Effect of sodium butyrate on doxorubicin resistance and expression of multidrug resistance genes in thyroid carcinoma cells

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ATP-binding cassette (ABC) transporters [P-glycoprotein and multidrug resistance (MDR)-associated proteins (MRPs)] confer MDR to tumor cells. In this work, we investigated doxorubicin resistance in three thyroid carcinoma cell lines. The effects of sodium butyrate (NaB) on doxorubicin-induced cytotoxicity and on transcription of three MDR genes were also studied. Thyroid cell lines established from anaplastic (8505C) and two poorly differentiated follicular (FTC 238 and FTC 133) cancers were cultured for 24 or 48 h in the presence of NaB (0, 0.25, 0.5 and 1 mM) alone or combined with increased doses of doxorubicin. Cytotoxicity was assessed using the MTT test. MDR1, MRP1 and MRP2 mRNA expression was studied by RT-PCR. After a 24- or 48-h incubation, doxorubicin alone induced cytotoxicity in the three cell lines. NaB significantly ($p < 0.0001$) increased the doxorubicin-induced cytotoxicity. MRP1 transcripts were expressed in the three non-treated cell lines. MDR1 and MRP2 mRNAs were both present in 8505C, but absent in FTC 133 or FTC 238 cell lines, respectively. Treatment with NaB for 24 or 48 h induced no change in MRP1 and MRP2 levels, but increased MDR1 expression in 8505C and FTC 238 cell lines comparably to alkaline phosphatase activity. In conclusion, MRP1 and sometimes MDR1 and MRP2 are expressed in the tested cell lines. NaB potentiates doxorubicin-induced cytotoxicity independently of the ABC transporters. The combination of doxorubicin and NaB might have clinical implications for thyroid cancer therapy. *Anti-Cancer Drugs* 16:255–261 © 2005 Lippincott Williams & Wilkins.

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Introduction

Thyroid carcinoma, the most common endocrine malignancy, accounts for the majority of deaths from endocrine cancers [1]. Conventional therapy consists of surgical resection and radioiodine ^{131}I therapy [2]. In anaplastic thyroid carcinoma (ATC) and in 20% of differentiated thyroid carcinomas (DTC) that eventually lose their ability to concentrate iodine, an additional chemotherapy is also used [1,3]. Current chemotherapy is based primarily on doxorubicin, a quinone-containing anthracycline, alone or in combination [4]. However, chemotherapy alone has been largely unsuccessful for all the cases of ATC and some DTC, possibly related to intrinsic or acquired multidrug resistance (MDR) [5–7]. The MDR to doxorubicin observed in several medullary thyroid carcinoma cell lines has been attributed to the overexpression of the MDR1 gene encoding for the plasma membrane P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter [8–11]. Other ABC transporters, such as the MDR-associated proteins (MRPs) have also been involved in resistance to doxorubicin in different carcinomas [12–15]. However, the role of the ABC transporters in chemotherapy failure in ATC and DTC remains poorly understood.

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Sodium butyrate (NaB), the major short-chain fatty acid produced by fermentation of dietary fibers in the colon [16], is known as an inhibitor of histone deacetylase (HDAC) [17]. It has been recently shown to inhibit proliferation and to induce apoptosis in anaplastic thyroid cancer cell lines [18,19]. However, its effects on thyroid MDR have yet to be determined.

In an attempt to investigate the potential mechanisms responsible for the chemoresistance to doxorubicin in thyroid carcinomas, we studied the presence of MDR1, MRP1 and MRP2 in an anaplastic and two poorly differentiated thyroid carcinoma cell lines. We also evaluated the effects of NaB on doxorubicin-induced cytotoxicity, on alkaline phosphatase (ALP) activity, and on the expression of MDR1, MRP1 and MRP2 genes.

Materials and methods

Chemical agents and culture media

Doxorubicin hydrochloride (Adriblastine) was purchased from Pharmacia (Guyancourt, France). All chemical reagents and fetal calf serum (FCS) were purchased from Sigma (St Louis, MO). NaB was prepared as a stock

solution (1.5 M) in culture medium and stored at –20°C until use.

