a \times b^2$$

where $a$ and $b$ are the longest and shortest diameters of the tumor mass (in mm), respectively. Calculated tumor volumes were expressed in percentages as relative tumor volume (*RV*s) from the equation:

$$RV = V_n/V_0 \times 100$$

where $V_n$ is the tumor volume at day $n$ and $V_0$ is the initial volume immediately before the first drug treatment. The criterion of antitumor activity was tumor growth inhibition. The T/C value (treated/control) value was calculated as a percentage by use of the following formula:

$$\text{T/C} = (\text{mean } RV \text{ of the treated animals})/(\text{mean } RV \text{ of the control animals}) \times 100$$

### Detection of mRNA by reverse transcription-PCR (RT-PCR)

Expression of *mdr1*, *MRP* and *LRP* mRNA in adriamycin-resistant sublines of HCT-15 was analyzed by competitive RT-PCR using PCR MIMIC, a heterologous internal standard. The PCR MIMICs were 580-bp *Bam*HI–*Eco*RI fragments of v-*erbB* conjugated with gene-specific composite primers of the target gene. The PCR MIMICs for *mdr1*, *MRP* and *LRP* (designated as *mdr1* MIMIC, *MRP* MIMIC and *LRP* MIMIC, respectively) were prepared using the PCR MIMIC Construction kit (Clontech). The primers for *mdr1/MRP/LRP* MIMIC consist of primer residues of the *Bam*HI–*Eco*RI fragment of v-*erbB* conjugated with additional gene-specific primer residues of *mdr1/MRP/LRP* (Table 1).[14–16] The *mdr1/MRP/LRP* MIMIC were amplified for 16 cycles in the presence of the compose primers for *mdr1/MRP/LRP* MIMIC, followed by secondary reactions for 18 cycles in the presence of the target-gene-specific primers (Table 1). The PCR cycle included heat denaturing at 94°C for 45 s, annealing at 60°C for 45 s and polymerization at 72°C for 90 s.

Total RNA was prepared from the tumor cells by using Isogen (Nippon Gene). cDNA was prepared from 4.5 $\mu$g of total RNA using the GeneAmp RNA PCR kit (PE Applied Biosystems). The reaction mixture was incubated at 42°C for 30 min and then heated for 5 min at 99°C to inactive MuLV reverse transcriptase. The cDNA derived from 0.15 $\mu$g of total RNA was mixed with 20 $\mu$l reaction mixture (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.25 mM $MgCl_2$, 188 nM gene-specific primers and 0.625 U AmpliTaq DNA polymerase) with the *mdr1* MIMIC (0.765 amol), *MRP* MIMIC (0.00765 amol) and *LRP* MIMIC (0.00765 amol). PCR reactions consisted of 24 cycles, and included 45 s of heat denaturation at 94°C, annealing at 55°C for 1 min and polymerization at 72°C for 2 min. The PCR products were analyzed by 1.5% agarose-gel electrophoresis and stained by 0.2 $\mu$g/ml ethidium bromide. The products were visualized on an UV transilluminater. Densitometry analysis was performed by the Electrophoresis Documentation and Analysis System (EDAS) and 1D Image Analysis Software (Kodak, Rochester, NY). Relative levels of *mdr1*, *MRP* and *LRP* mRNA for each cells were expressed as a MDR gene target to MIMIC ratio.

### Detection of resistance-associated markers expression by flow cytometry

P-gp, MRP and LRP expression were determined as described previously,[17,18] with minor modifications. For determination of P-gp expression, suspension of parental and resistant sublines {$3 \times 10^6$ cells in staining buffer [Dulbecco's phosphate-buffered saline (PBS) supplemented with 5% FCS and 0.2% sodium azide]} were incubated with 10 $\mu$g/ml of either MRK16 or an isotype-matched mouse IgG2a for 1 h at 4°C. The cells were washed twice with staining buffer and then incubated with 0.175 mg/ml of FITC-labeled goat anti-mouse IgG for 1 h at 4°C. After washing with staining buffer, the cells were passed through a 200-$\mu$m nylon

**Table 1.** Sequence of PCR primers

| cDNA | | Products (bp) |
|---|---|---|
| *mdr1* | 5′-CCCATCATTGCAATAGCAGG-3′ (sense)<br>5′-GTTCAAACTTCTGCTCCTGA-3′ (antisense) | 167 |
| *MRP* | 5′-TGAAGGACTTCGTGTCAGCC-3′ (sense)<br>5′-GTCCATGATGGTGTTGAGCC-3′ (antisense) | 237 |
| *LRP* | 5′-CCTCGAGATCCATTGTGCTGG-3′ (sense)<br>5′-CACAGGGTTGGCCACTGTGCA-3′ (antisense) | 300 |
| *mdr1* MIMIC | 5′-CCCATCATTGCAATAGCAGGCGCAAGTGAAATCTCCTCCG-3′ (sense)<br>5′-GTTCAAACTTCTGCTCCTGATTGAGTCCATGGGGAGCTTT-3′ (antisense) | 620 |
| *MRP* MIMIC | 5′-TGAAGGACTTCGTGTCAGCCCGCAAGTGAAATCTCCTCCG-3′ (sense)<br>5′-GTCCATGATGGTGTTGAGCCTTGAGTCCATGGGGAGCTTT-3′ (antisense) | 620 |
| *LRP* MIMIC | 5′-CCTCGAGATCCATTGTGCTGGCGCAAGTGAAATCTCCTCCG-3′ (sense)<br>5′-CACAGGGTTGGCCACTGTGCATTGAGTCCATGGGGAGCTTT-3′ (antisense) | 620 |

