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Effect of S9788 on the efficiency of doxorubicin *in vivo* and *in vitro* in medullary thyroid carcinoma xenograft

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In medullary carcinoma of the thyroid (MTC), drug resistance remains the major obstacle to effective chemotherapy. In this work, we studied the effect of S9788 on doxorubicin (DOX) efficiency in a MTC cell line (TT cells) injected in nude mice. After two passages, TT cells were injected in 40 nude mice divided into four groups [controls and groups receiving DOX alone (10 mg/kg), S9788 alone (50 mg/kg) or both DOX + S9788]. The weight of the mice, tumoral volume (TV), doubling time (DT) of the tumor and survival time of mice were evaluated in each group. In addition, the efficiency of DOX with or without S9788 was assessed by the inhibition of tumoral growth and specific growth delay. *In vitro*, glycoprotein P 170 (P-gp) was detected on tissular sections and on tumoral cells by immunocytochemistry or flow cytometry with several monoclonal antibodies: JSB1, MRK 16, C219 and UIC2. *In vivo* the weight of the mice decreased slightly with DOX and dropped dramatically with DOX + S9788. The DT of the tumors increased with DOX over controls (22.5 ± 8.5/12.7 ± 3.9 days) and showed a higher value with DOX + S9788 (29.2 ± 11.4 days). Inhibition of tumoral growth, 89% with DOX, fell to 47.6% with DOX + S9788. Specific growth delay increased with the double treatment (130 versus 75% with DOX alone). *In vitro*, P-gp was not detected on tissular sections and cells whatever the method and the antibody used. In conclusion, S9788 potentiates the efficiency of DOX treatment *in vivo*. The absence of P-gp may result from the absence of translation of the *MDR1* gene. The reversal effect of S9788 may involve another resistance mechanism such as the MDR Sister of MRP.

Key words: Doxorubicin, glycoprotein P 170, medullary carcinoma, nude mice, S9788, thyroid.

Introduction

In medullary carcinoma of the thyroid (MTC), chemotherapy must be considered in patients who relapse or who have metastasis after surgery. Unfortunately, this therapy is often ineffective.[1] Intrinsic (without prior chemotherapy) and acquired (developing during the course of chemotherapy) drug resistance remain the major obstacles to effective chemotherapy. It has been previously reported that several MTC cell lines, an established human MTC cell line (TT)[2] and other cell lines of MTC isolated in our laboratory[3,4] possess intrinsic multidrug resistance (MDR) to a few antineoplastic agents, especially to doxorubicin (DOX).[5–8] We have also shown that this resistance can be partly reversed by cyclosporin A, verapamil[6,7] and by S9788.[6]

In an attempt to investigate the potential mechanisms responsible for the chemoresistance to DOX in TT cells, we studied the effect of S9788 on the efficiency of DOX in MTC cells transplanted into immunodeficient mice. We also evaluated the presence of glycoprotein P 170 (P-gp) on the tumoral cells with four monoclonal antibodies (mAb) specific for different epitopes of P-gp.

Materials and methods

Chemicals and reagents

DOX hypochloride was purchased from Roger Bellon (Neuilly, France). S9788 was obtained from the Institute de Recherches Internationales Servier (IRIS), Courbevoie, France.

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

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

All chemical reagents were purchased from Sigma (St Louis, MO).

Cell Culture

The cells tested in this study were from the TT cell line of human MTC originally established by Leong *et al.*[2] The TT cell line was a gift from Dr Moukhtar

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(Inserm U 349, Paris, France). As controls we used normal thyroid cells and several tumoral cell lines: KB-3-1 epidermal carcinoma cell lines[9] and a 200-fold DOX-resistant human breast carcinoma subline (Adr200 MCF7), kindly provided by KH Cowan (National Cancer Institute, Bethesda, MA).[10]

TT and Adr200 MCF7 cell lines were cultured in RPMI 1640 medium, KB-3-1 cell line in Dulbecco's modified Eagle medium. The culture media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μg/ml streptomycin and 100 UI/ml penicillin. For Adr200 MCF7 cells, 10 μM DOX was also added to the culture medium. 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.

The preservation of the endocrine nature of the presently used passages of TT and of tumoral cells removed from the mice at the end of the protocol (TTM cells) was checked by radioimmunological measurements of calcitonin (CT) secreted in culture medium and by positive immunohistochemical staining on TTM cells and tumoral sections for CT as previously described for a mixed medullary and follicular thyroid carcinoma cell line.[4] For all techniques used, the cell lines were analyzed during the exponential phase of growth.

