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Cyclosporin A, verapamil and S9788 reverse doxorubicin resistance in a human medullary thyroid carcinoma cell line

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Multidrug resistance was investigated in TT cells, a human medullary thyroid carcinoma (MTC) cell line and in normal thyrocytes. MDR1 mRNA was revealed by polymerase chain reaction (PCR) analysis both in normal and neoplastic cells despite the absence of glycoprotein P (P-gp) by immunohistochemistry using JSB-1 monoclonal antibody. Glutathione-S-transferase mRNA was undetectable by Northern blotting in TT cells. Doxorubicin-induced cytotoxicity was evaluated in TT cells with MTT, lacticodehydrogenase (LDH), glutathione (GSH) assays and neutral red uptake. IC_{50} values obtained for MTT assays were higher than those obtained with the three other tests. Cyclosporin A (CSA) (3 μM), verapamil (10 μM) and S9788 (5 μM) partially reversed the resistance to doxorubicin after a 48 h co-incubation (followed by a 24 h post-incubation for the S9788). Under these conditions, GSH levels were altered by verapamil and S9788, whereas CSA decreased LDH activity. CSA and verapamil had no effect on MTT assay. In conclusion this MTC cell line exhibited over-expression of the MDR1 gene and its resistance to doxorubicin can be partially reversed by CSA, verapamil and S9788.

Keywords: Cyclosporin A, doxorubicin, medullary carcinoma of the thyroid, multidrug resistance, S9788, verapamil.

Introduction

Medullary carcinoma of the thyroid (MTC) is a neoplasm of thyroid C cells that can vary from benign and indolent diseases to highly virulent metastases.[1,2] However, unlike other thyroid cancer types, MTC is insensitive to external irradiation,[3] has a quite low capacity for the uptake of radioactive iodine[4,5] and is refractory to conventional chemotherapy.[3,6] Therefore surgery is the treatment of choice for MTC if an early diagnosis is established and if the tumoral ablation is complete.[7] However, chemotherapy must be considered in patients who relapse or who have metastasis after surgery. Unfortunately, this therapy is often ineffective.[8] Intrinsic (without prior chemotherapy) and acquired (developing during the course of chemotherapy) drug resistance remains the major obstacle to effective chemotherapy.

It has been previously reported that an established human MTC cell line (TT), cloned from a patient who had received no prior chemotherapy, possessed intrinsic multidrug resistance (MDR) to a few antineoplastic agents.[9,10] As doxorubicin has been shown to be the most efficient drug used in chemotherapy of MTC,[11–13] we tested it on TT cells with or without cyclosporin A (CSA), verapamil, S9788, nifedipine and quinidine—known chemoresistance modulators. Moreover, in an attempt to investigate the potential mechanisms responsible for the chemoresistance to doxorubicin in TT cells, we studied the presence of MDR1 and glutathione-S-transferase mRNAs and of glycoprotein P (P-gp) in TT cells as well as the correlations between doxorubicin toxicity and glutathione (GSH) metabolism.

Materials and methods

Chemicals and reagents

Doxorubicin hydrochloride and CSA were purchased from Roger Bellon (Neuilly, France) and from Sandoz (Basel, Switzerland), respectively. Verapamil, nifedipine and quinidine were purchased from Sigma (St Louis, MO). S9788 was obtained from the Institut de Recherches Internationales Servier (IRIS), Courbevoie, France.

S9788 was prepared as a stock solution (50 μg/ml) and stored at 4°C for 2 months or dissolved in water immediately before use.

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All culture media were obtained from Gibco (Grand Island, NY).

Cell culture

The cells tested in this study were the TT cell line of human MTC originally established by Leong *et al.*[14] The TT cell line was a gift from Dr Moukhtar (Inserm U349, Paris, France). As controls we used normal thyroid cells and several tumoral cell lines: KB-3-1 and KB-8 epidermal carcinoma cell lines,[15] and a 200-fold doxorubicin-resistant human breast carcinoma subline (Adr200 MCF7), kindly provided by KH Cowan (National Cancer Institute, Bethesda, MD).[16]

Normal human thyroid tissue was obtained surgically from patients undergoing cold nodule removal. Thyroid cells were isolated as previously described[17] and kept in Ham's F-12 medium containing 10% heat inactivated fetal calf serum (FCS), streptomycin (50 µg/ml) and penicillin (100 UI/ml).

TT and Adr200 MCF7 cell lines were cultured in RPMI 1640 medium, KB-3-1 and KB-8 cell lines in Dulbecco's modified Eagle's medium. The culture media were supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 µg/ml streptomycin and 100 UI/ml penicillin. Doxorubicin (10 µM) was added to the culture medium for Adr200 MCF7 cells. Medium used for KB-8 cells was supplemented with glucose and colchicin as described.[15] Cells were grown in 25 or 175 cm^2 flasks (Beckton Dickinson, Orangeburg, NY) at 37°C in a water-saturated atmosphere of 5% CO_2–95% air. The medium was changed on every third day and trypsinization was performed at confluence. Cells were then seeded either in 96-well culture plates (10^4 cells/well) for metabolic tests or in 25 cm^2 flasks (10^6 cells/flask) for RNA extraction.