Cell lines and culture conditions

Three thyroid human carcinoma cell lines were obtained from European Collection of Animal Cell Cultures (Salisbury, UK). An undifferentiated thyroid cancer cell line (8505C) was derived from primary culture of a human ATC tumor. Follicular thyroid carcinoma (FTC) cell lines 133 and 238 were established from the primary tumor (FTC 133) and a nodal metastasis (FTC 238) of a follicular thyroid carcinoma. As positive and negative controls for MDR1 studies, we used the KB-3-1 epidermal carcinoma cell line [20] and a 200-fold doxorubicin-resistant human breast carcinoma subline (Adr200 MCF7), kindly provided by K. H. Cowan (National Cancer Institute, Bethesda, MD) [21]. For MRP1 and MRP2 controls, we used human hepatocytes and MRP1-overexpressing lung tumoral GLC4/Sb30 cells kindly given by O. Fardel (INSERM U456, Rennes, France) as previously reported [22].

The thyroid cell lines were cultured in MEM with 1% non-essential amino acids (for 8505C) and in Dulbecco's modified Eagle's medium (DMEM):Ham F12 (1:1) (for FTC cell lines) purchased from Eurobio (Les Ulis, France). The culture media were supplemented with 10% (for 8505C and FTC 133) or 5% (for FTC 238) heat-inactivated FCS, 10 mM HEPES, 2 mM glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin.

Adr200 MCF7 and KB-3-1 cell lines were cultured in RPMI 1640 medium and in DMEM, respectively. The culture media were supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin (for both cell lines), and with 10 µM doxorubicin (for Adr200 MCF7 cells).

William's E medium or RPMI 1640 medium were used for primary hepatocytes or the GLC4/Sb30 cell line, respectively. The media were supplemented with hormones, chemicals and serum as previously described [22].

Cells were cultured in 175-cm^2 flasks (Falcon Plastics, Becton Dickinson, Orangeburg, NY) at 37°C in a 5% CO_2/95% air/water saturated atmosphere. Trypsinization was performed at confluence. Isolated cells were then cultured in 96-well culture plates for cytotoxicity studies and in 75-cm^2 flasks for ABC transporter analysis. The cell lines were examined during culture with an inverted microscope (Olympus, CK-2).

Doxorubicin sensitivity

Doxorubicin sensitivity was measured using the MTT assay as previously described [23]. Briefly, 8505C, FTC 238 and FTC 133 cell lines were plated at a density of 1.2×10^4 cells/well in 96-well plates and allowed to attach overnight. Then, the cells were incubated for 24 and 48 h at 37°C in a 5% CO_2/95% air/water saturated atmosphere with serial dilutions of doxorubicin (0.01, 0.1, 0.5, 1, 5 and 10 µM) and NaB (0.25, 0.5 and 1 mM) dispensed alone or combined. Controls without doxorubicin and NaB were also included in each experiment.

An aliquot of 100 µl of MTT working solution (0.5 mg/ml; Sigma) was added to each culture well and the cultures were incubated for 2 h at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and resulting formazan crystals were dissolved in 100 µl dimethylsulfoxide (Sigma). The absorbance was measured at 540 nm. All the measurements were performed with the absorbance values within the linear range of the assays. The absorbances of each well were measured with a microtiter plate reader (Multiskan RC; Thermo Life Sciences, Cergy Pontoise, France). Results were calculated as percent control as follows: (experimental absorbance/untreated control absorbance) × 100.

In all the experiments, cells were cultured at 70% of confluence. Three replicate wells were analyzed for each test and each assay was repeated 3 or 4 times. IC_{50} values were defined as the mean concentrations reducing absorbance by 50% (IC_{50}). The effect of NaB was quantified by the reversal factor: RF = IC_{50} (doxorubicin only)/IC_{50} (doxorubicin associated with NaB).