Oligonucleotide sequence of primers used for RT-PCR. *mdr1*, *MRP* and *LRP* gene-specific primers were from Noonan *et al.*,[14] Zaman *et al.*[15] and Schadendorf *et al.*[16]

mesh and resuspended in staining buffer. Fluorescence intensity (excitation wavelength=488 nm) was determined by means of Epics flow cytometer (Coulter Electronics, Hialeah, FL).

To estimate MRP or LRP expression, cells were fixed with formalin (FIX & PERM cell permeabilization kits; Caltag, Burlingame, CA) by gentle mixing for 10 min at room temperature. The cells were then incubated with 10 $\mu$g/ml of MRPr1, LRP-56 and a isotype-matched rat IgG2a (for MRPr1) or mouse IgG2b (for LRP-56) for 1 h at 4°C. After washing, cells were incubated with 0.175 mg/ml of FITC-labeled goat anti-rat IgG (for MRPr1) or FITC-labeled goat anti-mouse IgG (for LRP-56) for 1 h at 4°C. Fluorescence intensity was determined by a flow cytometer.

The specific binding of primary antibody was estimated by subtracting the fluorescence intensity of samples incubated with isotype-matched IgG from the fluorescence of samples incubated with primary antibody. The ratio of the specific fluorescence intensity of the resistant sublines to that of parental cells indicated the relative levels of expression.

### P-gp function assay

P-gp function was analyzed by the rhodamine 123 assay. Cells in growth medium were seeded in 24-well tissue culture plates ($10^6$ cells/well) and incubated for 24 h. The growth medium was removed and replaced by incubation medium (Hanks' balanced salt solution supplemented with 10 mM HEPES buffer and 10% FCS), and then the cells were incubated for 15 min at 37°C. Rhodamine 123 was added to a final concentration of 10 $\mu$g/ml and the cells were further incubated for 30 min. After washes with ice-cold PBS, the cells were harvested with trypsin and suspended in PBS. Intracellular fluorescence intensity (accumulation fluorescence, emission wavelength=530 nm, excitation wavelength=485 nm) was determined with a fluorescence plate reader (CytoFluor 2350; Millipore, Bedford, MA).

To estimate rhodamine 123 retention, the cells were further incubated in rhodamine 123-free incubation medium for 30 min at 37°C. After washing, fluorescence intensity (retention fluorescence) was determined. Cell surface-associated fluorescence was determined after adding ice-cold rhodamine 123 to the cells and then washing immediately. The retention (%) is the percentage ratio of the retained fluorescence divided by the accumulated fluorescence of the cells.

### Statistical analysis

Statistical analysis was performed by repeated measure analysis of variance (ANOVA) (Bonferroni/Dunn test or Bonferroni's multiple comparison test). Correlations were performed by Pearson's correlation coefficient test. These analyses were done by StatView II software (version 4.02 for Macintosh; Abacus Concepts, Berkeley, CA) and GraphPad PRISM software (version 2.0 for Windows; GraphPad, San Diego, CA).

## Results

### Characterization of resistance *in vitro*

The tumor doubling times as parameters of proliferation capacity were unchanged in both parental and resistant sublines. In initial experiments in the present study, the $IC_{50}$ values for adriamycin were determined in parental as well as three adriamycin-resistant sublines. The HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 showed a 2.23-, 4.40- and 7.77-fold increase in $IC_{50}$ values for adriamycin in comparison with the parental

**Table 2.** Resistance profile to adriamycin and relative expression of resistance-associated markers in HCT-15 and HCT-15/ADM cells

| | $IC_{50}$ values (ng/ml), relative resistance and expression of resistance-associated markers | | | |
|---|---|---|---|---|
| | HCT-15 | HCT-15/ADM1 | HCT-15/ADM2 | HCT-15/ADM2-2 |
| Adriamycin | 252 ± [a] (1)[b] | 562 ± 219 (2.23) | 1110 ± 295*** (4.40) | 1960 ± 810*** (7.77) |
| P-gp level | 1 ± 0.06[c] | 1.69 ± 0.24 | 2.33 ± 0.63** | 4.73 ± 0.68*** |
| MRP level | 1 ± 0.10 | 1.13 ± 0.08* | 1.37 ± 0.15 | 1.43 ± 0.16** |
| LRP level | 1 ± 0.06 | 0.825 ± 0.01* | 0.985 ± 0.04 | 0.771 ± 0.02** |

Cell growth was measured by means of the XTT assay, as described in Materials and methods.
[a]The $IC_{50}$ values were defined as the concentration of cells inhibiting growth at 50%.
[b]Relative resistance of adriamycin was determined by dividing $IC_{50}$ values of HCT-15/ADM by that of HCT-15 cells.
[c]The relative expression level of resistance-associated markers in intact or permeabilized cells was determined by flow cytometry using monoclonal antibody MRK16, MRPr1 and LRP-56, respectively. These monoclonal antibodies recognize extracellular (MRK16 and MRPr1) or intracellular (LRP-56) domains of the markers.[17,18] The relative expression level was expressed as the ratio of the specific fluorescence intensity of HCT-15/ADM sublines to those of HCT-15. The values are means and SD of at least three determinations.
Significant difference from $IC_{50}$ values or expression of resistance-associated markers of HCT-15 cells: *$P<0.05$, **$P<0.01$, ***$P<0.001$ [Bonferroni/Dunn (two-tail)].