The preservation of the endocrine nature of the presently used passages of TT cells was verified by radioimmunological measurements of calcitonin in culture medium and by positive immunohistochemical staining for calcitonin, as previously described for a mixed medullary and follicular thyroid carcinoma cell line[18] (data not shown). For all techniques used, the cell lines were analyzed during the exponential phase of growth.

Drug sensitivity testing

Doxorubicin cytotoxicity in TT cell line was tested with four metabolic tests: MTT assay, neutral red (NR) uptake, GSH and lacticodehydrogenase (LDH) measurements.

TT cells (10^4 cells/well) were cultured in monolayers in 96-well plates. After 24 h in culture, different concentrations of doxorubicin were added at the following final concentrations: 0.010, 0.100, 0.500, 1, 5, 10 and 100 µM. The plates were then incubated for 6, 16 or 48 h at 37°C in a 5% CO_2–95% air–water-saturated atmosphere with or without modulators (3 µM CSA, 10 µM verapamil, 35 µM nifedipine, 20 µM quinidine, or 1, 2 or 5 µM S9788). In other experiments cells were exposed for periods of 48 h to doxorubicin with or without reversal products followed by a 24 h post-incubation with reversal agents. Drug-free controls were also included in each experiment.

Thyroid cell damage was then evaluated with the following tests:

MTT assay. Cytotoxicity was evaluated by the following modification of the original MTT assay initially described by Mosmann:[19] 100 µl MTT was added and the plates were incubated for 2 h at 37°C. The medium was then removed and the formazan crystals were solubilized by 100 µl dimethylsulfoxide. The OD was measured at 540 nm.

NR uptake. The incorporation of NR into the lysosomes of viable cells was assessed according to the method described by Borenfreund and Puerner.[20]

LDH measurement. Cell monolayers were harvested with 200 µl 0.2% (v/v) Triton X-100. The LDH activity was immediately determined as previously described.[21]

GSH assay. Triton X-100 (200 µl 0.2%, v/v) was added to each well and the total reduced and oxidized GSH measurement was performed according to the method previously described.[22] The OD was read at 414 nm.

For the study of GSH metabolism, the total GSH concentration was determined by comparison with a standard curve and expressed as nmol GSH/10^4 cells.

In these four metabolic tests, OD was measured in a microtiter plate reader (MCC Multiscan 240, Labsystem). Results were calculated as percent control = average OD of wells/average OD of control wells.

Four replicate wells were analyzed for each test and each assay was repeated at least twice. The drug concentration required to inhibit metabolic functions of cells by 50% (IC_{50}) was determined with or without reversal products.

The activity of the reversal modulators was quantified by the reversal factor: RF = IC_{50} (cytotoxic

agent only)/IC_{50} (cytotoxic agent + reversal modulator). Any RF value $\geqslant 2$ was considered as positive.

Fixation and immunochemistry

Immunohistochemical staining was performed on TT cells and on normal culture thyrocytes using KB-3-1 and Adr200 MCF7 cell lines as negative and positive controls, respectively. The different cell lines were briefly washed with phosphate buffer, pH 7.4, for 1 min, then fixed in absolute ethanol for 10 min at room temperature. Endogenous peroxidase activity was then blocked by a 5 min treatment with 3% hydrogen peroxide in absolute methanol. After incubation with 10% normal goat serum, a monoclonal anti-Pgp antibody (mAb), JSB-1 (Tebu Laboratories, Le Perray en Yvelines, France), used at the working dilution of 1:30, was applied for 1 h at room temperature. Revelation was performed with an avidin–biotin peroxidase technique using the LSAB kit purchased from Dako (Trappes, France) (Universal LSAB kit/HRP). Negative (for cell lines) or positive (for normal thyrocytes) controls also included substitution of the primary antibody by an anti-thyroglobulin mAb (Dako).

Isolation of RNA

RNA was extracted from normal human tissues or from TT cells with RNA^{zol} (Bioprobe System, Montrevil sous Bois, France) according to the method described by Chomczynski and Sacchi[23] and was quantified by absorbance at 260 nm. The intactness of the RNA was confirmed by agarose formaldehyde gel electrophoresis.

Northern blot analysis for GSTπ mRNA detection

Total RNAs (10 µg) extracted from TT cells and from Adr200 MCF7 cells (as positive control) were subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto a nylon membrane (Hybond N^+; Amersham, les Ulis, France). RNA amounts in each lane and transfer efficiency were verified by staining the gel with ethidium bromide. The sheets were prehybridized at 42°C for 2 h and then hybridized with specific ^{32}P-labeled probes in a hybridization solution containing 50% formaldehyde for 24 h at 42°C. GSTπ and β-actin (as internal standard) were analyzed with the rat cDNA GSTπ probe (GSTP1-1) obtained from the American Type Culture Collection (Rockville, MD) and with a cDNA actin probe purchased from Clontech (Ozyme Society, Montigny le Bretonneux, France), respectively. After hybridization, the sheets were washed initially twice with 2 × standard saline citrate (SSC) for 5 min at room temperature, followed by two washes in 2 × SSC, 1% SDS for 30 min at 55°C and finally washed twice in 0.1 × SSC for 30 min at room temperature. The sheets were then dried and autoradiographed at −80°C.