TT cell xenograft

Monolayer cultures were harvested with trypsin/EDTA and resuspended in a phosphate buffer saline (PBS). About 10^6 cells were injected s.c. into the right flank of two athymic female nude mice (Swiss *nu/nu*; Iffa Credo, Saint-Germain, France). After about 2 moths, the tumors measured about 450 mm^3 and were aseptically removed from the mice, disaggregated by mechanical means and reinjected into two other mice. After another passage, the tumors were transplanted into 40 mice. The 40 mice were randomized into four groups of 10 animals and were peritoneally injected according to Table 1 when the tumors measured about 450 mm^3. The animals were weighed and the tumors were measured twice a week. The survival of the animals was checked every day.

In vivo studies

The following parameters were determined on the animals:

Body weights measured biweekly.

Tumoral volume (TV) calculated from the two diameters of the tumor: $VT = a^2 \times b/2$ mm^3 (a and b were the smaller and the larger diameter, respectively).

Doubling time (DT) of the tumor.

Survival time of mice.

Efficiency of DOX without or with S9788 was also assessed on day 50 by specific growth delay (%) = (DT of treated animals − DT of controls/DT of controls) × 100.

Inhibition of tumoral growth (%) = T/C (mean tumoral volume of the treated group/mean tumoral volume of the control group) × 100.

Results were analyzed with Student's t-test (StatView II, Abacus Concept, USA) with $p < 0.05$ as criterion.

In vitro studies

Tumoral markers. CT was measured with a RIA kit (purchased from CIS Bio, Gif sur Yvette, France) in the culture medium of TT cells before the transplantation and in the culture supernatants of TTM cells at the end of the protocol. The presence of CT was also evaluated on tumoral sections and on TTM cells at each passage and at the end of the study with immunohistochemical staining using a peroxidase method (LSAb kit/HRP) and a polyclonal antibody purchased from Dako (Trappes, France) as described.[4]

P-gp detection. P-gp detection was performed by immunocytochemistry and by flow cytometry on TTM cells at the end of the protocol.

Immunocytochemistry. TT cell line, TTM cells and tissue sections 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, three mAb, MRK 16 (Valbiotech Laboratories, Paris, France) used in a dilution of 1:200, JSB1 (Tebu Laboratories, Le Perray en Yvelines, France) used in the working dilution of 1:30 and C219 without dilution (Centocor, Malvern, PA) were applied for 1 h at room temperature. Revelation was performed with an avidin–biotin peroxidase technique using the LSAB kit from Dako. KB-3-1 and Adr200 MCF7 cell lines were used as negative and positive controls, respectively.

Flow cytometry. For this analysis, we used four mAb, three for immunocytochemistry (MRK16, JSB1 and C219) and UIC2, a new P-gp-specific mAb for an external epitope from Immunotech (Marseille, France). MRK16 and UIC2 mAbs were incubated on fresh cells, whereas JSB1 and C219 mAbs were used on fixed and permeabilized cells. KB-3-1 and Adr200 MCF7 cell lines were also used here as controls.

Cell preparations. TTM cells in exponential growth were trypsinized and 5×10^5 fresh cells were directly incubated with **MRK16 or UIC2** mAbs.

For **JSB1** (25 μg/ml) and **C219** (50 μg/ml) mAbs, four different fixation and permeabilization protocols were used.

(1) *Iced methanol at −20°C.* The cells were exposed to ice-cold methanol for 30 min at 4°C: this step was followed by two consecutive washings with PBS with the addition of 10% FCS.

(2) *Paraformaldehyde (PFA)–triton.* PFA diluted to 4% in PBS followed by 0.1% Triton X-100 were incubated with the cells for 5 min at 4°C before two washings as for protocol (1).

(3) *Formol–saponin.* The cells were fixed with formol (3.7% in PBS) for 5 min at 4°C, then two successive incubations were performed at 4°C with the primary antibody and the secondary antibody in the presence of 0.1% saponin containing 1% bovine serum albumin for permeabilization.

(4) *Commercial cell permeabilization kit (Seralab CPK 50).* A fixation reagent of the kit was placed in contact with the cells for 15 min at room temperature and then the permeabilization reagent was added simultaneously to the incubation steps with the first and secondary antibodies.

Addition of the primary and secondary antibody. The fresh cells in suspension in plastic tubes were incubated with 10 μg/ml **MRK16** or 50 μg/ml **UIC2** antibody at 4°C for 45 min and then washed twice with PBS. Whatever the cell preparation method, **JSB1** or **C219** mAbs were incubated at 4°C for 45 min or 12 h, respectively. Then the cells were exposed for 45 min at 4°C to the secondary antibody (FITC-conjugated rabbit anti-mouse IgG serum; Silenus, Hawthorn Laboratories, Victoria, Australia) diluted 1:100 in the presence of saponin or permeabilization reagent was described above. The cells were then stored on ice for a period ranging from 30 min to 2 h and analyzed on the flow cytometer (Cytoron-Absolute, Ortho Diagnostic Systems, Roissy, France). Laser excitation was at 488 nm. Emitted green (FITC) fluorescence was measured at 515 nm. A minimum of 10 000 events (cells) was recorded for each fluorescence histogram. A subclass-matched mAb (IgG2a) was routinely used as negative control of fluorescence.

