Effect of all-*trans* retinoic acid and sodium butyrate *in vitro* and *in vivo* on thyroid carcinoma xenografts

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Histone deacetylase (HDAC) inhibitors have been shown to reverse repression of some genes, including retinoic acid (RA) receptor β2. In this work, we studied the effects of RA alone or combined with the HDAC inhibitor sodium butyrate (NaB) in a poorly differentiated thyroid carcinoma cell line (FTC-133) cultured *in vitro* or transplanted into nude mice. *In vitro*, the action of the xenobiotics on cell differentiation was evaluated by the measurement of alkaline phosphatase (ALP) activity. *In vivo*, FTC cells were injected in nude mice divided into four groups: controls, RA (1 mg/kg), NaB (50 mg/kg) in two daily injections or both RA plus NaB. Body weight, tumoral volume (TV), doubling time of the tumor, specific growth delay and inhibition of tumoral growth at day 35 were determined in each group. *In vitro*, RA increased the NaB-induced increase in ALP activity. *In vivo*, body weight and TV decreased with RA or NaB. Specific growth delay significantly increased with RA (72.5%; $P < 0.001$) and with NaB (31.3%; $0.02 < P < 0.01$). Inhibition of tumoral growth significantly fell to 50.3% ($0.02 < P < 0.01$) and 42.9% ($0.05 < P < 0.02$) with RA and NaB, respectively. The combination of RA with NaB induced no significant change in the different parameters observed in mice treated with RA alone. In conclusion, a synergistic effect between RA and NaB was observed *in vitro* on cell differentiation. The addition of NaB to RA induces no change in growth inhibition observed with RA alone in nude xenografts. *Anti-Cancer Drugs* 17:559–563 © 2006 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]. They are mainly represented by differentiated papillary and follicular thyroid cancer (DTC) [2]. Conventional therapy for these tumors consists of total thyroidectomy and radioiodine ^{131}I therapy [3]. However, about 10–15% of patients with DTC develop local recurrence and distant metastasis as a consequence of their inability to take up and to concentrate iodine [1,4]. In these patients, tumors progressively lose the expression of markers of thyrocyte differentiation, such as thyroid-stimulating hormone/thyrotropin receptor, thyroglobulin, thyroperoxidase and sodium iodide symporter [5,6]. External radiotherapy and conventional chemotherapy have all been tried with limited success [7–9]. Differentiation therapy represents a novel and alternative therapeutic approach in the treatment of cancer. Among differentiation therapy, restoration of iodide trapping with retinoic acid (RA) [10–12] or with histone deacetylase (HDAC) inhibitors [13–15] has been proposed with limited success [16,17].

Sodium butyrate (NaB), the major short-chain fatty acid produced by fermentation of dietary fibers in the colon [18], is known as an inhibitor of HDAC [19]. NaB has been shown to induce expression of differentiated markers [20], to increase radioiodine uptake [14,15,21] and to arrest cell cycle [22,23] in thyroid carcinoma cells. It has been recently reported that NaB could restore retinoid response of RA-resistant human acute promyelocytic leukemia [24], prostate [25,26], breast [27] or renal [28,29] cancer cells. However, the effects of NaB combined with RA in thyroid carcinomas have yet to be determined.

In an attempt to improve the therapeutic efficacy of thyroid cancer, we evaluated the effects of RA used in combination with NaB *in vitro* on alkaline phosphatase (ALP) activity in DTC cultured cells. We also studied the action of RA and NaB on DTC cells transplanted into immunodeficient mice.

Materials and methods

Chemical agents and culture media

All chemical reagents and fetal calf serum (FCS) were purchased from Sigma (St Louis, Missouri, USA). RA was dissolved in DMSO at a concentration of 100 mmol/l, stored at −20°C and protected from light until use. NaB

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was prepared as a stock solution (136 mmol/l) in NaCl (0.9%) and stored at −20°C.