Complementary cDNA PCR analysis of MDR1 mRNA

Synthesis of first strand cDNA. Total RNA (1 µg) of TT cells, normal thyrocytes, KB-3-1 cells and KB-8 cells (chosen as negative and positive control, respectively) were mixed, in a total volume of 20 µl, with a solution containing 50 pmol poly(dT) primer, 1 mM of each of the four deoxynucleotides, 10 units of RNAse inhibitor (Promega, Paris, France), 50 mM KCl, 20 mM Tris–HCl (pH:8.4), 2.5 mM $MgCl_2$ and 200 units of MMLV reverse transcriptase (BRL, Cergy Pontois, France). The reaction mixture was incubated at 23°C for 10 min then at 42°C for 45 min and subsequently stopped by heating to 95°C for 7 min 30.

PCR. We used β-actin as the internal control. Amounts of cDNA preparations providing the same yield of the β-actin PCR product were determinated by these assays and were then used for MDR1-specific PCR.

PCR buffer (80 µl; Promega) containing 50 pmol each of upstream- and downstream-specific primers and 2 units of Taq DNA polymerase (Promega) were added to 20 µl reverse transcriptase reaction mixture. Primers used for amplification of MDR1- or β-actin-specific sequences were prepared according to Noonan *et al.*[24] or Funk *et al.*,[25] respectively. Amplification was performed on a programmable heater (Braun Sciencetec, les Ulis, France) for 25 cycles. The reaction began with a denaturation step at 94°C for 30 s, followed by annealing at 55°C for 1 min and elongation at 72°C for 2 min (5 min for the last cycle).

Analysis of amplified cDNA products. Amplified cDNA products were analyzed on agarose gel (1%) and were visualized by two methods.

(i) The gel containing ethidium bromide (0.5 µg/

ml) was photographed under UV light at 302 nm using positive/negative instant pack film.

(ii) Southern blot: the amplified products on the agarose gel were transferred to a Hybond N^+ membrane (Amersham). The blots were prehybridized at 42°C for 2 h and then hybridized for 24 h at 42°C with specific probes in a hybridization solution containing 50% formamide. MDR1 mRNAs were detected with a 3 kB cDNA *Eco*RI fragment corresponding to the 3′ end of MDR1 cDNA. This probe, termed pAdr1, was cloned into the *Eco*RI restriction site of the plasmid pGEM3.[26] It was a gift from KH Cowan. β-actin mRNAs were revealed with a cDNA-specific probe purchased from Clontech. After hybridization the membrane was washed, dried and autoradiographed as described above for Northern blotting.

Statistical analysis

IC_{50}, RF and GSH values obtained in metabolic studies were analyzed by the Mann–Whitney test with a 5% limit for statistical significance. Relationships of GSH levels with TT cell sensitivity to doxorubicin and with reversion factors were determined by Spearman correlation analysis.

Results

Determination of MDR1 and GSTπ mRNAs

cDNA PCR analysis showed the presence of MDR1 mRNA both in normal human thyroid cells and in TT cell line when compared with KB-3-1 and KB-8 cells as negative and positive controls, respectively (Figure 1). The expression of MDR1 mRNA was higher in TT cells than it was in normal thyrocytes. No expression of GSTπ mRNA was detected in the TT cell line, whereas Adr200 MCF7 clearly demonstrated a GSTπ mRNA band (Figure 2).

Immunohistochemical staining for P-gp

Adr200 MCF7 cells showed a strongly positive staining, whereas the TT cell line, normal thyrocytes and KB-3-1 cells remained negative (Figure 3a–d).

Evaluation of cytotoxic effects of doxorubicin on the TT cells

The cytotoxic effects of doxorubicin added at final concentrations of 10 nM to 10 μM on the TT cells were not observed after 6 or 16 h incubation periods (data not shown). On the contrary, a dose-dependent cytotoxic effect of the drug was found with the four metabolic tests after a 48 h incubation or after a 48 h incubation followed by a 24 h post-incubation without doxorubicin (Figure 4). These drug incubation times were then selected from comparative chemosensitivity studies.

IC_{50} values for 48 h exposures to doxorubicin with and without a 24 h post-incubation are reported in Table 1. Similar IC_{50} values were obtained with NR uptake, GSH assay and LDH measurement

Figure 1. Autoradiogram of MDR1- and β-actin-specific PCR products from TT cell line (lane 1) and normal human thyrocytes (lanes 2–5), and from KB-8 (lane 6) and KB-3–1 (lane 7) cell lines. Time exposure of autoradiograms was 1 and 22 h for β-actin and MDR1, respectively. Arrows indicate the specific product bands for β-actin (463 bp) and MDR1 (167 bp) sequences.