ALP activity

Cells were cultured in 96-well plates as described above for the MTT test. After a 48- and 72-h incubation, Triton X-100 (50 µl 0.2%, v/v) was added to each well. ALP activity (EC 3.1.3.1) was measured with a kit (Enzyline PAL Optimisé) purchased from Biomérieux (Marcy l'Etoile, France). The absorbance was read at 405 nm. The ALP activity was quantified using a positive control (Multiqual 3) obtained from Bio-Rad (Marnes la Coquette, France).

RNA isolation and RT-PCR experiments

Total RNA was extracted from cell lines using GenElute mammalian Total RNA miniprep kit (Sigma) and was quantified by absorbance at 260 nm. A sample of 2 µg of total RNAs were subjected to electrophoresis in a denaturing 37% (v/v) formaldehyde/1.2% (w/v) agarose gel. Total RNA (2 µg) of thyroid carcinoma cell lines and cells chosen as positive or negative controls (Adr200 MCF7 or KB-3-1, GLC4/Sb30 or primary hepatocytes and primary hepatocytes or GLC4/Sb30 for MDR1, MRP1 and MRP2, respectively) were used for synthesis of first-strand cDNA, using the Superscript first-strand synthesis system for RT-PCR (Gibco/BRL, Grand Island, NY). The reaction mixture was incubated at 42°C for 50 min and subsequently stopped by heating to 70°C for 15 min.

For PCR, we used GAPDH as the internal control. Amounts of cDNA preparations providing the same yield of the GAPDH PCR product were determined by these assays and were then used for MDR1-, MRP1- and MRP2-specific PCR. PCR Master Mix (10 µl; Promega, Madison, WI), containing 20 pM each upstream- and downstream-specific primers were added to 2 µl reverse transcriptase reaction mixture in a total volume of 20 µl. Primers (upstream 5′ to 3′, downstream 5′ to 3′, in all cases) used for amplification were CCCATCATTGCAATAGCAGG/GTTCAAACTCTGCTCCTGA for MDR1 [24] (to amplify a 167-bp product), GGACCTGGACTTCGTTCTCA/GTGTTCCGGATGGTGGACTG for MRP1 [22] (to amplify a 500-bp product), CTAGCAGCCATAGAGCTGGC/TGGCTCCAGAGTTCTGCTGG for MRP2 [22] (to amplify a 600-bp product) and GTCAGTGGTGGACCTGACCT/TGAGCTTGACAAAGTGGTCG for GAPDH (accession number in GenBank 002046) (to amplify a 212-bp product).

Amplification was performed on a programmable heater (DNA Engine; MJ Research) for 30 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). Amplified cDNA products were analyzed on agarose gel (2%) containing ethidium bromide (1 µg/ml).

Statistical analysis

The results of IC_{50} and ALP activity were compared using Student's *t*-test (Stat-View 4.11) with a 5% limit for statistical significance.