**Table 3.** Cross-resistance profile to various MDR-related antitumor drugs in HCT-15 and HCT-15/ADM cells

| Drugs | $IC_{50}$ values (ng/ml) and relative resistance | | | |
|---|---|---|---|---|
| | HCT-15 | HCT-15/ADM1 | HCT-15/ADM2 | HCT-15/ADM2-2 |
| Epirubicin | 1980 ± 384[a] (1)[b] | 3130 ± 935 (1.58) | 6600 ± 1490*** (3.34) | 8550 ± 2340*** (4.33) |
| Mitoxantrone | 87 ± 16 (1) | 124 ± 32 (1.42) | 470 ± 54*** (5.40) | 272 ± 133*** (3.12) |
| Vincristine | 192 ± 138 (1) | 589 ± 277 (3.07) | 943 ± 531*** (4.91) | 1040 ± 342*** (5.41) |
| Etoposide | 1740 ± 110 (1) | 3940 ± 544*** (2.27) | 4420 ± 1250*** (2.55) | 5480 ± 1270*** (3.16) |
| Taxol | 326 ± 37 (1) | 916 ± 500** (2.81) | 859 ± 162** (2.63) | 1570 ± 426*** (4.81) |
| Mytomycin C | 3530 ± 1710 (1) | 2840 ± 544 (0.80) | 6230 ± 109** (1.77) | 901 ± 538 (0.26) |

[a]$IC_{50}$ values and [b]relative resistance to MDR-related antitumor drugs were determined.
Significant difference from $IC_{50}$ values of HCT-15 cells: *$P<0.05$, **$P<0.01$, ***$P<0.001$ [Bonferroni/Dunn (two-tail)].

HCT-15 (Table 2). For other MDR-related drugs, the low and intermediate resistant HCT-15/ADM1 and HCT-15/ADM2 showed a moderate elevation of a 1.42- to 5.40-fold increase in the resistance factor to epirubicin, mitoxantrone, vincristine, etoposide and taxol (Table 3). These antitumor drugs were shown to be transported by P-gp or MRP.[5,19] The highly adriamycin-resistant HCT-15/ADM2-2 showed the highest *in vitro* change in drug response, leading to a 3.12- to 5.41-fold increase in $IC_{50}$ values depending on the different MDR-related drugs. These results demonstrated that these three sublines were clearly cross-resistant to MDR-related drugs. However, no significant differences in $IC_{50}$ values between the parental and resistant sublines were obtained for the non-MDR-related drug, mytomycin C.

## Characterization of resistance *in vivo*

Resistance of parental HCT-15 and highly adriamycin-resistant HCT-15/ADM2-2 cells were evaluated *in vivo* by use of established xenografts in athymic mice. The HCT-15 and HCT-15/ADM2-2 xenografts achieved a volume of more than 100 $mm^3$ on day 11 or 7. At that time, mice were treated i.v. once with the 80% of maximum tolerated doses of adriamycin (8 mg/kg body weight) known from a pilot study. Tumor volumes in nude mice transplanted with HCT-15 or HCT-15/ADM2-2 cells were decreased by treatment with adriamycin (Figure 1). The T/C values of single adriamycin treatment in the HCT-15 or HCT-15/ADM2-2 xenografts were 60.0 or 69.0%, as measured 4 weeks after the treatment. Thus, there was no significant difference in the adriamycin resistance in the HCT-15/ADM2-2 as compared to the parental HCT-15 using the nude mice system.

## Co-expression of different resistance-associated markers

Parental HCT-15 cells naturally co-express P-gp, MRP and LRP.[11,20] To examine whether acquisition of adriamycin resistance was altered at the molecular level, *mdr1*, *MRP* and *LRP* mRNA levels in the cells

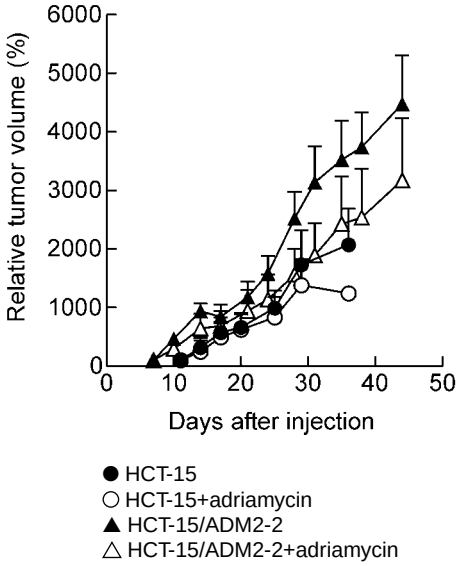


**Figure 1.** Response of HCT-15 or HCT-15/ADM2-2 cells of nude mice to administration of adriamycin. Athymic mice were injected s.c. with $10^7$ HCT-15 or HCT-15/ADM2-2 cells on day 0. Adriamycin (8 mg/kg body weight ) was given i.v. on day 11 (HCT-15 xenografts) or day 7 (HCT-15/ADM2-2 xenografts). Symbols and bars denote the means and SD from 10 nude mice for the control group or five nude mice for the treatment group.