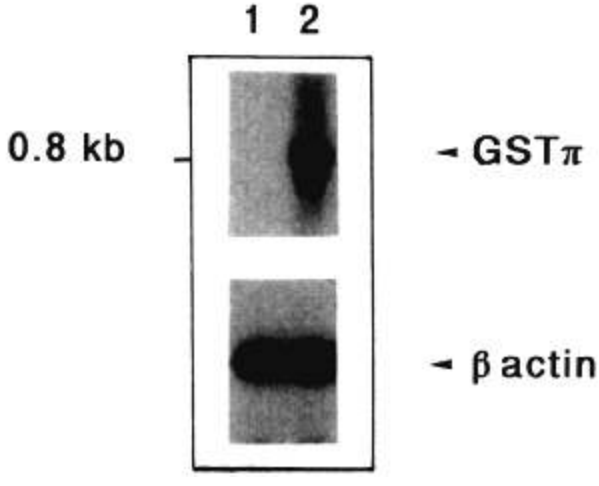

Figure 2. Northern analysis of mRNA from TT (1) and Adr200 MCF7 (2) cells with a GSTπ-1 cDNA probe. Each blot was rehybridized to a β-actin cDNA probe. Time exposure of autoradiograms was 22 and 4 h for GSTπ and β-actin, respectively.

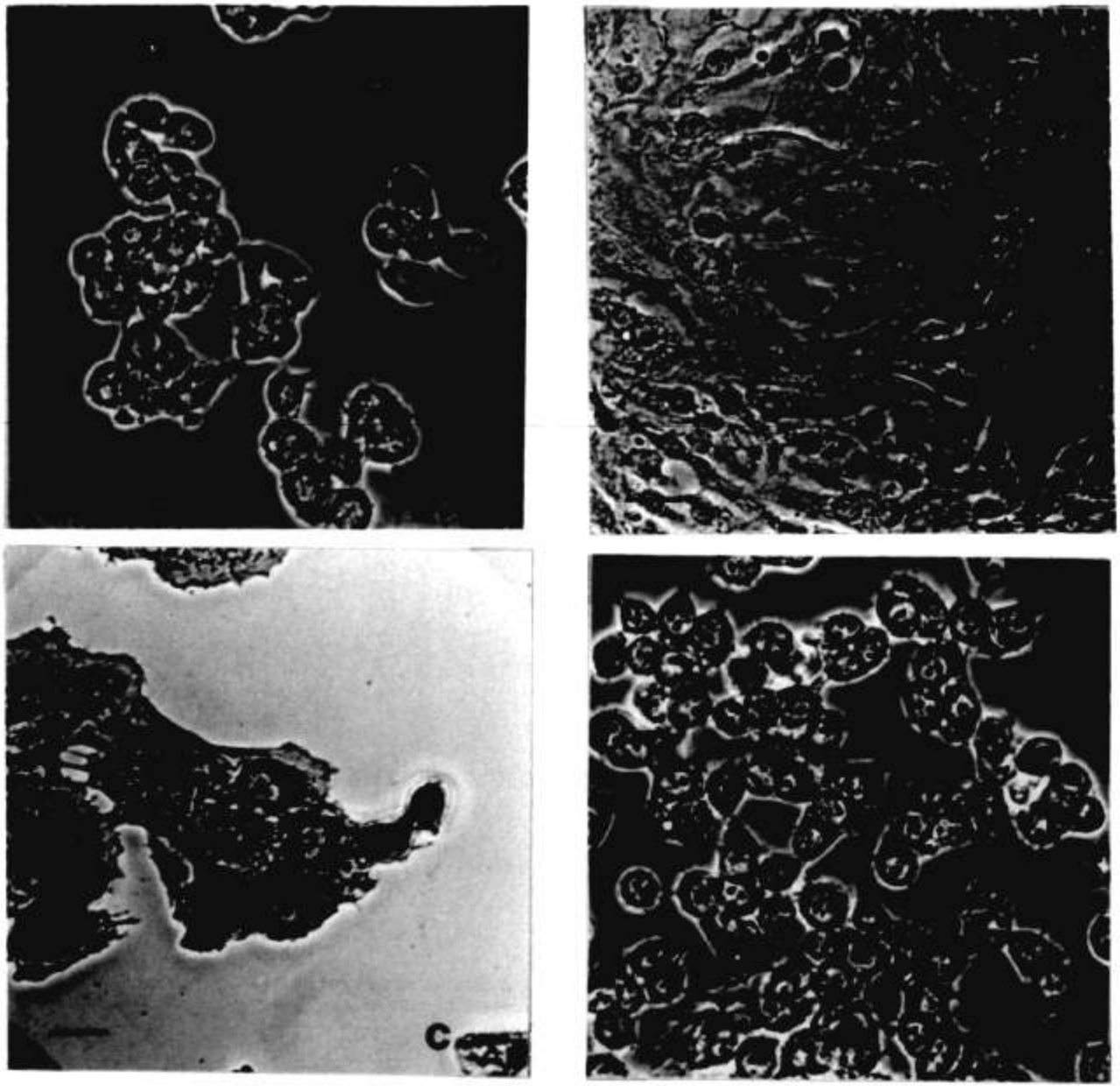

Figure 3. Immunocytochemical localization of P-gp in TT cell line (a) and in normal thyrocytes (b). Adr200 MCF7 (c) and KB-3-1 (d) cell lines were chosen as positive or negative control, respectively. Bar = 25 μm.

for the two incubations, whereas higher values were found for the MTT test: the MTT assay gave an IC_{50} approximately 2.3–2.6 times higher than the other three tests for 48 h co-incubation and 3.6–4.6 times higher for 48 h incubation with a 24 h post-incubation.