Results

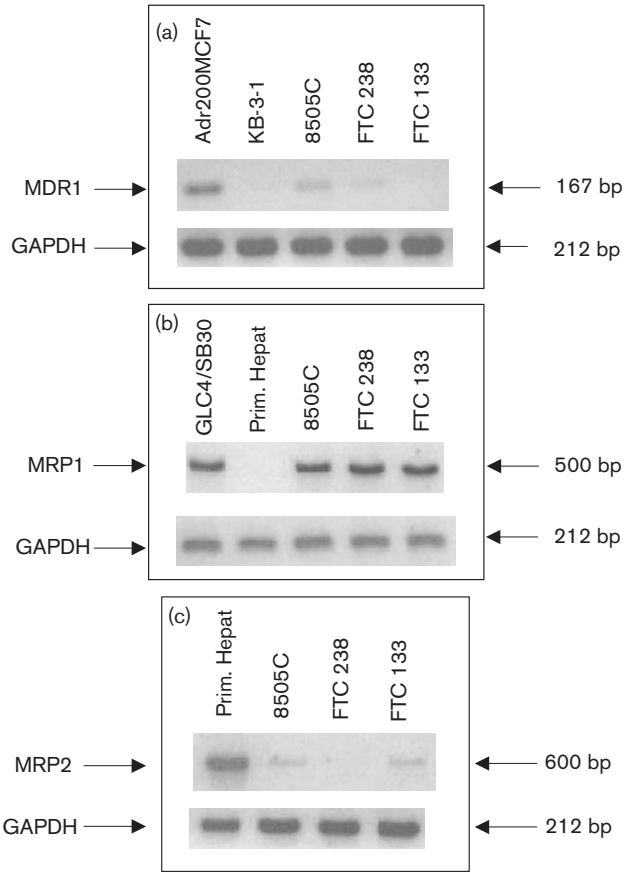
Expression of ABC transporters

RT-PCR revealed the presence of MDR1 mRNA only in 8505C and in FTC 238 cell lines (Fig. 1a). MRP1 mRNA was found in the three cell lines, whereas MRP2 mRNA was not detected in FTC 238 cells (Fig. 1b and c).

Doxorubicin toxicity

Cytotoxic effects of doxorubicin on the cells were analyzed using the MTT assay as described above. Attached cells were incubated with different doses of doxorubicin for 24 and 48 h. Dose-dependent effects of doxorubicin at final concentrations of 0.01–10 µM were observed only for the two FTC cell lines after a 24-h incubation as previously reported [25] (Fig. 2a). For the 8505C line, doxorubicin-induced cytotoxic effects were found after a 48-h incubation (Fig. 2b). The dose–response curves obtained after a 24-h incubation showed that the FTC 238 cells were the most sensitive to the action of doxorubicin (IC_{50} values, mean ± SD = 2680 ± 212, 9650 ± 1105 and $> 10^4$ nM for FTC 238, FTC 133 and 8505C cell lines, respectively.

Fig. 1



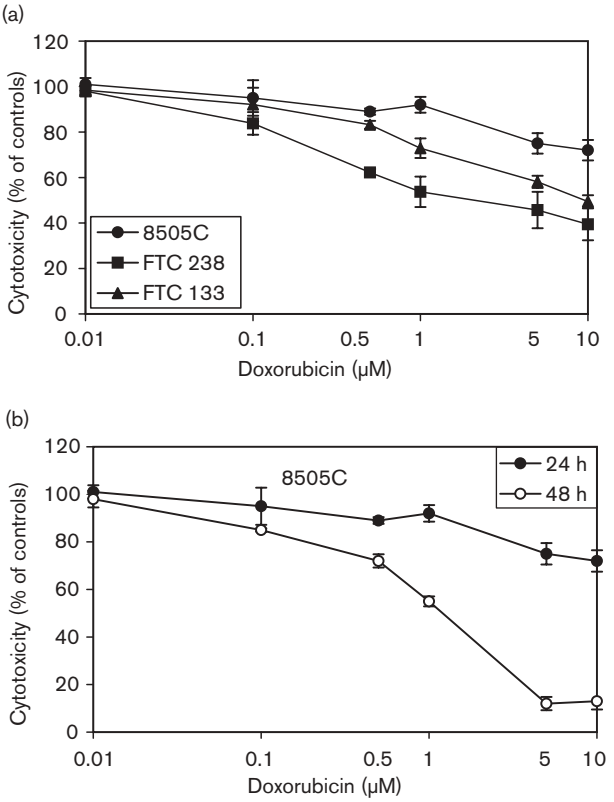
Expression of MDR1, MRP1 and MRP2 mRNAs in 8505C, FTC 238 and FTC 133 cell lines. Each lane contains 2 µg total RNA isolated from cells cultured for 24 h after seeding. RT-PCR was performed, using primers specific for MDR1 and GAPDH (a), MRP1 and GAPDH (b) or MRP2 and GAPDH (c). Arrows on the right indicate the specific product bands for MDR1 (167 bp), GAPDH (212 bp), MRP1 (500 bp) and MRP2 (600 bp) sequences.

Effect of NaB on doxorubicin sensitivity

The cytotoxic effects of doxorubicin combined with NaB (0.5 or 1 mM) administered on the cells for 24 or 48 h were assessed with MTT assay as described above.