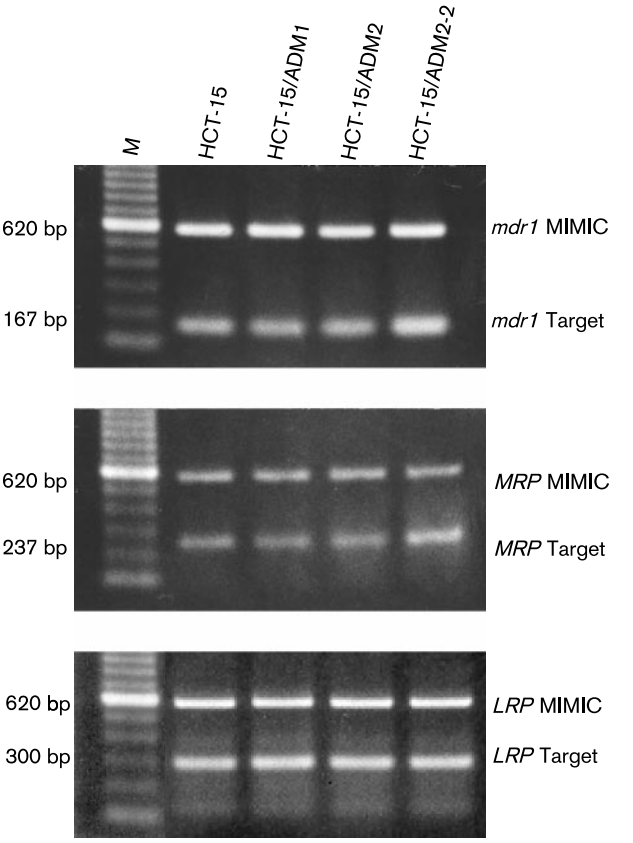


**Figure 2.** Target and MIMIC PCR products of *mdr1*, *MDR* and *LRP*. Total RNA (0.15 $\mu$g) extracted from parental and resistant sublines, and *mdr1* MIMIC (0.765 amol), *MRP* MIMIC and *LRP* MIMIC (0.00765 amol) were used in the PCR reactions. After 24 amplification cycles, the products were resolved on a 1.5% agarose gel and stained with ethidium bromide. Lane M represents a 100-bp DNA ladder as a size marker.

were evaluated by a RT-PCR method using PCR MIMIC as a heterologous internal standard. As demonstrated in Figure 2, *mdr1*, *MRP* and *LRP* mRNAs were detected in parental as well as in all resistant sublines. Whereas the *LRP* expression level was unaffected by adriamycin treatment, *mdr1* and *MRP* mRNA increases were observed in adriamycin-resistant sublines. When analyzing RT-PCR products specific for the MDR gene in comparison with those for the MIMIC, the ratios were 0.751, 0.953, 1.05 and 2.33 (*mdr1*), and 0.410, 0.597, 0.719 and 1.28 (*MRP*) in HCT-15, HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 cells. In addition, concerning the absolute MDR gene expression in the cells, approximately equal amounts of *mdr1* gene and MIMIC were amplified in the presence of 0.765 amol of *mdr1* MIMIC, while equal amounts of gene and MIMIC were amplified in the presence of 0.00765 amol of *MRP* or *LRP* MIMICs (Figure 2). This suggested that the absolute amounts of *MRP* or *LRP* mRNA were significantly very small compared to *mdr1* mRNA.

Flow cytometry analysis was further performed to evaluate the involvement of the MDR-associated genes *mdr1*, *MRP* and *LRP* on the protein level in a quantitative manner in the cells. The analysis showed that parental HCT-15 co-expresses the resistance-associated markers, P-gp, MRP and LRP (Figure 3). Adriamycin induced a significant increase in both P-gp and MRP expression levels in the resistant sublines using the MRK16 antibody ($p < 0.01$ in HCT-15/ADM2; $p < 0.001$ in HCT-15/ADM2-2) and the MRPr1 antibody ($p < 0.01$ in HCT-15/ADM2-2), whereas no significant drug-induced increase in LRP expression was observed using the LRP56 antibody (Table 2). The specific fluorescence intensities were compared with those obtained with the parental cells, and the relative expression of HCT-15, HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 was 1, 1.69, 2.33 and 4.73 for P-gp, and 1, 1.13, 1.37 and 1.43 for MRP (Table 2). These results obtained with monoclonal antibodies using flow cytometry are in agreement with the results of