No significant difference was found between IC_{50} values obtained for 48 h or 48 + 24 h incubation periods as measured with the MTT assay and RN uptake, whereas a significant decrease was observed for GSH and LDH IC_{50} values ($p = 0.0196$, $Z = -2.333$ and $p = 0.0477$, $Z = -1.98$, respectively).

Evaluation of the cytotoxic effects of the reversal modulators

The cytotoxicity of each modulator is given in Table 2. The results clearly indicate that none of the modulators used was cytotoxic at the concentrations used for the MTT assay and NR uptake as they re-

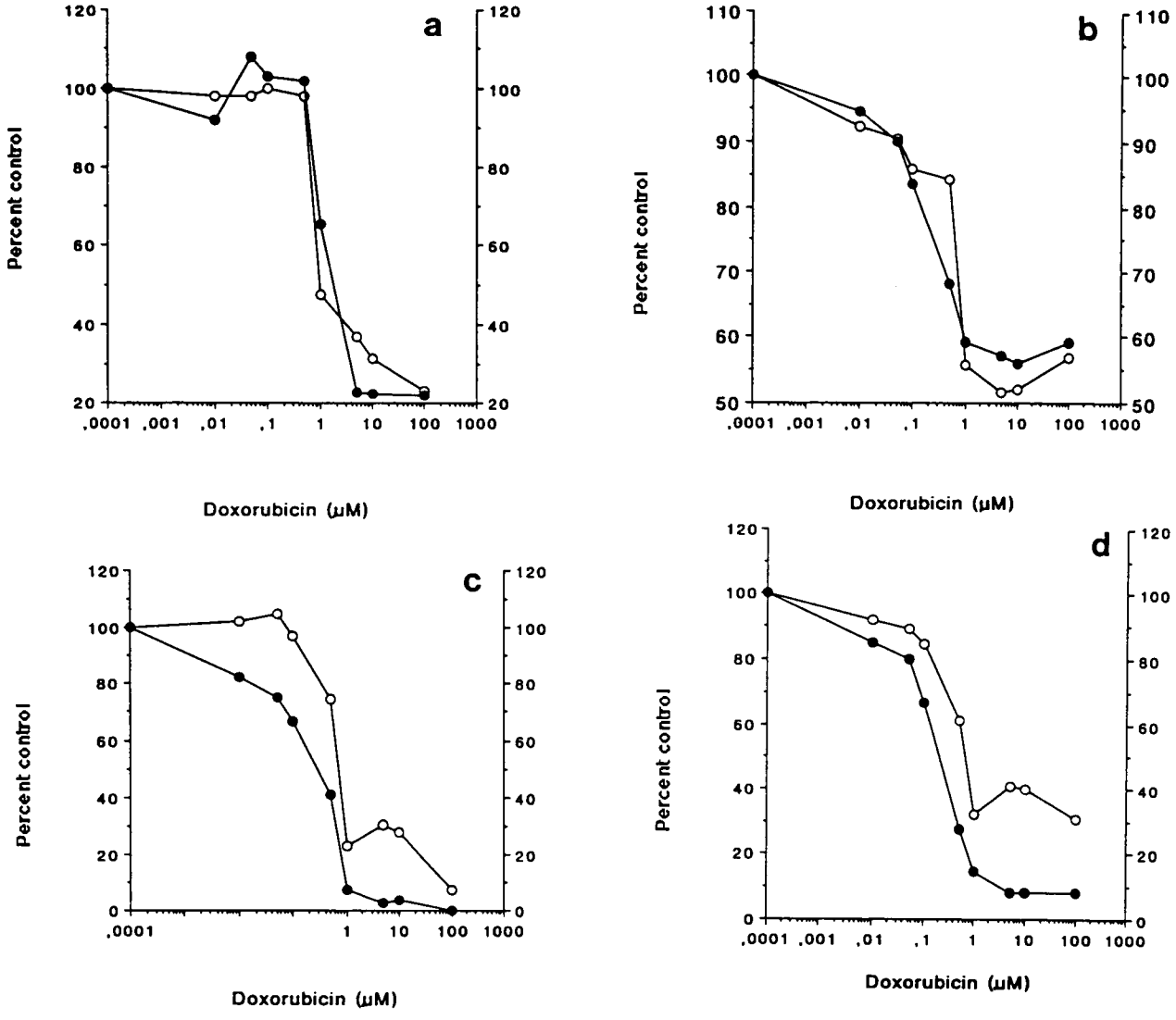

Figure 4. Doxorubicin-induced cytotoxicity on TT cells for a 48 h incubation (○) or for a 48 h incubation followed with a 24 h post-incubation (●) and measured by MTT assay (a), NR uptake (b), GSH (c) and LDH (d) assays. The results are expressed as percent control and represent one typical experiment.

duced metabolic functions by less than 10% of control values.