Results

In vivo

In each group, we observed only slight variations between the *weight* of the different mice since the standard deviations (SD) were less than 15% at day 71 and less than 25% after 100 days of treatment (data not shown). Therefore the mean growth curves were shown without SD to obtain a clearer figure (Figure 1a). Treatment by S9788 alone did not affect weight since the growth curve was identical to that obtained with controls. We found a significant decrease in the weight of the mice treated with DOX alone ($0.025 < p < 0.05$ between day 25 and day 99; $p < 0.005$ between day 43 and day 71). The addition of S9788 to DOX gave the same statistical results. As for the growth curve, the SD were not presented for *TV* (Figure 1b) since they were less than 15%. We observed that DOX alone slightly affected TV. On the contrary, the addition of S9788 to DOX induced a dramatical decrease in TV as early as day 43 ($0.005 < p < 0.025$).

The *survival curves* showed the toxicity of DOX or S9788 administered alone since as soon as day 50 several mice in these groups had already died (Figure 2). The double treatment with DOX and the modulator induced a dramatic drop in the survival rate. The *doubling time of the tumor* was higher in the presence of DOX alone (22.2 ± 8.5 versus 12.7 ± 3.9 days for the controls; $p = 0.0058$) and especially with the double treatment with S9788 (29.2 ± 11.4 days; $p = 0.0012$) (Table 1). The *specific growth delay* increased with DOX alone (75% as compared with controls) and particularly with DOX and S9788 (130%)(Table 2). The administration of DOX alone induced only a slight fall in the *inhibition of the tumoral growth* at day 50 (89%) whereas the addition of the modulator decreased this index to 47.6% (Table 2).

In vitro

Morphological and hormonal characteristics of TTM cells. At the end of the protocol, the TTM cells

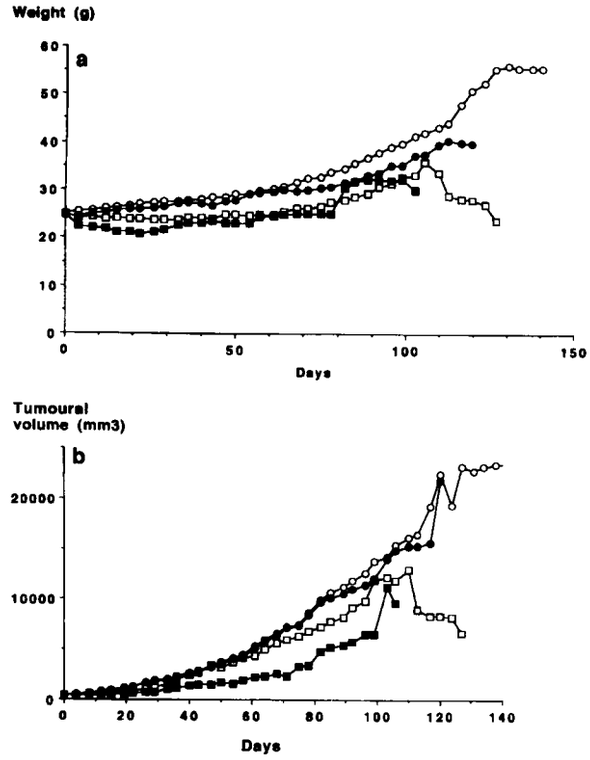

Figure 1. Time course of weights of the nude mice (a) and tumoral volumes (b) in the controls (○), groups receiving DOX alone (□), S9788 alone (●) or DOX + S9788 (■).

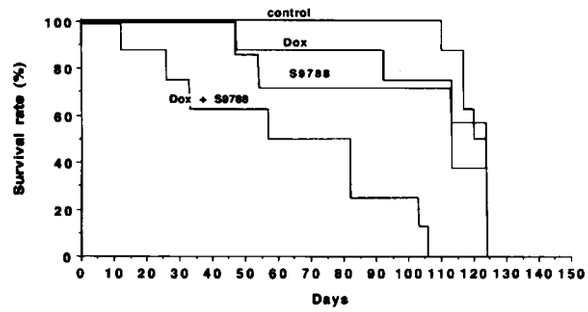

Figure 2. Effect of DOX treatment with or without S9788 on the survival rate of nude mice.

