Bexarotene via CBP/p300 induces suppression of NF-kappaB-dependent cell growth and invasion in thyroid cancer

Audrey Cras¹, Béatrice Politis¹, Nicole Baltrand¹, Diane Darsin-Bettinger¹, Pierre Yves Boelle², Bruno Cassinat¹, Marie-Elisabeth Toubert³, Christine Chomienne¹

¹ UMR-S 940, INSERM, Université Denis Diderot, Institut Universitaire d’Hématologie, Paris 75010 France.
² UMR-S 707, INSERM, Université Pierre et Marie Curie, Paris, 75010 France.

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Abbreviations: ATRA, all-trans-retinoic acid; DTC, differentiated thyroid cancer; FTC, follicular thyroid carcinoma; NIS, Na⁺/I⁻ symporter; NF-kappaB, nuclear factor kappaB; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; TG, thyroglobuline; TPO, thyroid peroxidase; TSHR, thyroid stimulating hormone receptor; PAX8, paired box 8; PPARγ, peroxisome proliferator-activated receptor gamma; PMA, phorbol myristate acetate; 13-cis RA, 13-cis retinoic acid; DIO1, type I iodothyronine 5’-deiodinase.
Translational relevance

After thyroidectomy, monitoring and treatment of thyroid cancer metastases relies on the uptake of radioactive iodine by the malignant thyroid cells. Unfortunately, some patients have signs of relapse with no detectable metastases on radioiodine whole body scan. This has been attributed to an absence of sodium-iodide symporter (NIS) and subsequent loss of iodine uptake. Retinoids, inducers of differentiation during embryogenesis are equally important for thyroidogenesis and NIS and the 5’ deiodase genes are retinoic acid target genes. We confirm previous reports of the ability of retinoids to restore differentiation in thyroid cancer in vitro and in vivo and present novel data showing that retinoid resistant cells and patients may respond to rexinoids via inhibition of cell growth and invasion. This results from the decrease of NF-kappaB target gene expression by modification of promoter permissiveness. This highlights a novel mechanism by which bexarotene may control cancer progression.
ABSTRACT

Purpose:
Retinoic acid (RA) treatment has been used for redifferentiation of metastatic thyroid cancer with loss of radioiodine uptake. The aim of this study was to improve the understanding of RA resistance and investigate the role of bexarotene in thyroid cancer cells.

Experimental design:
A model of thyroid cancer cell lines with differential response to RA was used to evaluate the biological effects of retinoid and rexinoid and to correlate this with RA receptors levels. Subsequently, thyroid cancer patients were treated with 13-cis RA and bexarotene and response evaluated on radioiodine uptake re-induction on post-therapy scan and conventional imaging.

Results:
In thyroid cancer patients, 13-cis RA resistance can be bypassed in some tumours by bexarotene. A decreased tumour growth without differentiation was observed confirming our in vitro data. Indeed we show that ligands of RARs or RXRs exert different effects in thyroid cancer cell lines through either differentiation or inhibition of cell growth and invasion. These effects are associated with restoration of RARβ and RXRγ levels and down-regulation of NF-kappaB targets genes. We show that bexarotene inhibits the transactivation potential of NF-kappaB in an RXR-dependent manner through decreased promoter permissiveness without interfering with NF-kappaB nuclear translocation and binding to its responsive elements. Inhibition of transcription results from the release of p300 co-activator from NF-kappaB target gene promoters, and subsequent histone deacetylation.

Conclusion:
This study highlights dual mechanisms by which retinoids and rexinoids may target cell tumorigenicity not only via RARs and RXRs as expected, but also via NF-kappaB pathway.
INTRODUCTION

Thyroid cancers are the most common malignancy of endocrine organs (1). Well-differentiated thyroid carcinomas (DTC) derived from follicular cells account for around 80% of thyroid cancers and include papillary and follicular cancer (respectively PTC and FTC) which are differentiated on the basis of histological parameters. Most of these cancers have an excellent prognosis and can be effectively managed by total thyroidectomy, ablative doses of radioiodine and suppressive treatment. The follow-up is based on measuring serum thyroglobulin (TG) and imaging with radioiodine (RI) therapeutic scans. It has been estimated that about 20% of these patients will develop a local or distant recurrence. In some cases, TG and RI scan are discordant, suggesting the presence of thyroid cancer metastases which have lost the ability to trap RI (2). This is challenging for thyroid cancer management, as anatomical localization of recurrences cannot be assessed and treatment with RI therapeutic doses is not effective, predicting a poorer outcome.