On the contrary, 10 μM verapamil, 35 μM nifedipine and 5 μM S9788 induced perturbations in GSH metabolism in the 48 h incubation period. In the 48 h co-incubation followed by a 24 h post-incubation, 1 and 2 μM S9788 also altered GSH metabolism. The decrease in GSH levels induced by verapamil, nifedipine and S9788 are reported in bold type in Table 3. Therefore, in the experiments using these reversal modulators, GSH measurement may be considered with care. CSA modified the LDH assay since percent control values were below 90%. Thus, for experiments concerning CSA, the results of this test may be subject to caution.

Relation between GSH levels and doxorubicin toxicity

No significant correlation was observed between GSH levels and any IC_{50} obtained with the four metabolic tests.

Table 1. Comparison of GSH levels and IC_{50} values obtained with four metabolic tests performed on TT cells exposed to doxorubicin for 48 or 48 + 24 h post-incubation

Incubation (h)	GSH levels (nmol/10^4 cells)	IC_{50} (nM)			
		MTT	NR	GSH	LDH
48	30 ± 4.5 (10)	1434 ± 399 (7)	595 ± 156 (9)	621 ± 71 (10)	550 ± 174 (10)
48 + 24	31.8 ± 2.6 (4)	1520 ± 420 (4)	375 ± 188 (4)	427 ± 116 (4)	327 ± 187 (4)

The results are expressed as the means ± SD of several experiments, the number of which is noted in brackets.

Table 2. Cytotoxic effects of reversal products on TT cells

Incubation period (h)	Reversal modulator	MTT	NR	GSH	LDH
48	CSA 3 μM	94.1 ± 5.7	104 ± 3.8	110 ± 3.2	**79.4 ± 5**
	verapamil 10 μM	96.2 ± 6.9	105 ± 2.5	**70.2 ± 4.4**	101 ± 4.2
	nifedipine 35 μM	95 ± 8	94.6 ± 8	**58.5 ± 4**	96.6 ± 5
	quinidine 20 μM	101 ± 7.8	102 ± 1.5	109 ± 4	92.7 ± 8
	S9788 1 μM	90.7 ± 0.1	96.6 ± 1.6	90.8 ± 5.9	95.6 ± 2.8
	2 μM	90.2 ± 2	90.7 ± 2.5	90 ± 3	95.3 ± 4.2
	5 μM	92.5 ± 2.7	100 ± 9.7	**68.9 ± 0.3**	92.4 ± 2 .5
48 + 24	CSA 3 μM	92.7 ± 3	106 ± 8.1	108 ± 5.2	**69.1 ± 0.9**
	verapamil 10 μM	96.8 ± 4.3	101 ± 7.9	**71 ± 1**	96.4 ± 2.8
	S9788 1 μM	91 ± 3.2	102 ± 1.7	**81.7 ± 2.4**	90.9 ± 1.8
	2 μM	104 ± 4.1	95.4 ± 7.2	**80.4 ± 3.2**	90.3 ± 4.5
	5 μM	107 ± 14.3	112 ± 15.6	**72.4 ± 15.9**	90.2 ± 1.8

Results are expressed as the ratio of OD with reversal product/OD without reversal product and are the mean ± SD of two to four experiments. Ratios <90% are in bold type

Table 3. Effect of reversal modulators on GSH levels

Reversal modulator	48 h incubation		48 + 24 h post-incubation	
	GSH levels	ratio	GSH levels	ratio
CSA 3 μM	29.3 ± 0.5	0.97	31 ± 3.4	0.97
Verapamil 10 μM	**19.8 ± 1.55**	**0.66**	**20.5 ± 2.8**	**0.64**
Nifedipine 35 μM	**17.8 ± 2.1**	**0.59**		
Quinidine 20 μM	35.4 ± 3.2	1.18		
S9788 1 μM	29.3 ± 3.7	0.98	**26.7 ± 3.1**	**0.84**
2 μM	28.6 ± 2.9	0.95	**24.5 ± 2.8**	**0.77**
5 μM	**20.7 ± 3.4**	**0.69**	**23.3 ± 7.8**	**0.73**

The GSH results are expressed as the means ± SD of two to four experiments. The ratios are GSH levels with modulator/GSH levels without modulator. The ratios <0.90 are reported in bold type.

Cytotoxic effects of doxorubicin with resistance modulators on the TT cell line (Tables 4 and 5)

After a 48 h incubation period, 3 μM CSA and 10 μM verapamil had significant effects on the cytotoxicity of doxorubicin in TT cells with positive RFs of 2.33–4 as evaluated by NR uptake, GSH and LDH assays. With these three tests, RF values obtained for CSA were higher than those obtained for verapamil. Thus CSA enhanced the action of doxorubicin more than verapamil. On the contrary, MTT assay gave negative RF values in both types of incubation. We found no significant difference between RF values for CSA and verapamil with or without post-incubation.

Nifedipine (35 μM) and quinidine (20 μM) had no significant effect on the doxorubicin-induced toxicity since the four metabolic tests were negative.