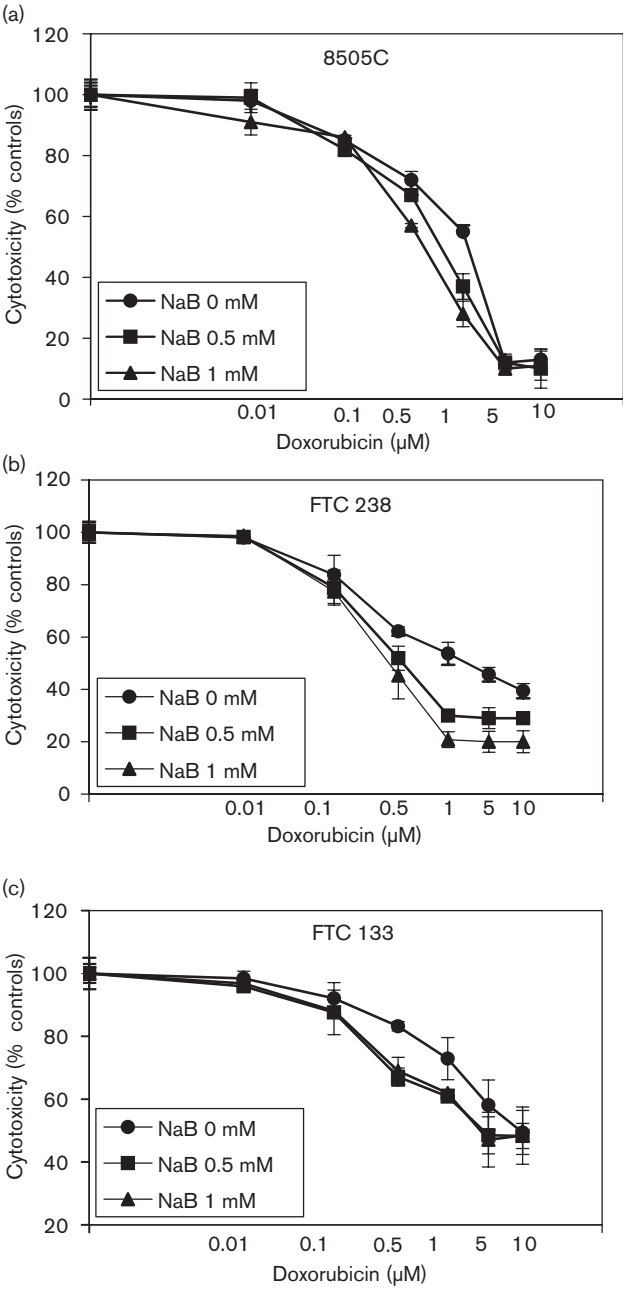
The results clearly indicate that NaB administered alone at the doses of 0.5 or 1 mM induced no cytotoxicity. In contrast, these non-toxic concentrations increased the doxorubicin-induced cytotoxicity (Fig. 3). The IC_{50} values decreased significantly ($p < 0.001$) in the three cell lines in the presence of doxorubicin combined with NaB as compared with cells treated with doxorubicin alone after a 24- (for the FTC cells) or 48-h (for the 8505C cells) incubation (Table 1). The effect of NaB was higher in FTC 238 cells than that obtained in the two other lines as indicated by RF values (Table 1).

Fig. 2



Effect of doxorubicin on viability of 8505C, FTC 238 and FTC 133 cell lines. The MTT test was performed after a 24-h incubation for the three cell lines (a) or after a 24- and 48-h incubation for the 8505C cells (b). Results are expressed as percent control and represent means ± SD of three or four independent experiments.

Fig. 3



Effect of doxorubicin alone or combined with NaB on viability of 8505C (a), FTC 238 (b) and FTC 133 (c) cell lines for a 24- (FTC 238 and FTC 133) or 48-h (8505C) incubation. Results are expressed as percent control and represent means ± SD of three or four independent experiments.