Signalling through growth factors and their receptors and defects in regulation of cell-cycle control elements are considered essential for cancer cell proliferation. Recent advances have improved the understanding of the thyroid oncogenesis. Inappropriate activation of the mitogen-activated protein kinase (MAPK)-pathway effectors may lead to tumour initiation and transformation. Indeed mutations or rearrangements of tyrosine kinase receptor (RET and NTRK1), BRAF and Ras are found in most of thyroid cancers (3). Cancer progression is associated with a loss of the differentiated characteristics that define mature follicular thyroid cells with the full capacity for normal physiological function such as iodine uptake. Iodine is transported across the basolateral plasma cell membrane of thyrocytes via the $\text{Na}^+/\text{I}^-$ (sodium iodine) symporter (NIS) (4). NIS and other proteins linked to the synthesis of thyroid hormones, such as TG, thyroid peroxydase (TPO) and type I iodothyronine 5'-deiodinase (DIO1), have been reported to be poorly expressed in thyroid cancer patients (5). Reduced expression of these differentiation proteins results from Ras mutations and alterations of transcription factors involved in thyroid differentiation such as loss of TTF1 expression or chromosomal translocation of paired box 8 (PAX8) with peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) (6, 7).

Retinoids, a group of structural and functional derivatives of vitamin A are known to regulate a large number of essential biological processes such as cell growth, differentiation and death. The effects are mainly mediated by two types of nuclear receptors – retinoic acid receptors (RARs) and retinoid X receptors (RXRs) - which act as ligand-dependent transcription factors. By qualitative mRNA detection assays,
expression of retinoic acid receptors (RARA, RARB, RARG and RXRA and RXRB) has been detected in normal thyrocytes suggesting that absence of a tissue-specific retinoic acid receptor may participate in differentiation arrest and thyroid oncogenesis (8). Reduced expression of RARβ has indeed been observed in human thyroid carcinomas (9). RA therapy in malignant cells is based on functional alternative RA signalling pathways which, upon pharmacological concentrations of a given retinoid, restore control of cell death, differentiation and proliferation. Various mechanisms are involved including the transcriptional control of the tissue specific retinoic acid receptor gene via endogenous receptors. We have shown the up-regulation of RARβ in neuroblastoma and glioblastoma by various natural and synthetic retinoids (10), and the transcriptional control of the normal RARA gene expression by all-trans RA (ATRA) treatment in acute promyelocytic leukemia cells (11). RA efficacy in patients with thyroid cancer is supported by in vitro data, showing that retinoids induce up-regulation of RARβ and differentiation of cells, accompanied by the up-regulation of NIS and TG expression and DIO1 activity (12, 13). Treatment with 13-cis retinoic acid (13-cis RA) was shown to restore RI uptake in thyroid cancer patients in clinical trials (14-16). However, only one third of the patients respond, and further trials have failed to confirm 13-cis RA efficacy in these patients (17, 18). A clinical study showed that treatment with bexarotene, a RXR agonist can induce a radioiodine uptake by metastases in some patients (19, 20). These data prompted for a better understanding of RA signalling pathways and resistance in thyroid cancer cells.

We have previously reported that lack of RA-responsiveness in a follicular thyroid cancer cell line was attributable to an altered histone acetylation pattern and could be relieved by combination of ATRA and trichostatin A, a histone deacetylase inhibitor, underscoring the ongoing clinical trials based on differentiation and transcriptional therapy combination (21). In order to investigate the role of RXR agonist in these patients, we took advantage of the RA-resistant thyroid cancer cell line, FTC238, derived from the metastasis of the patient whose cells, at diagnosis, allowed the establishment of the RA-sensitive FTC133 cell line (22). In this study, we show that ligands of RAR or RXR exert different effects in thyroid cancer cells through either differentiation or inhibition of growth and invasion and may thus provide different therapeutic approaches in DTC patients. Drug effects were associated with restoration of RARβ and RXRγ levels. Interestingly, bexarotene inhibits the transactivation potential of NF-kappaB through the involvement of nuclear co-activator p300 without interfering with either nuclear translocation or binding of p65 to NF-kappaB responsive elements. This study highlights in a given cancer, dual mechanisms by which retinoids may target cell
tumorigenicity not only via targets of retinoids such as RARs and RXRs as expected, but also via targets of the NF-kappaB pathway.

MATERIALS AND METHODS

Reagents and chemicals

Bexarotene (LGD1069) was kindly provided by Ligand Pharmaceuticals (San Diego, CA). ATRA and 13-cis RA were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The powdered retinoid was dissolved in ethanol at an initial stock concentration of $10^{-2}$ M, stored protected from light at $-80^\circ$C, and further diluted before use. JSH-23 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was dissolved in DMSO at an initial stock concentration of $10^{-2}$ M.

Cell culture and treatment

Human follicular thyroid-carcinoma cell lines FTC133 and FTC238 were kindly provided by C. Schmutzler (Dusseldorf, Germany). Human breast cancer cell line MCF7 and FTC cell lines were propagated in Dulbecco’s modified Eagle’s medium/Ham’s F12 (1:1)(DMEM-F12) (Invitrogen, Cergy Pontoise, France) supplemented with 10% (v/v) or 5% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin (100 units/ml), streptomycin (100 ng/ml) and L-glutamine (2 mM). Human acute promyelocytic leukemia NB4 was propagated in RPMI-1640 medium supplemented with 10% (v/v) FBS and penicillin (100 units/ml) and streptomycin (100 ng/ml) and L-glutamine (2 mM). All cultures were incubated at 37°C in a 5% CO$_2$ humidified atmosphere. For retinoids treatment, cells were split the day before treatment and cultured with serum-containing medium. On the next day, the medium was replaced with retinoid-containing or retinoid-free medium with serum.