Table 1. Treatment of the four groups of 10 mice

Groups	One injection at day 0	One injection at day 0 2 h later
Control	NaCl (100 μl)	NaCl (100 μl)
DOX	DOX (10 mg/kg)	DOX (10 mg/kg)
DOX + S9788	DOX (10 mg/kg) S9788 (50 mg/kg)	S9788 (50 mg/kg)
S9788	S9788 (50 mg/kg)	S9788 (50 mg/kg)

Table 2. Tumoral doubling time at day 50

Treatment	No. of days	Student's *t*-test
Controls	12.7 ± 3.9	
DOX	22.2 ± 8.5	$p = 0.0058$***
DOX + S9788	29.2 ± 11.4	$p = 0.0012$***
S9788	13.9 ± 4	NS

The results are expressed as the means ± SD of results obtained in all the mice of each group

removed from the mice were cultured and took on different aspects according to the treatments given to the animals. The TTM cells from the control group and for the group DOX alone or DOX + S9788 had the same morphology as that of the TT cells (Figure 3a): they were small, compact and spindle-shaped (Figure 3b). Sometimes they joined together to form spheroids (Figure 3c). The levels of CT measured in the culture media were high and remained stable (450–950 pg/ml) for several weeks. On the contrary, the cells from mice treated, with *S9788 alone* were very different since they had an extremely large cytoplasm (Figure 3d). The concentrations of CT secreted by these cells decreased to reach low levels after a few days in culture (15–54 pg/ml). Therefore these cells seemed to lose their capacity to synthesize CT and to dedifferentiate. The immunoenzymatic labeling with specific anti-CT antibody showed high staining in the TTM cells from control and DOX groups (Figure 4a and b). Staining in the large cells from S9788-treated mice was also positive, though less so (Figure 4c). The CT immunostainings performed on tissular sections showed positive results (Figure 4d).

Detection of P-gp. Immunostaining. When P-gp was assessed by JSB1 and C219 mAbs, Adr200 MCF7 cells showed a strongly positive staining, whereas TTM cells and tumoral sections remained negative (Figure 5a–f). With MRK16 MAb, P-gp was detected in Adr200 MCF7, TTM cells and in tissular sections but also in the KB-3-1 cell line (Figure 6a–d).

Flow cytometry. We obtained negative staining with MRK16 and UIC2 mAbs used on fresh cells, the positive control with MRK16 giving results above 95%. Whatever the fixation protocol used, we also found negative results with the use of JSB1 (Table 3). Positive results were obtained with C219 MAb but these results were excluded since KB-3-1 cells were also stained in all the fixation protocols with this mAb (67–100%).