Effects of NaB on ALP activity

NaB (0.25, 0.5 or 1 mM) significantly increased ALP activity in 8505C cells after a 48- or 72-h incubation (Fig. 4). A significant rise in ALP activity was observed in the two FTC cell lines treated with 1 mM NaB (Fig. 4).

Effects of NaB on the expression of ABC transporters

NaB (0.25, 0.5 or 1 mM) induced a dose-dependent increase of MDR1 mRNAs in 8505C and FTC 238 cell lines after a 24-h incubation (Fig. 5). MDR1 mRNA undetected in the FTC 133 cell line cultured alone was not found in these cells cultured with NaB (data not shown).

No change was observed for MRP1 and MRP2 mRNAs in the cell lines treated for 24 h with different doses of NaB (Fig. 6). MRP2 mRNA absent in the FTC 238 cell line cultured alone was not observed in cells in the presence of NaB (data not shown).

Discussion

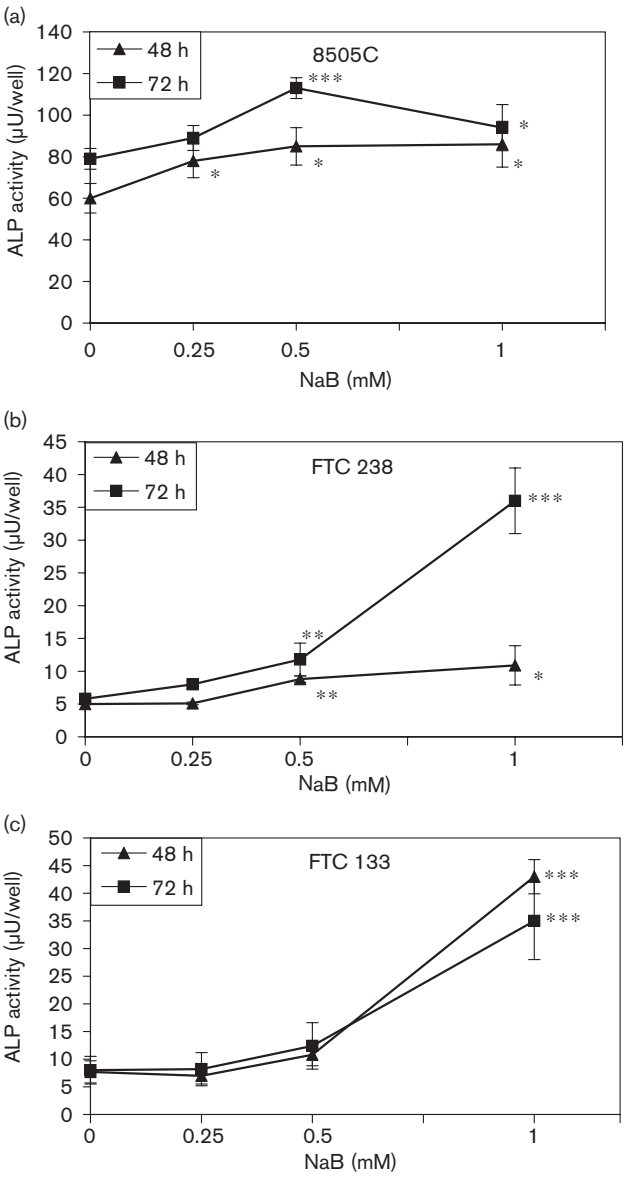
Few reports have been published on MDR in thyroid carcinomas. We have previously demonstrated the overexpression of MDR1 mRNA and the reversion of MDR phenotype by several modulators in medullary thyroid cancer cell lines [10,26,27]. In the present work, the

Table 1 Comparison of IC_{50} and RF values obtained with the MTT test performed on the three cell lines exposed to doxorubicin alone or combined with NaB for a 24- (for FTC cell lines) or 48-h (for 8505C line) incubation

Cell line	Doxorubicin alone	Doxorubicin combined with NaB (0.5 mM) [IC_{50} (RF)]	Doxorubicin combined with NaB (1 mM) [IC_{50} (RF)]
8505C	1250 ± 354	755 ± 21[a] (1.65)	470 ± 42[a] (2.66)
FTC 238	2680 ± 212	540 ± 40[a] (4.96)	460 ± 10[a] (5.83)
FTC 133	9650 ± 1105	4510 ± 730[a] (2.10)	4210 ± 840[a] (2.29)

The IC_{50} results are expressed as the means ± SEM of three or four experiments each carried out in triplicate.
[a]$p < 0.001$ from the values obtained with doxorubicin alone.