For the in-vitro experiments, the stock solutions of RA and NaB were diluted with culture medium (DMEM: Ham F12; 1:1) purchased from Eurobio (Les Ulis, France). The final concentration of DMSO in medium was 0.005 or 0.001% for 1 or 5 µmol/l RA, respectively.

For the in-vivo experiments, dilutions of stock solutions were performed in PBS (pH 7.4) or in NaCl (0.9%) for RA or NaB, respectively. The end concentration of DMSO in PBS was 1%.

Cell culture conditions

A follicular thyroid carcinoma (FTC) cell line 133, established from the primary tumor of a FTC was obtained from European Collection of Animal Cell Cultures (Salisbury, UK). The FTC-133 cell line was cultured in DMEM:Ham F12 (1 : 1) purchased from Eurobio. The culture medium was supplemented with 10% heat-inactivated FCS, 10 mmol/l HEPES, 2 mmol/l glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin.

Cells were cultured in 175-cm^2 flasks (Falcon Plastics; Becton-Dickinson, Orangeburg, New York, USA) at 37°C in a 5% CO_2/95% air/water-saturated atmosphere. Trypsinization was performed at confluence.

In-vitro studies

FTC-133 cells 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 72 h at 37°C in a 5% CO_2/95% air/water-saturated atmosphere with RA (1 or 5 µmol/l) and NaB (0.5, 1, 2 or 3 mmol/l) dispensed alone or combined. Controls without RA and NaB or with dilution solvent alone were also included in each experiment. Triton X-100 (50 µl 0.2%, v/v) was then added to each well. ALP activity (EC 3.1.3.1) was measured with a kit (Enzyline PAL Optimisé) purchased from Biomerieux (Marcy l'Etoile, France). The absorbance was read at 405 nm using a microtiter plate reader (Multiskan RC; Thermo Life Sciences, Cergy Pontoise, France). The ALP activity was quantified using a positive control (Multiqual 3) obtained from Bio-Rad (Marne la Coquette, France).

In all the experiments, cells were cultured at 70% of confluence. Culture medium was changed every day. Three replicate wells were analyzed for each test and each assay was repeated 3 or 4 times.

Cell xenograft and in-vivo studies

Monolayer cultures were harvested with trypsin/EDTA and resuspended in PBS. About 10^6 cells were injected s.c. into the right flank of athymic female nude mice (Swiss *nu/nu*; Charles River Laboratories, Larbresle France). After about 2 months, the tumors measured about 350 mm^3, and were aseptically removed from mice, disaggregated by mechanical means and re-injected 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 injected according to Table 1 when the tumors measured about 350 mm^3. The animals were weighed and the tumors were measured biweekly for 49 days. The survival of the animals was checked every day.

Table 1 Treatment of the four groups of 10 mice

Groups	Treatment
Controls	NaCl peritoneally twice a day/DMSO in PBS (1%, v/v) p.o. daily
RA	RA (1 mg/kg) p.o. daily
NaB	NaB (50 mg/kg) peritoneally twice a day
RA + NaB	RA (1 mg/kg) p.o. daily/NaB (50 mg/kg) peritoneally twice a day

The following parameters were determined on the animals as previously reported [30]: survival time of the mice evaluated daily; body weight measured biweekly; tumoral volume (TV) calculated from the two diameters of the tumor ($TV = a^2 \times b/2$ mm^3, where a and b were the smaller and the larger diameter, respectively), and doubling time of the tumor. Efficiency of RA and NaB alone or combined was also assessed by specific growth delay (%) = [(doubling time of treated animals–doubling time of controls)/doubling time of controls] × 100. Inhibition of tumoral growth (%) = T/C (mean tumoral volume of the treated group/mean tumoral volume of the control group) × 100 was evaluated at day 35.

Statistical analysis

Results were analyzed using the non-parametric Mann–Whitney *U*-test (Stat-View 4.11; Abacus Concept, Piscataway, New Jersey, USA) with a 5% limit for statistical significance.