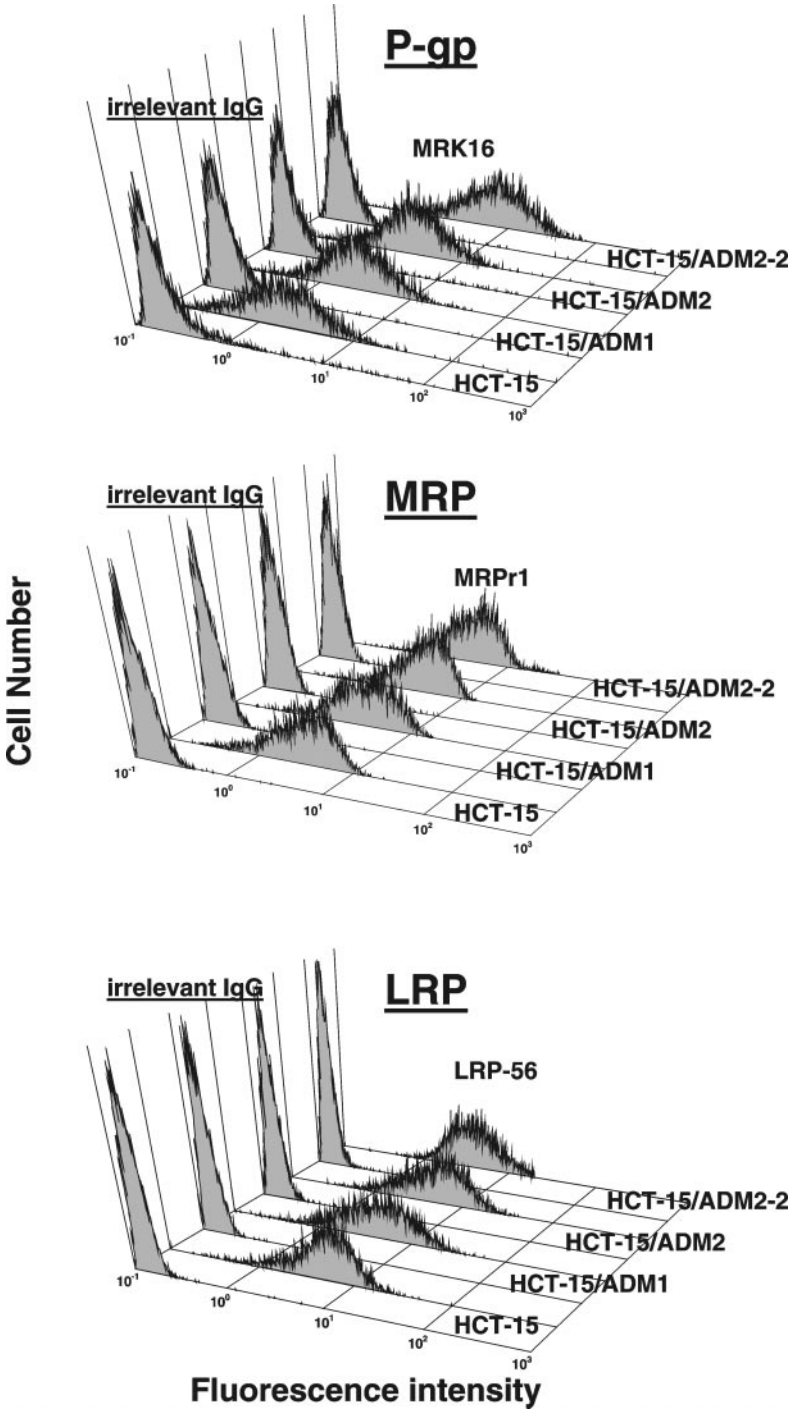


**Figure 3.** Expression of resistance-associated markers on parental and resistant sublines. P-gp, MRP or LRP expression was determined by flow cytometry using monoclonal antibodies MRK16, MRPr1 and LRP-56, respectively. The fluorescence of 5000 cells was measured at an excitation wavelength of 488 nm. The vertical and horizontal axes indicate the relative cell number and fluorescence intensity.

mRNA expression analyses by RT-PCR, indicating that adriamycin induces mainly an increase in the *mdr1* gene and a slight increase in the *MRP* gene. In addition, the relative expressions of P-gp and MRP were well correlated (Figure 4, $r=0.508$, $p<0.0458$), while a relationship between P-gp and LRP had no statistical correlation (Figure 4, $r=-0.651$, $p<0.0404$).

## Correlation of P-gp, MRP and LRP expression with drug sensitivity

To elucidate the contribution of the resistance-associated markers with MDR, we examined the relationship between the expression of the three resistance markers and *in vitro* drug resistance. As shown in Figure 5, statistically significant correlations were observed between P-gp expression and resistance to MDR-related drugs, i.e. adriamycin ($r$=0.819, $p<0.0001$), vincristine ($r$=0.582, $p$=0.0003), mitoxantrone ($r$=0.364, $p$=0.0317), taxol ($r$=0.769, $p<0.0001$), epirubicin ($r$=0.8245, $p<0.0001$) and etoposide ($r$=0.728, $p<0.0001$). A similar positive correlation was shown for MRP expression, i.e. adriamycin ($r$=0.777, $p<0.0001$), vincristine ($r$=0.691, $p<0.0001$), mitoxantrone ($r$=0.727, $p<0.0001$), taxol ($r$=0.687, $p<0.0001$), epirubicin ($r$=0.876, $p<0.0001$) and etoposide ($r$=0.776, $p<0.0001$). These results indicated that changes in P-gp and MRP expression were associated with drug resistance in these sublines. However, no direct correlation was observed between P-gp, MRP and response to mytomycin C (P-gp; $r=-0.501$, $p$=0.0972, MRP; $r=-0.0526$, $p$=0.871), and between LRP and resistance to all MDR-related drugs (data not shown).