When the cells were incubated with doxorubicin for 48 h or for 48 + 24 h post-incubation, S9788 (administered at 1 and 2 μM) had no significant effect on toxicity since RFs of 0.91–2 were found with the different tests. With 5 μM of S9788, RFs obtained with the four tests for 48 h incubation were negative. They became positive when the co-incubation with doxorubicin was followed by a 24 h post-incubation with the reversal product. RFs found with NR uptake and GSH assay were similar to those obtained for verapamil but lower than those obtained for CSA.

Discussion

To our knowledge, only few and partial reports have been published on MDR in MTC whereas none have concerned cultured normal thyrocytes. Certain authors have cytochemically studied P-gp expression in TT cells,[10] others the presence of MDR1 mRNA by Northern blotting.[27] In our present work, we evaluated the expression of both the MDR1 gene and P-gp in the TT cell line and in normal human thyroid tissues.

Our immunohistochemical results showed the absence of P-gp in the thyroid cells (normal or neoplastic). The lack of P-gp with JSB1 mAb has already been reported in TT cells[10] and in human normal thyroid sections,[28,29] but never in cultured normal thyrocytes.

This study demonstrated the presence of MDR1 mRNA both in normal and TT cells by PCR. Overexpression of MDR1 mRNA detected by Northern blotting has already been shown in TT cells.[27] On the contrary, to our knowledge, the presence of non-negligible amounts of MDR1 mRNA in normal thyroid cells has never been reported. These results contrasted with the absence of P-gp. We cannot exclude that normal and TT cells contain very low concentrations of P-gp that cannot be detected by a cytochemical method. On the contrary, the cDNA PCR, a very sensitive technique, allowed the detection of low concentrations of MDR1 mRNA. The absence of P-gp may also result from the absence of translation of the MDR gene.

In many cancer cells, resistance to doxorubicin has been associated with P-gp over-expression, although different mechanisms such as GSH-mediated detoxification have also been reported. Our findings suggest that GST metabolism does not play a major role in TT cell resistance to doxorubicin since GSTπ mRNA was not detected. Moreover, we did not observe a positive correlation between GSH levels and doxorubicin-induced cytotoxicity. This is in agreement with some studies reporting that alterations in GSH metabolism were not principally involved in chemoresistance in renal cell carcinoma cell lines[30] and in small cell lung cancer.[31]

In this study, we used four metabolic tests to study doxorubicin-induced cytotoxicity. In the previous works on TT cells either a fluorimetric assay[10] or a method based on the inhibition of colonies formation[9] were employed for the evaluation of doxorubicin toxicity. In our study we used MTT, LDH and GSH assays and NR uptake for this assessment. The MTT assay has been used in several studies for the evaluation of chemotherapeutic drug toxicity on colon adenocarcinoma cell line LoVo,[32] on human leukemic cell line CCRF-CEM,[32,33] on murine L1210 leukemia cells,[34] on neuroblastoma cell lines[35] and on human cell lung cancer cell lines.[36] Moreover, it has been previously described that NR uptake, and GSH and LDH assays are common tests performed to assess drug cytotoxicity,[21,37–39] particularly intracellular LDH for evaluating doxorubicin-induced cytotoxicity.[40,41] Our results showed similar IC_{50} values with NR uptake, and GSH and LDH assays. Yang *et al.*[27] tested drug cytotoxicity in TT cells with a 48 h growth inhibition assay and they found a similar IC_{50} value (870 ± 42 nM). However the IC_{50} value that we obtained with the MTT assay was slightly higher than those obtained with NR, GSH and LDH assays. We are in agreement with Marks *et al.*[33] who observed that IC_{50} concentrations obtained for several cell lines were higher by MTT assay than [^{3}H]leucine incorporation and Trypan blue exclusion.

Table 4. Modulation of doxorubicin cytotoxicity on TT cells by co-incubation with different reversal products for 48 h

Reversal modulator	MTT		NR		GSH		LDH	
	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
CSA 3 μM	760 ± 5	1.61 ± 0.50	203 ± 38	**3.21 ± 0.39**	173 ± 6	**4 ± 0.12**	(213 ± 102)	**(3.65 ± 0.92)**
Verapamil 10 μM	1275 ± 601	1.06 ± 0.08	240 ± 30	**2.33 ± 0.49**	(230 ± 30)	**(2.90 ± 0.39)**	200 ± 14	**3.01 ± 0.21**
Nifedipine 35 μM	1300 ± 707	1.32 ± 0.09	625 ± 21	1.16 ± 0.31	(800 ± 57)	(0.77 ± 0.06)	680 ± 70	0.88 ± 0.19
Quinidine 20 μM	1230 ± 523	0.94 ± 0.11	530 ± 14	1.36 ± 0.35	600 ± 71	1.03 ± 0.07	350 ± 140	1.13 ± 0.09
S9788 1 μM	1490 ± 721	1.31 ± 0.45	375 ± 177	1.14 ± 0.08	(475 ± 205)	(0.91 ± 0.02)	325 ± 64	1.21 ± 0.20
2 μM	1800 ± 715	1.11 ± 0.2	400 ± 102	1.35 ± 0.3	(600 ± 74)	(0.97 ± 0.09)	250 ± 17	1.20 ± 0.4
5 μM	995 ± 555	1.85 ± 0.86	435 ± 262	1.43 ± 0.80	(550 ± 71)	(1.14 ± 0.24)	525 ± 289	1.18 ± 0.12

Results are expressed as the mean ± SD of two to four experiments. Positive RFs are in bold type. Results in brackets must be interpreted with caution.