Results

Effects of RA combined with NaB on ALP activity

NaB (1, 2 and 3 mmol/l) significantly increased ALP activity in FTC cells after a 72-h incubation (Fig. 1a). The addition of RA (1 or 5 µmol/l) to NaB potentiated the NaB-induced increase in ALP concentrations (Fig. 1b). Thus, the combination of RA and NaB led to synergistic induction of ALP secretion.

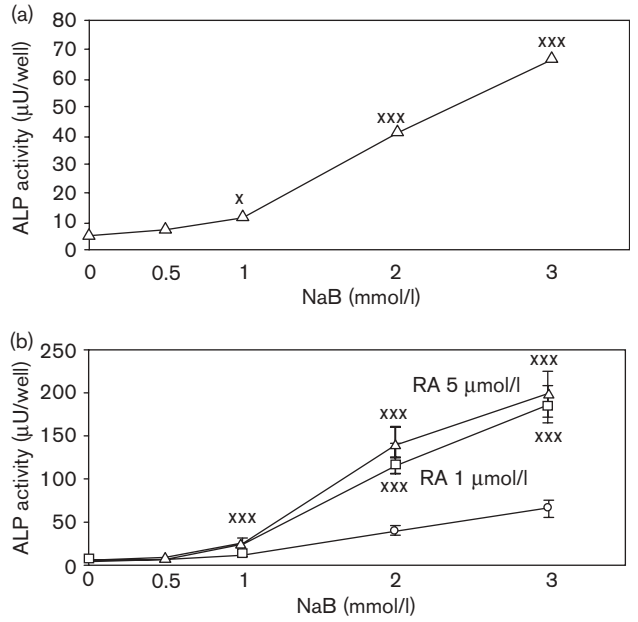
Survival time of the mice

The survival curves showed the absence of toxicity of RA and NaB administered alone. The combination of RA with NaB did not reveal any significant toxicity of the double treatment (Fig. 2).

Weight of the mice

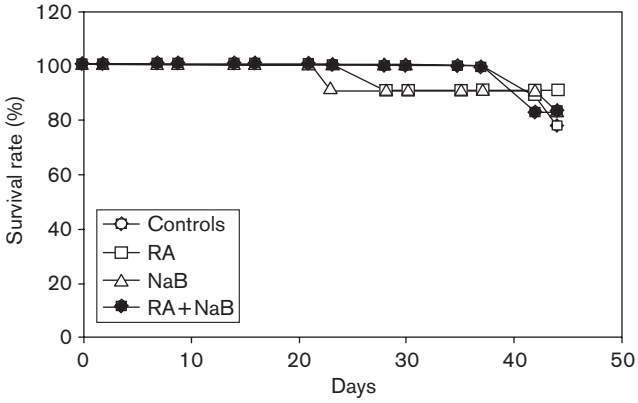
The mean growth curves are reported in Fig. 3(a). A significant decrease in the weight of the mice treated

Fig. 1



Effect of NaB alone (a) (○) or combined (b) with 1 (□) or 5 µmol/l (Δ) RA on ALP activity in FTC-133 cell lines. Results represent means ± SD of three independent experiments. (a) *$0.02 < P < 0.01$; ***$P < 0.001$ from control values (without NaB); (b) ***$P < 0.001$ from values obtained with NaB without RA.