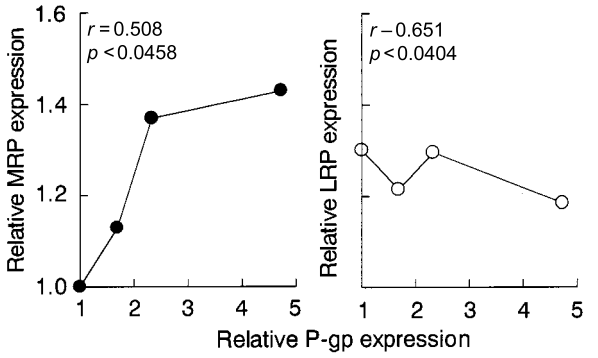


**Figure 4.** Relationship between expression level of resistance-associated markers. The expression levels of P-gp, MRP and LRP measured by flow cytometry were expressed as the ratio of resistant sublines to that of parental cells (see also Table 2). The correlation coefficients between P-gp, MRP and LRP were determined by Pearson's correlation test. The P-gp expression level correlated well with MRP, whereas the no correlation was observed with LRP.

## P-gp function assay

Rhodamine 123 was used as a molecular probe for a functional assay. Since this fluorescent dye is considered to be a relatively specific substrate of P-gp,[21] reduced retention of rhodamine 123 in the cells gives an indication of the function of P-gp. Rhodamine 123 retention was evaluated after accumulation for 30 min and 30 min efflux in dye-free medium. As shown in Figure 6, adriamycin-resistant sublines HCT-15/ADM2 and HCT-15/ADM2-2 significantly reduced retention of rhodamine 123 compared with the parental cells ($p<0.001$ for HCT-15/ADM2; $p<0.001$ for HCT-15/ADM2-2). The rhodamine 123 retention in the adriamycin-resistant sublines correlated with the relative P-gp expression (correlation coefficient; $r=-0.760$, $p<0.001$).

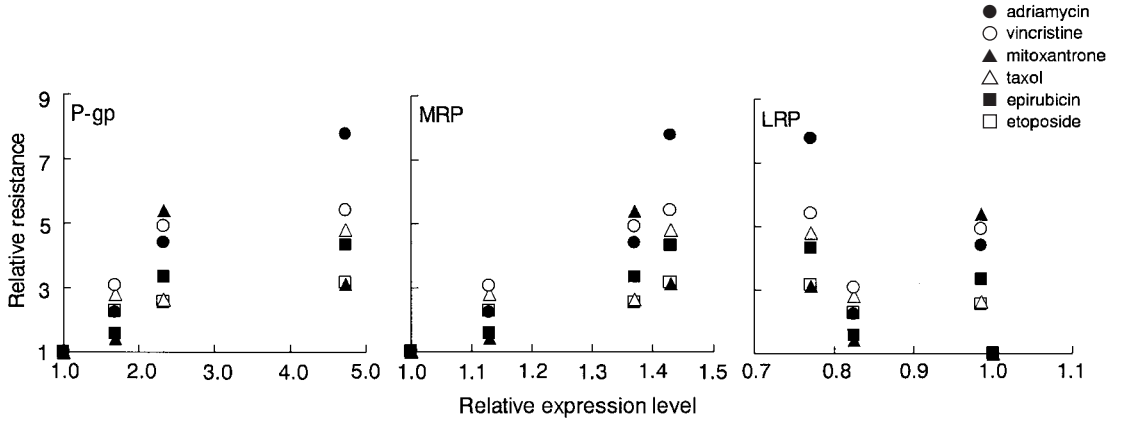


**Figure 5.** Expression of P-gp, MRP and LRP in relation to the relative resistance to MDR-related antitumor drugs. Cell growth of HCT-15 and HCT-15/ADM was measured by means of the XTT assay. The relative resistance was expressed as the ratio of $IC_{50}$ values of resistant sublines to that of parental cells (see also Tables 2 and 3). The relative resistance was plotted against the relative P-gp, MRP and LRP expression levels.

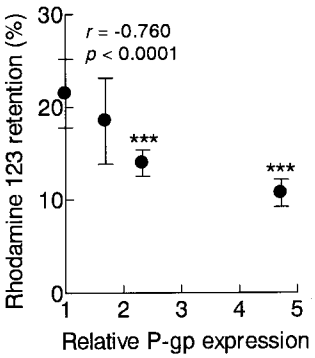


**Figure 6.** P-gp expression-dependent reduction of rhodamine 123 retention (%). Rhodamine 123 retention (%) of parental and resistant sublines was estimated by 30-min accumulation at 10 $\mu$g/ml rhodamine 123. Symbols and bars denote the means and SD from at least four determinations. Significant difference from HCT-15: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by ANOVA [Bonferroni (two-tail)].