Table 5. Modulation of doxorubicin cytotoxicity on TT cells by co-incubation with different reversal products for 48 h followed with a 24 h post-incubation period

Resistance modulator	MTT		NR		GSH		LDH	
	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
CSA 3 μM	620 ± 155	1.58 ± 0.15	105 ± 55	**5.7 ± 0.95**	170 ± 82	**3.41 ± 0.4**	(230 ± 57)	**(2.61 ± 0.32)**
Verapamil 10 μM	760 ± 156	1.29 ± 0.18	180 ± 62	**3.67 ± 0.33**	(190 ± 81)	**(3.05 ± 0.4)**	270 ± 120	**2.22 ± 0.28**
S9788 1 μM	960 ± 28	1.67 ± 0.35	150 ± 39	1.07 ± 0.03	(230 ± 56)	(1.87 ± 0.4)	160 ± 45	1.31 ± 0.06
2 μM	1000 ± 115	2 ± 0.45	250 ± 23	1.20 ± 0.32	(180 ± 36)	(1.67 ± 0.09)	210 ± 32	1.43 ± 0.4
5 μM	430 ± 42	2.75 ± 0.71	225 ± 78	**2.36 ± 0.32**	(185 ± 7)	**(2.63 ± 0.59)**	280 ± 82	**2.14 ± 0.23**

Results are expressed as the mean ± SD of two to four experiments. Positive RFs are in bold type. Results in brackets must be interpreted with caution.

We found that 3 μM CSA, 10 μM verapamil and 1, 2 or 5 μM S9788 altered LDH or GSH levels in TT cells. This is consistent with a few studies showing that 10 μM verapamil was toxic in human leukemic lymphoblasts[42] and decreased total GSH in human colorectal cell lines.[43] Moreover, Hill *et al.*[37] found that verapamil and S9788 exerted considerable toxicity at high concentrations on lymphoblastoid T cell leukemia or ovarian carcinoma cell lines.

We did not obtain a MDR reversion with nifedipine and quinidine in TT cells. These results paralleled those of Wigler *et al.*,[44] who considered quinidine as a weak inhibitor of the MDR pump. They are also in agreement with those of Ramu *et al.*,[45] who have shown that nifedipine had no effect in potentiating doxorubicin in leukemia cell lines.

Our results also showed that 3 μM CSA, 10 μM verapamil and 5 μM S9788 partially reversed resistance to doxorubicin in the TT cell line. Verapamil, a calcium channel blocker, is known to reverse resistance to chemotherapeutic drugs, especially to doxorubicin in TT cells.[27] However, to our knowledge, neither CSA nor S9788 has been tested in the reversion of doxorubicin toxicity in MTC cells. It has been shown that CSA enhanced the cytotoxic effect of vincristine, daunorubicin and VP-16 in TT cells,[10] and of doxorubicin in Chinese hamster ovary cells[46] or in resistant leukemic cells.[47] Our findings showed that CSA potentiated the action of doxorubicin more than verapamil did. Moreover, we found that CSA and verapamil had no effect on the MTT assay, and thus are in agreement with Marks *et al.*[33] who obtained the same negative result with verapamil in human leukemic cell lines. Therefore, the MTT assay must be used with care for the evaluation of resistance modulators. On the contrary, NR uptake, and GSH and LDH measurements are very rapid and simple techniques that might be commonly used in clinical drug sensitivity testing of human tumours.

We also tested several concentrations of S9788, a new triazinoaminopiperidine derivative,[48,49] on TT cells. Our results showed that 5 μM S9788 reversed the resistance in TT cells only after a 24 h post-incubation following the 48 h co-incubation, and with RFs lower than CSA and equivalent to those obtained with verapamil. Hill *et al.*[35] also reported that S9788 could induce a resistance modulation comparable to that obtained with verapamil in several other cell lines. However, CSA and verapamil are cytotoxic to normal and tumoral tissues at doses that are needed to reverse MDR.[42,50] Therefore S9788, a less toxic chemosensitizing agent than verapamil and CSA, may be clinically tested in MTC. It remains to be established whether plasma concentrations of S9788 will be sufficiently high to permit efficient modulation without increasing toxic side-effects in normal tissues.

Conclusion

The TT cell line represents an interesting cell model of thyroid drug resistance to doxorubicin. We have shown that 3 μM CSA, 10 μM verapamil and 5 μM S9788 partially reversed the doxorubicin-induced resistance. Work is underway in our laboratory to investigate the action of these resistance modulators *in vivo* in nude mice and *in vitro* on five other cell lines isolated from patients with MTC.

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