Fig. 2



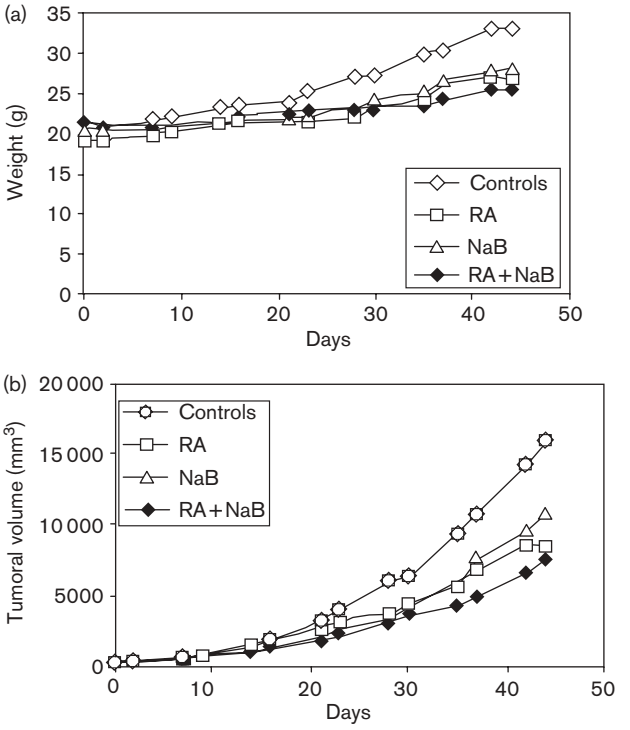
Effect of RA or NaB treatment alone or combined on the survival rate of nude mice.

with RA alone or NaB alone was observed between day 24 and 42 ($P < 10^{-3}$). The addition of NaB and RA gave similar results ($P < 10^{-3}$) (Fig. 3a).

Tumoral volumes

The administration of RA alone or NaB alone significantly reduced the TV between day 31 ($0.05 < P < 0.02$) and 42 ($P < 0.001$) (Fig. 3b). The combined treatment gave identical results in TV decrease between day 31 and 42 (Fig. 3b).

Fig. 3



Time course of weights of the nude mice (a) and tumoral growth volumes (b) in the control and groups receiving RA alone, NaB alone or RA plus NaB.

Table 2 Tumoral doubling time

Treatment	Mean doubling time (days ± SD)
Controls	8 ± 1
RA	13.8 ± 3.7[b]
NaB	10.5 ± 1[a]
RA + NaB	13.3 ± 4.2[b]

The results were compared to those obtained for controls ([a]$0.02 < P < 0.01$; [b]$P < 0.001$).

Efficiency of RA and NaB alone or combined

The doubling time of the tumors was higher in the presence of RA alone (13.8 ± 3.7 versus 8 ± 1 days for the controls; $Z = -2.781$; $P < 0.001$) or with NaB (10.5 ± 1 days; $Z = -2.556$; $0.02 < P < 0.01$) (Table 2). The combination of RA with NaB induced an increase in the doubling time (13.3 ± 4.2 days) identical to that obtained with RA alone. The specific growth delay increased slightly with NaB (31.3%; $0.05 < P < 0.02$) and dramatically with RA alone (72.5%; $P < 0.001$) as compared with controls. The double treatment induced an increase in the specific growth delay identical to that observed for RA alone (66.3%; $P < 0.001$). The inhibition of the

Table 3 Efficacy of RA and NaB alone or combined on tumoral growth

Treatment	Specific growth delay (%) at day 23	Inhibition of tumoral growth (%) at day 35
RA	72.5[c]	50.3[b]
NaB	31.3[a]	42.9[a]
RA + NaB	66.3[c]	53[b]

The results were compared to those obtained for controls ([a]$0.05 < P < 0.02$; [b]$0.02 < P < 0.01$; [c]$P < 0.001$).

tumoral growth at day 35 significantly decreased with RA (50.3%) and NaB (42.9%) alone when compared with tumoral growth obtained for the controls ($Z = -2.357$; $0.02 < P < 0.01$ and $Z = -2.282$; $0.05 < P < 0.02$ for RA and NaB, respectively) (Table 3). The association of RA and NaB gave a similar value to that found for RA alone (53%, $Z = -2.549$; $0.02 < P < 0.01$) (Table 3).

Discussion

We studied the effect of RA combined with NaB *in vitro* on DTC cell differentiation and *in vivo* in DTC xenografts in immunodeficient mice for the first time.