It remains unclear whether MRP-mediated transport exists under these conditions, since longer incubation times (more than 60 min) are needed to detect MRP-mediated rhodamine 123 transport.[21]

## Discussion

In this study, we established three adriamycin-resistant sublines from the human colorectal adenocarcinoma HCT-15 by continuous exposure and selection with 25–200 ng/ml adriamycin. These sublines were showing 2.23-, 4.40- and 7.77-fold resistance to adriamycin compared with the parental HCT-15 cells, and cross-resistance to MDR-related drugs, epirubicin, mitoxantrone, vincristine, etoposide and taxol, but not the MDR-unrelated drug, mytomycin C (Tables 2 and 3). The development of resistance did not lead to a remarkable change in proliferation characteristics such as tumor doubling time. They were transplanted to athymic nude mice and the *in vivo* growth rates were roughly similar.

Since some cancer cells in tumors are likely to co-express a variety of genes involved in the development of resistance, we analyzed the expression of different MDR-associated genes on the mRNA as well as the protein level. Although our RT-PCR method was semi-quantitative, the induction by adriamycin of *mdr1* and *MRP* gene expression was observed in the three adriamycin-resistant sublines. A similar situation was apparent for the antibody-labeled protein level. P-gp and MRP expression determined by flow cytometry were 1.5- to 5-fold up-regulated following the adriamycin-selected steps (Figure 3 and Table 2). Since flow cytometry gives quantitative data, the results obtained by RT-PCR on the mRNA level were confirmed on the protein level as well. On the other hand, LRP expression determined by flow cytometry and RT-PCR in our sublines remained unaffected by the developing adriamycin resistance, although LRP over-expression has frequently been observed in adriamycin-selected cells.[11]

Furthermore, we also confirmed in our study that antibody-labeled P-gp and MRP expression were significantly correlated with the resistance of the cells to adriamycin (Figure 4). Thus, P-gp and MRP expression is considered to be induced by developing adriamycin-resistance phenotype. The acquired MDR during chemotherapy can be explained based on the two previous reports that stated MDR-related antitumor drugs such as adriamycin possess the ability to modulate transcription of the *mdr1* gene.[22,23] In addition, several drug-responsive elements have been identified within the *mdr1* gene promoter and the inducibility of reporter genes through the *mdr1* gene promoter gene sequence by classical MDR-related antitumor drugs including adriamycin, vincristine or actinomycin D has been shown.[24] On the other hand, drug inducibility of MRP expression could also have been expected since MRP has mainly been identified in adriamycin (or other anthracycline)-selected cell lines.[25] Although there is data on the *MRP* gene promoter and its transcription regulation,[26] the identification of putative response elements remained to be investigated in adriamycin resistance. In the present study, we do not know whether our adriamycin-resistant sublines of HCT-15 might be developed by a transcriptionally similar regulation of *mdr1* and *MRP* genes. However, it is clear that the induction of these genes by adriamycin resulted in the changes of P-gp and MRP expression.

Moreover, the expression of MRP was slightly increased in the adriamycin-resistant sublines as compared with P-gp expression as measured on an mRNA as well as protein level (Figure 2 and Table 2). In addition, since taxol is not a good substrate transported by MRP,[19,27] it is remarkable that a possible taxol resistance in the sublines is mediated by P-gp. Therefore, our data lead to the conclusion that the adriamycin-induced MDR phenotype in HCT-15 cells may be mainly caused by an increase in the *mdr1* gene and partially caused by an increase in the *MRP* gene.

Since almost all investigations have been performed in *in vitro* systems, it remains unclear whether the resistance phenotypes could be maintained in *in vivo* systems as well. Therefore, we decided to perform the

adriamycin sensitivity studies *in vivo*. The adriamycin-resistant subline HCT-15/ADM2-2 did not change adriamycin resistance *in vivo* (Figure 1). Since HCT-15 are colon carcinoma cells that have intrinsically high resistance as reported in previous studies,[11] the additional resistance to adriamycin might be limited *in vivo* by use of the established xenografts in athymic nude mice. Furthermore, the difference between the sensitivity *in vitro* and *in vivo* is likely explained by the assumption that the contributions of other mechanisms [e.g. via DNA topoisomerase II, ATP-dependent glutathione S-conjugate transporter (GS-X pump)] to the resistance phenotype could exist in our HCT-15 sublines. Such an assumption would support the previous observation that the overexpression of MRP has recently been shown to increase the GS-X pump,[28] and decreased DNA topoisomerase II activity.[29] Therefore, rhodamine 123 efflux and sensitivity data obtained *in vitro* is partially possible in our study. It must be concluded from these examples of acquired resistance, that other mechanisms and resistance markers than those that we have determined probably contribute to the resistance phenotype.