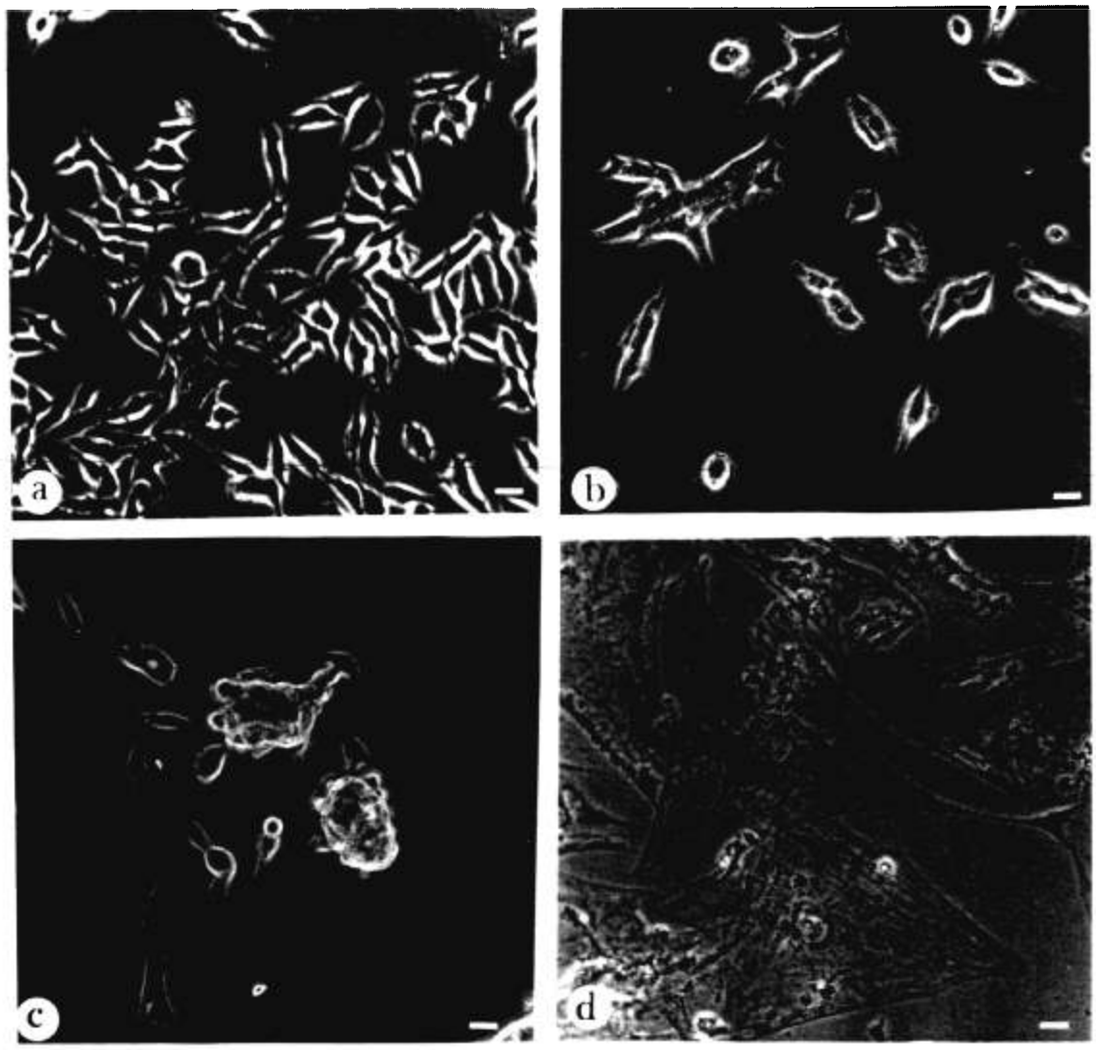

Figure 3. Morphology of cells cultured on plastic (phase-contrast microscopy). TTM cells from mice treated with DOX alone, with DOX + S9788 or without drug showed the same morphology as that of TT cells (a). (b) TTM cells were small, compact and spindle shaped. (c) Sometimes they joined together to form spheroids. (d) TTM cells from mice which have been treated by S9788 alone had an extremely large cytoplasm. Bars indicate 25 μm.

Discussion

To our knowledge, only few reports have been published on *in vitro* drug resistance in MTC: certain authors have studied the P-gp expression cytochemically in MTC cells,[5–7] others the presence of MDR1 mRNA by northern blot[8] or by polymerase chain reaction (PCR).[6,7] No *in vivo* study has concerned MTC xenografts in immunodeficient mice. Therefore in this work we performed *in vivo* experiments in nude mice which all developed a tumor when the TT cell line was injected in large quantities.[2]

We observed that the administration of DOX alone induced different effects according to the part of the curve studied and the parameter used: TV slightly changed, whereas doubling time of the tumor and specific growth delay dramatically increased. On the contrary, the addition of S9788, a new triazino-aminopiperidine derivative,[11,12] to DOX clearly proved the tumoral regression. These results are in agreement with our previous studies showing that S9788 potentiated the action of DOX on TT cell cultures.[6] Other workers have also reported the efficiency of this modulator on the resistance to different chemotherapeutic