Fig. 4



Effect of NaB on the alkaline phosphatase activity in (a) 8505C, (b) FTC 238 and (c) FTC 133 cell lines. Results represent means ± SD of three independent experiments. *$p < 0.05$; **$0.02 < p < 0.01$; ***$p < 0.001$ from control values (without NaB).

expression of both MDR1 and MRPs in ATC and DTC cell lines were studied for the first time.

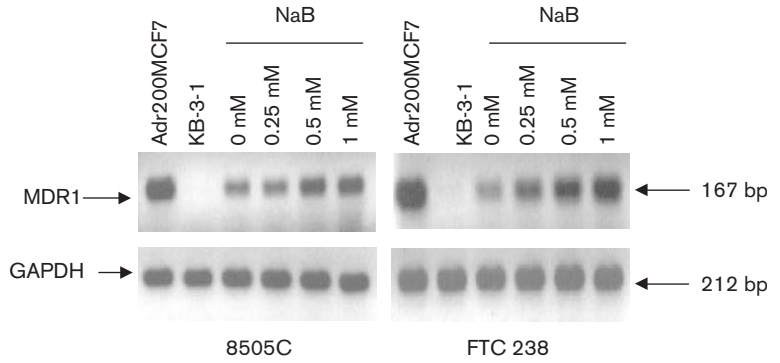
In the first part of this work, we evaluated the expression of the three MDR genes in the cell lines.

In the literature, conflicting results have been published on MDR1 mRNA detection by RT-PCR in ATC cell lines. Certain authors found a significant MDR1 expression in 60% of their ATC cell lines [14], whereas others failed to detect it [28,29]. In agreement with Satake *et al.* [14], our results showed a significant MDR1 expression in two of our three cell lines. The MTT assay showed intensive doxorubicin resistance in 8505C cells which was associated with the highest MDR1 expression. However, for the FTC 133 cell line, we found a higher resistance to doxorubicin than that obtained for FTC 238, but we failed to detect MDR-1 mRNA. Thus, contrary to ATC lines, doxorubicin sensitivity was not correlated with the MDR1 expression in the FTC cell lines.

We have also studied MRP expression in our cell lines. This family of ABC transporters includes MRP [30], also known as MRP1, and apical MRP, now widely termed MRP2 [31]. MRP1 and MRP2 have been demonstrated to confer drug resistance on tumoral cells from various tissues through increased efflux of anticancer drugs [13,31]. In the literature, the few reports concerning thyroid carcinomas showed an increase of MRP expression in ATC. Using RT-PCR some authors were able to detect MRP mRNA in all the ATC cell lines investigated [14], whereas others using immunohistochemistry techniques found MRP expression in 52% of the cell lines studied, which was higher than in other types of cancers [15]. In concordance with these findings, we found MRP1 in the three cell lines, whereas the expression of MRP2 was observed in only two of them. Thus, the expression of MRP1 in the three cell lines may at least partly explain the chemoresistance and the failure of chemotherapy in patients with thyroid cancer.