In the first part of this work, we evaluated the action of NaB alone or combined with RA *in vitro* on ALP activity, which known as a differentiation marker of epithelial thyroid carcinoma cells. We showed for the first time that the combination of RA and NaB led to synergistic induction of thyroid cell differentiation. Previous studies have shown that these individual agents restore the expression of thyroid-specific genes [12,14,15,31] or ALP activity [30] in thyroid cancer cells. However, this is the first time that NaB and RA were tested together on thyroid carcinoma cells and found to have an additive stimulating effect on cell differentiation. This finding is in agreement with previous reports showing the synergistic effect of HDAC inhibitors and retinoids in differentiation of promyelocytic leukemia [24]. Several mechanisms may be involved in this synergistic effect by keeping chromatin in a hyperacetylated state, upregulating the expression of RA receptor (RAR) [25,27,32], and/or removing the co-repression and allowing the transcription of retinoic acid response elements [24].

In the second part of the present work, we studied the in-vivo effect of RA and NaB alone or combined in nude xenografts. We found a growth inhibition induced by these agents administered alone or combined without any synergistic effect. Controversial data have been reported on the RA-induced growth inhibition in DTC cells cultured *in vitro*. Some authors [6,31,33] found a RA-induced decrease in DTC cell proliferation, others observed this phenomenon only in anaplastic thyroid carcinoma cells [34], whereas others found a growth increase [35]. These discrepancies have been reported to depend on the experimental conditions (duration of treatment with RA and the RA doses administered) [6]. These controversies observed *in vitro* led us to perform in-vivo studies in nude mice xenografts. To our knowledge, only one study has been reported on the effect of RA in DTC xenografts in immunodeficient mice [31]. When pre-treated FTC-133 cells were injected to nude mice, a clearly reduced tumor growth was detected, as compared with animals transplanted with untreated cells [31]. Our present study differs from this work as we injected the same FTC cells into animals that were further treated by RA as for use in clinical trials. We found a tumoral regression, and an increase in the doubling time and the specific growth delay in the mice treated with RA alone as compared with the controls, confirming the RA-induced growth inhibition previously shown by some authors [6,31,33]. This inhibitory effect has been reported to be mediated by RARβ in thyroid cells [6,31,34]. We also demonstrated that NaB induced an inhibition of tumoral growth as shown by the decrease of tumoral growth, and the increase in doubling time and specific growth delay. This finding is in agreement with previous works showing a HDAC inhibitor-induced reduction in tumor growth in nude mice injected with renal [29] or prostate [26] carcinoma cells. The mechanisms involved in these inhibitory effects may be related to NaB-induced cell cycle arrest and apoptosis [22,23] or to cell re-differentiation [6,14,15,20]

We also evaluated the effect of RA combined with NaB *in vivo* in nude mice xenografts. We found that the association of the two xenobiotics did not result in increased toxicity as evaluated by the survival rate. Our results also demonstrated that the association of RA with NaB did not potentiate the RA-induced inhibiting effects in tumoral growth. Several explanations may be suggested in order to understand the lack of synergism that we observed *in vivo*: a ceiling effect obtained with RA administered alone, which sometimes occurred in in-vivo experiments [36]; factors related to intercellular interactions and signals present in the in-vivo experiments, but lacking under the in-vitro culture conditions; and transplantation-induced modifications of the thyroid cells remaining sensitive to each molecule administered alone in nude mice, but resistant when combined. The discrepancies between the in-vitro and in-vivo results suggest that caution should be taken when extrapolating data from in-vitro to in-vivo systems. Moreover, since no effect of the double treatment was observed in nude xenografts, additional in-vivo study is justified to define the optimal way to combine RA and NaB to maximize the therapeutic efficacy.

In conclusion, a synergistic effect between RA and NaB was observed *in vitro* on thyroid cell differentiation. RA or NaB treatment induces a significant in-vivo growth inhibition in nude xenografts. NaB does not potentiate the effect of RA administered alone in nude mice. The

absence of efficacy of the double treatment *in vivo* on cell growth may justify further experimentation for its use in clinical trials.

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