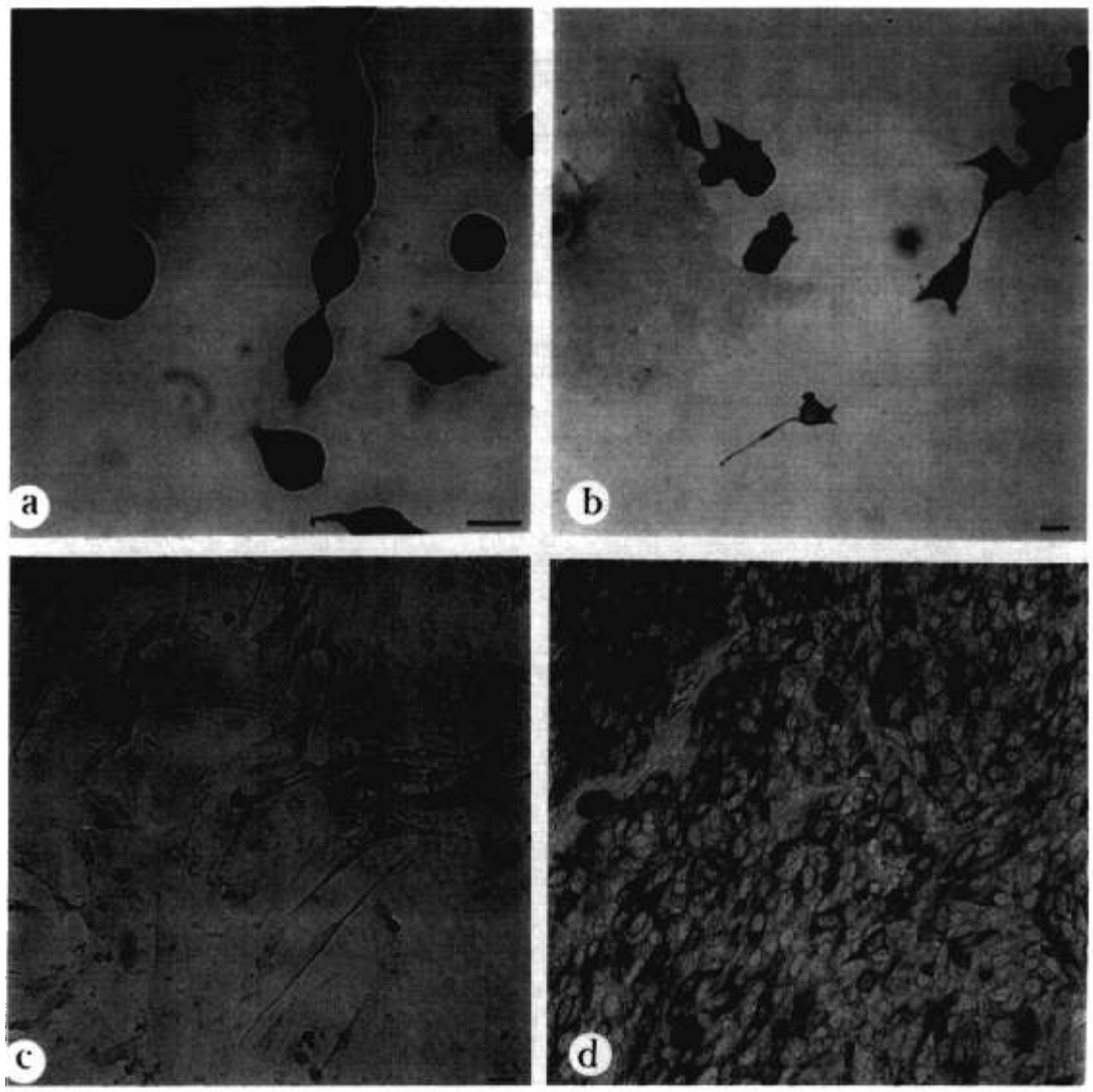

Figure 4. CT immunostaining in cultured TTM cells (a–c) or in tumoral sections (d) (light microscopy). (a) TTM cells from mice without treatment (bar indicates 25 μm) or (b) treated with DOX alone (bar indicates 25 μm) showed high stainings, whereas (c) TTM cells from mice treated with S9788 alone (bar indicates 50 μm) were slightly stained. (d) Positive immunostaining was also observed in tumoral sections (bar indicates 25 μm).

drugs.[13–16] Moreover we found that S9788 did not induce toxicity in the animal when administered alone, which contrasts with some *in vitro* studies showing toxicity at high concentrations.[6,17]

In vitro, we first analyzed the morphology and the secretion of TTM cells and we observed that these cells retained the shape of TT cells and their ability to form spheroids as previously observed.[18] However, TTM cells removed from mice treated with S9788 alone were very large and secreted less CT than cells from animals of other groups. It has previously been shown that S9788 exerts *in vitro* toxicity at high concentrations on MTC cells,[6] on lymphoblastoid T cell leukemia or ovarian carcinoma cell lines.[17] The change of the shapes and the dedifferentiation of the cells may reveal the toxicity of S9788.

We also studied *in vitro* the presence of P-gp on TTM cells with two methods. We used different MAb as C219 or JSB1, mAbs specific of intramembrane or intracytoplasmic epitopes, respectively; we also employed MRK16 and UIC2 mAbs acting with external epitopes.[19,20] Our cytochemical findings using three mAbs induced conflicting results showing the absence of P-gp in TTM cells and in tissular sections, whereas MRK16 mAb induced positive stainings in