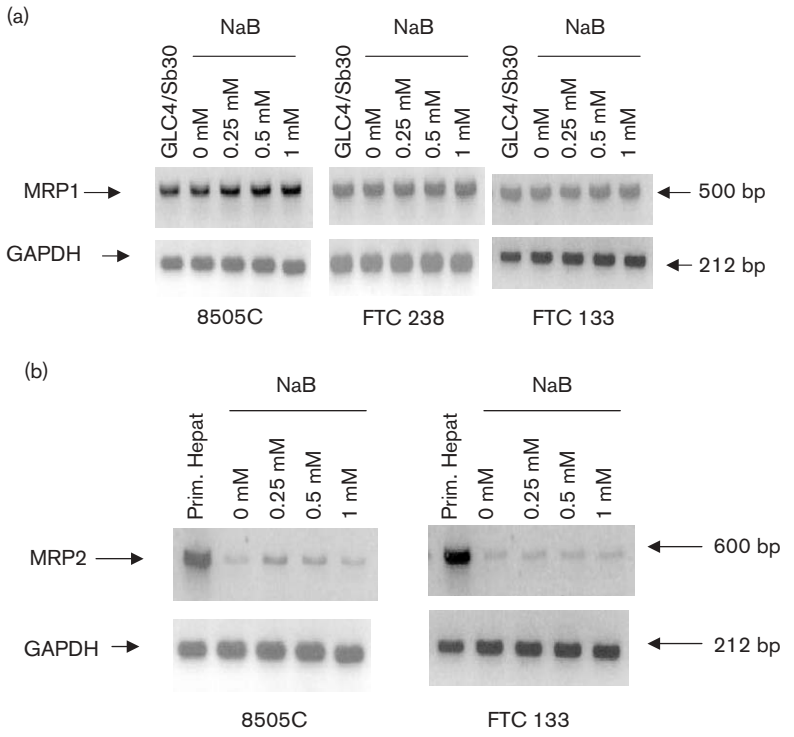
In the second part of our work, we studied the effects of NaB on doxorubicin-induced cytotoxic effects and on ABC transporter expression in thyroid carcinoma cells. We demonstrated that NaB used in combination with doxorubicin potentiated doxorubicin-induced cytotoxicity in the three tumor cells, in agreement with a previous report on ATC cell lines [32]. In an attempt to explain this increase in doxorubicin sensitivity, we studied the NaB-mediated effects on the expression of the ABC transporters. In the literature, few and conflicting results have been published on HDAC inhibitor action, promoting either down [33] or up [34] P-gp regulation in leukemia or colon carcinoma cell lines, respectively. Our results demonstrated no action of NaB on MRP1 and MRP2 mRNAs, and a rise in MDR1 expression, in

Fig. 5



Effect of NaB on the expression of MDR1 mRNA in 8505C and FTC 238 cell lines. Each lane contains 2 μg total RNA isolated from cells cultured for 24 h without or with different concentrations (0.25, 0.5 and 1 mM) of NaB. Arrows on the right indicate the specific product bands for MDR1 (167 bp) and GAPDH (212 bp) sequences.

Fig. 6



Effect of NaB on the expression of MRP1 (a) and MRP2 (b) mRNAs in the three (a) or in two (8505C and FTC 133) (b) cell lines. Each lane contains 2 μg total RNA isolated from cells cultured for 24 h without or with different concentrations (0.25, 0.5 and 1 mM) of NaB. Arrows on the right indicate the specific product bands for MRP1 (500 bp) and GAPDH (212 bp) (a) or MRP2 (600 bp) and GAPDH (212 bp) (b) sequences.

agreement with results on colon carcinoma cells [34]. This dose-dependent increase in MDR1 mRNA paralleled the stimulation of ALP activity known as a differentiation marker of epithelial thyroid carcinoma cell lines. Thus, the mechanisms involved in NaB-mediated effects include an increase in cell differentiation. This is in agreement with previous reports showing that HDAC inhibitors restore radioiodine uptake and the expression of thyroid-specific genes in thyroid cancer cells [35,36]. However, another resistance mechanism

such as lung resistance (LRP) or breast resistance (BCRP) proteins cannot be excluded.

In conclusion, MRP1, MDR1 and MRP2 are expressed in all or some cell lines. MDR1 expression increased in NaB-treated cell lines in parallel to cell differentiation. NaB enhanced cell sensitivity to doxorubicin independently to MDR1, MRP1 and MRP2 expression. Work is underway in our laboratory to investigate the role of LRP and BCRP in the NaB-mediated effects.

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