Our established adriamycin-resistant sublines of HCT-15 showed 2.2- to 7.8-fold resistance to adriamycin, and represent mainly a P-gp- and partially MRP-mediated MDR after acquired additional resistance *in vitro*. Since several cell lines selected in the laboratory showed high drug resistance, it may be difficult to extrapolate the results to that observed in the clinic. Thus, detailed characterization as shown in this study will provide clinically relevant tools to investigate how to overcome drug resistance and elucidate possible mechanisms of acquired MDR in human colon cancer.

## References


1. Silverberg E, Lubera JA. Cancer statistics 1988. *CA Cancer J Clin* 1988; **38**: 5–22.
2. Haller DG. Chemotherapy in gastrointestinal malignancies. *Semin Oncol* 1988; **15**(suppl 4): 50–64.
3. Bradley G, Ling V. P-glycoprotein, multidrug resistance and tumor progression. *Cancer Metast Rev* 1994; **13**: 223–33.
4. Tsuruo T. Mechanisms of multidrug resistance and implications for therapy. *Jpn J Cancer Res* 1988; **79**: 285–96.
5. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993; **62**: 385–427.
6. Monks A, Scudiero D, Skehan P, *et al*. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991; **83**: 757–66.
7. Cole SP, Bhardwaj G, Gerlach JH, *et al*. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; **258**: 1650–4.
8. Scheper RJ, Broxterman HJ, Scheffer GL, *et al*. Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res* 1993; **53**: 1475–9.
9. Scheffer GL, Wijngaard PL, Flens MJ, *et al*. The drug resistance-related protein LRP is the human major vault protein. *Nat Med* 1995; **1**: 578–82.
10. Hill BT. Differing patterns of cross-resistance resulting from exposures to specific antitumor drugs or to radiation *in vitro*. *Cytotechnology* 1993; **12**: 265–88.
11. Izquierdo MA, Shoemaker RH, Flens MJ, *et al*. Overlapping phenotypes of multidrug resistance among panels of human cancer-cell lines. *Int J Cancer* 1996; **65**: 230–7.
12. Dexter DL, Barbosa JA, Calabresi P. *N,N*-Dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. *Cancer Res* 1979; **39**: 1020–5.
13. Scudiero DA, Shoemaker RH, Paull KD, *et al*. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 1988; **48**: 4827–33.
14. Noonan KE, Beck C, Holzmayer TA, *et al*. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci USA* 1990; **87**: 7160–4.
15. Zaman GJ, Versantvoort CH, Smit JJ, *et al*. Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. *Cancer Res* 1993; **53**: 1747–50.
16. Schadendorf D, Makki A, Stahr C, *et al*. Membrane transport protein associated with drug resistance expressed in human melanoma. *Am J Pathol* 1995; **147**: 1545–52.
17. Heike Y, Okumura K, Tsuruo T. Augmentation by bispecific $F(ab')_2$ reactive with P-glycoprotein and CD3 of cytotoxicity of human effector cells on P-glycoprotein positive human renal cancer cells. *Jpn J Cancer Res* 1992; **83**: 366–72.
18. Flens MJ, Scheffer GL, van der Valk P, *et al*. Identification of novel drug resistance-associated proteins by a panel of rat monoclonal antibodies. *Int J Cancer* 1997; **73**: 249–57.
19. Zaman GJ, Flens MJ, van Leusden MR, *et al*. The human multidrug resistance-associated protein MRP is a plasma membrane drug efflux-pump. *Proc Natl Acad Sci USA* 1994; **91**: 8822–6.
20. Wu L, Smythe AM, Stinson SF, *et al*. Multidrug-resistant phenotype of disease-oriented panels of human tumor cell lines used for anticancer drug screening. *Cancer Res* 1992; **52**: 3029–34.
21. Feller N, Kuiper CM, Lankelma J, *et al*. Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* 1995; **72**: 543–9.
22. Kohno K, Sato S, Takano H, *et al*. The direct activation of human multidrug resistance gene (*mdr1*) by anticancer drugs. *Biochem Biophys Res Commun* 1989; **165**: 1415–21.
23. Stein U, Walther W, Shoemaker RH. Vincristine induction of mutant and wild-type human multi-drug resistance promoters is cell-type-specific and dose-dependent. *J Cancer Res Clin Oncol* 1996; **122**: 275–82.

24. Uchiumi T, Kohno K, Tanimura H, *et al*. Enhanced expression of the multidrug resistance 1 gene in response to UV light irradiation. *Cell Growth Different* 1993; **4**: 147–57.
25. Loe DW, Deeley RG, Cole SPC. Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer* 1996; **32A**: 945–57.
26. Zhu Q, Center MS. Cloning and sequence analysis of the promoter region of the MRP gene of HL60 cells isolated for resistance to adriablastin. *Cancer Res* 1994; **54**: 4488–92.
27. Parekh H, Simpkins H. Species-specific differences in taxol transport and cytotoxicity against human and rodent tumor cells. Evidence for an alternate transport system. *Biochem Pharmacol* 1996; **51**: 301–11.
28. Muller M, Meijer C, Zaman GJ, *et al*. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci USA* 1994; **91**: 1303–37.
29. Volm M, Kastel M, Mattern J, Efferth T. Expression of resistance factors (P-glycoprotein, glutathione S-transferase-pi, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. *Cancer* 1993; **71**: 3981–7.

*(Received 12 May 2001; accepted 7 August 2001)*