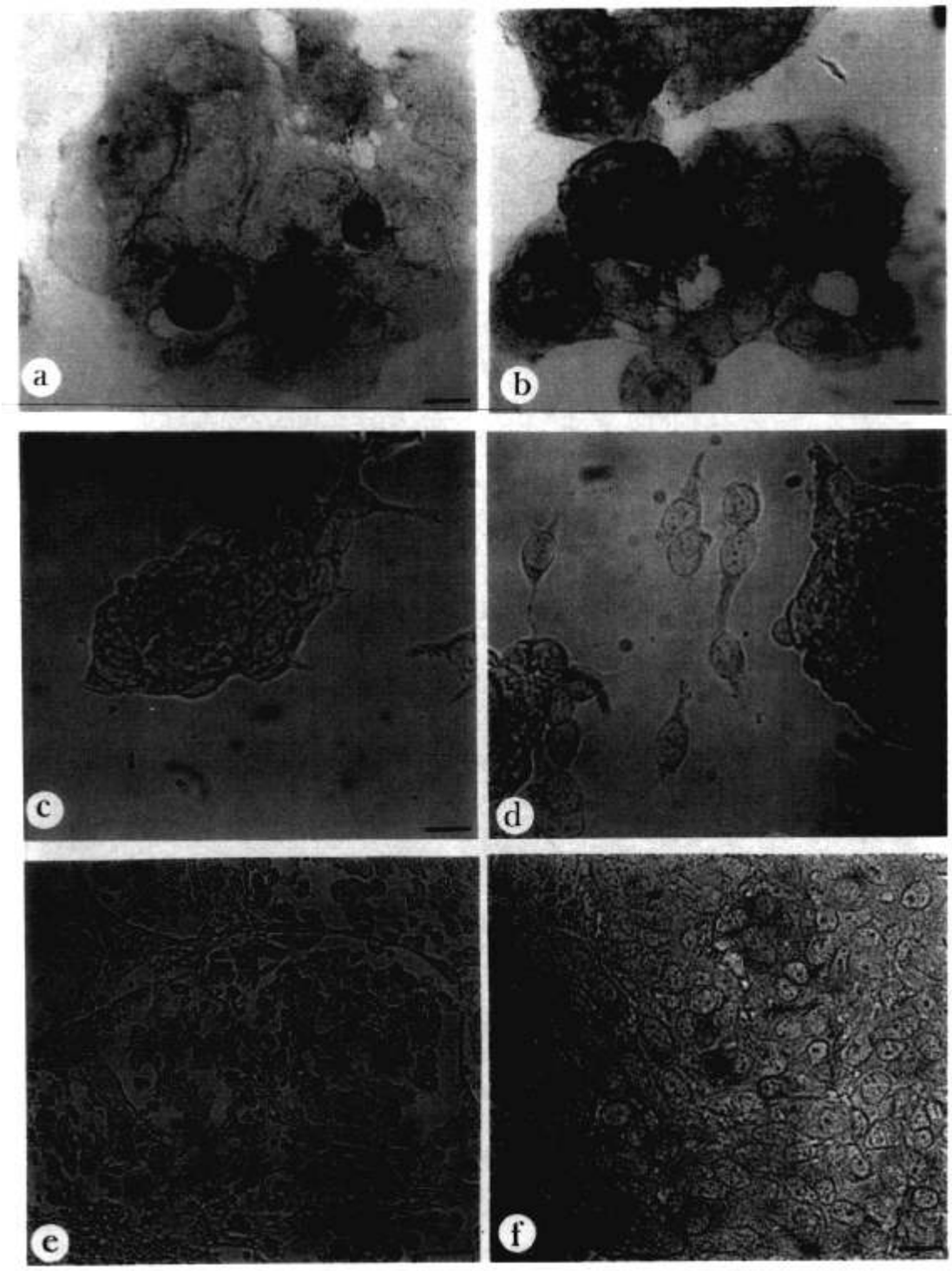

Figure 5. Immunocytochemical localization of P-gp in TTM cells and in tumoral sections. Adr200 MCF7 cells, TTM cells and tumoral sections were fixed in ethanol and incubated with monoclonal P-gp antibodies: JSB1 or C219 (mAbs). A positive staining was observed with JSB1 (a) and C219 (b) in Adr200 MCF7 cells, whereas negative results were obtained for TTM cells with JSB1 (c) and C219 (d) and for tumoral sections (e–f). Bars indicate 25 μm.

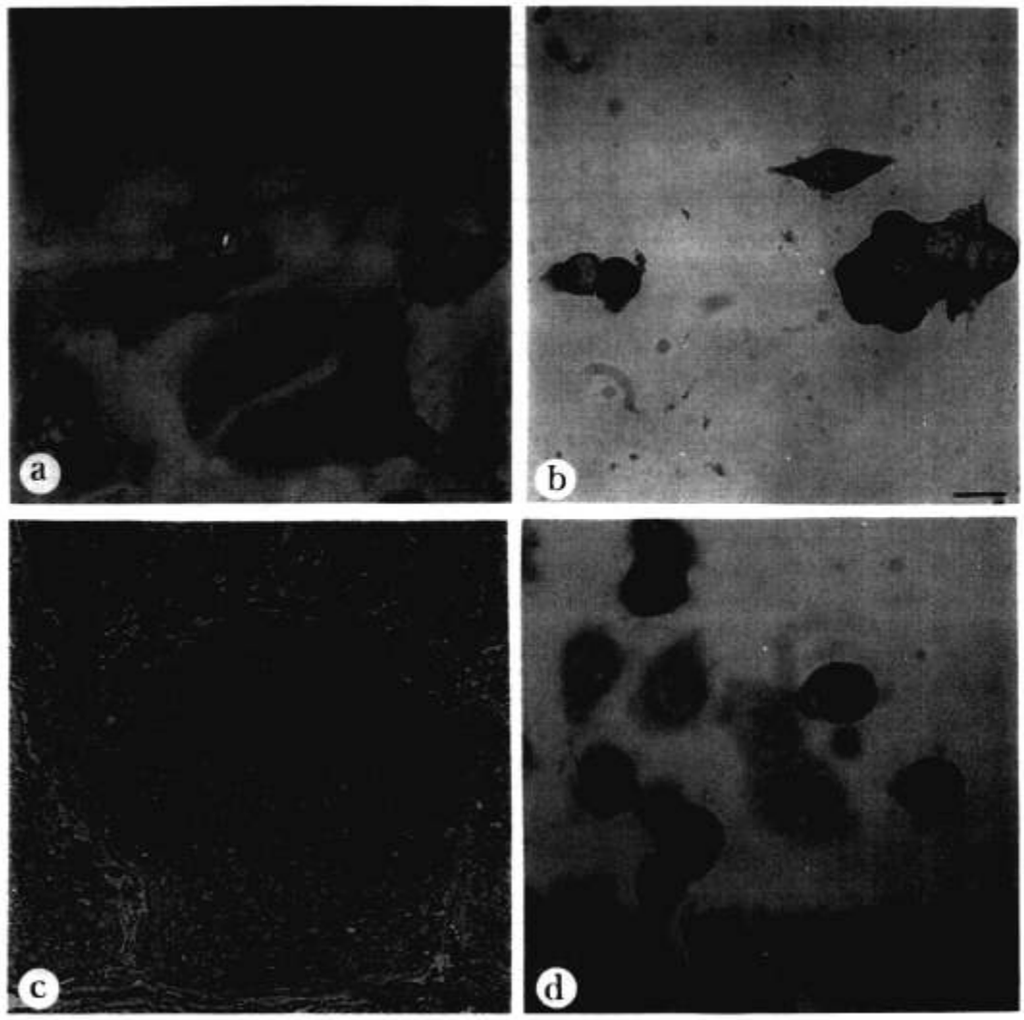

Figure 6. Immunocytochemical localization of P-gp in TTM cells and in tumoral sections. Adr200 MCF7 cells, TTM cells and tumoral sections were fixed in ethanol and incubated with MRK16 mAb: a positive staining was observed in Adr200 MCF7 cells (a), TTM cells (b), tumoral sections (c) and KB-3-1 cell line (d). Bars indicate 25 μm.

Table 3. Efficiency of S9788 on DOX toxicity at day 50

Treatment	Inhibition of tumoral growth (%)	Specific growth delay (%)
DOX	89	75
DOX + S9788	47.6	130

all the samples. Negative immunostaining with JBS1 mAb has already been reported in TT cells,[5,6] which contrasts with the presence of MDR1 mRNA in these cells.[6,8] On the contrary, the positive staining obtained with MRK16 mAb is questionable since KB-3-1 cells, considered as a negative control, as shown by PCR analysis, do not express MDR1

Table 4. Stainings in flow cytometry

	MRK16	ULC2	JSB1
Control	1.90 ± 0.54 (5)	0.70 ± 0.52 (2)	1.05 ± 0.41 (5)
DOX	2.27 ± 2.30 (4)	0.65 ± 0.07 (2)	1.85 ± 1.02 (2)
DOX + S9788	1.90 ± 1.01 (4)	0.55 ± 0.35 (2)	1.50 ± 0.17 (4)
S9788	2.27 ± 1.48 (4)	0.70 ± 0.42 (2)	1.10 ± 0.43 (4)

The numbers in parentheses indicate the number of experiments

mRNA.[6] We have also previously obtained in TT cells positive immunocytochemical results with MRK16 mAb (data not shown). Therefore the positive staining obtained with MRK16 mAb may be due to interactions with proteins other than P-gp, as has been suggested by Van der Valk *et al*[21] and Vergier *et al.*,[22] working on sections from different normal and neoplastic tissues or on human sarcomas, respectively.

It has been suggested that immunohistochemical techniques provide a quantification dependent on the subjective assessment of the examiner and often hampered by low-antigen expression.[23] Thus these authors recommend the use of flow cytometry, a more sensitive method than immunocytochemistry. However, we observed that this technique raises the same type of problem since positive and negative results were found according to the mAb. The results obtained with C219 mAb must be discarded since the negative control (Kb-3-1) cells were found positive, which could explain why this mAb is rarely used in flow cytometry. Certain authors[24–26] used this mAb on fixed cells with methanol for 24 h (and not 12 h as us) which is too injurious for our TTM cells. The fixation method performed for JSB1 was less drastic and UIC2 and MRK16 mAbs were incubated with fresh cells, which permits the use of these mAbs in several flow cytometry studies.[27–30] We observed the absence of P-gp in CMF with the use of these three mAbs, which corroborates the cytochemical results with JSB1. We cannot exclude that TT cells contain very low amounts of P-gp that cannot be detected by a cytochemical method or flow cytometry. The absence of P-gp may also result from the absence of translation of the MDR1 gene. The reversal effect of S9788 may also involve another resistance mechanism such as MRP[31,32] or MDR Sister.[33]

In conclusion, S9788 potentiates the efficiency of DOX treatment in nude xenografts. The absence of P-gp may be due to the lack of sensitivity of the techniques used or to the absence of translation of the *MDR1* gene. Another reversal mechanism such as MDR Sister or MRP cannot be excluded.

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