Cell viability assay (MTS assay)

Cells were plated in triplicate at 500 cells for FTC238 cells, at 1000 cells for FTC133 and MCF7 cells and at 10000 cells for NB4 cells per well in 96-well plates. Cell viability was measured by the Cell Titer 96 Aqueous One Solution Assay® (Promega, Madison, WI) according to the manufacturer’s instructions.
Invasion assay

The invasiveness of FTC cells was tested using the QCM Collagen Cell Invasion Assay® (Chemicon, Millipore). Cells were seeded in serum-free medium containing bexarotene to the upper collagen-coated transwell. The lower chambers were filled with culture medium containing 10% FBS as a chemoattractant. After a 72 h incubation period, the number of cells that migrated through the filters into the lower chamber was evaluated by a colorimetric method. Briefly invaded cells on the bottom of the insert membrane are incubated with Cell Stain Solution, then subsequently extracted and detected on a standard microplate reader (560 nm).

Cell cycle and apoptosis analysis

For cell cycle analysis, FTC238 cells were permeabilized with 0.1% triton in 1X Phosphate Buffer Saline (PBS) and incubated with 0.5 mL of a 50 µg/mL PI solution containing 20 U/mL RNase-A for 30 minutes (Sigma-Aldrich, Saint Quentin Fallavier, France). For apoptosis, cells were double-stained with FITC-conjugated annexin V and propidium iodide for 15 minutes at 4°C in a calcium-enriched binding buffer (Beckman Coulter, France). All analysis was performed on a FACS Calibur Flow Cytometer with Cell Quest software (Becton Dickinson, France).

RNA isolation and Real-Time PCR

Total RNA was extracted as described by the manufacturer (RNA-PLUS®, Qbiogene, Illkirch, France). Reverse transcription was performed on 1µg of total RNA using the High Capacity RT kit® (Applied Biosystems, Courtaboef, France). cDNAs were quantified using a fluorescence-based real time detection method (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Courtaboef, France) with the TaqMan Universal Master Mix®. The primers and probe sequences for RARs and RXRs are provided as supplementary information (Table 1). Others primers and probe were provided by Applied Biosystems (Gene Expression Assay®, Courtaboef, France). For each sample, parallel PCR reactions were performed for each gene of interest and the porphobilinogene deaminase (PBGD) reference gene to normalize for input cDNA. To quantify the gene expression profile, we used the comparative threshold cycle (Ct) method.
Western blot analysis

Nuclear extracts were obtained as previously described (23) and were quantified by the BCA protein assay® (Pierce, Rockford, IL). Proteins were run in SDS-PAGE gels, transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in 1X PBS at room temperature. The blots were incubated overnight at 4°C with the primary antibody anti-p65 (Upstate, Millipore). After washes with 1X PBS with 0.1% Tween 20, antigen-antibody complexes were detected by means of peroxysdase-conjugated secondary antibody and an enhanced fluoro-chemiluminescence system (ECL-plus®, Amerscham Biosciences, Arlington Heights, IL). Equivalent loading of lanes was controlled by an anti-actin antibody (Sigma-Aldrich, Saint Quentin Fallavier, France). Band intensities were quantified using a ChemiDoc XRS and Quantity One version 4.4.0 software.

Small interfering RNA transfection

Small interfering RNA against RXRG gene (siRXRG) and non-target control small interfering RNA (siCRT) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). siRNAs were transfected into cells at a final concentration of 50 nM by using the transfection reagent Lipofectamine RNAiMax® (Life Technologies, Invitrogen, France) according to the manufacturer’s instructions. Analyses were realized after 48 h of transfection.

Promoter activity assay

For cells transfections, FTC238 cells were seeded in 24-wells plates. After reaching about 80% confluence, the cells were transfected with luciferase gene in the reporter plasmid controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for nuclear factor kappaB (pNF-kappaB-luc) (Stratagene, CA) using lipofectamine (Lipofectamine Reagent Plus®, Invitrogen, France) according to the manufacturer’s instructions. Co-transfection with a β-galactosidase plasmid (pSV-β-gal control vector®, Promega, WI) was used to normalize luciferase activity. After transfection, the medium was replaced by fresh normal growth medium with retinoid and the cells were incubated for 24h. Luciferase (Luciferase assay system®, Promega, WI) and β-galactosidase (β-Gal Reporter Gene Assay®, Promega, WI) assay were performed by a standard procedure.
DNA-binding assay

Assay for NF-kappaB binding was done with the colorimetric Universal EZ-Transcription Factor Assay® (Upstate, Millipore) according to the manufacturer's instructions. Briefly, the capture probe, a double strand biotinylated oligonucleotide containing a NF-kappaB consensus binding site, is mixed with nuclear extract in the streptavidin coated 96-well plate. Bound NF-kappaB was detected by incubation of samples with primary antibody against the p65 subunit provided with the kit. An HRP-conjugated secondary antibody is then used for detection. Quantification of binding levels was evaluated by optical intensities at 450 nm with a microplate reader. A specific unlabeled competitor oligonucleotide was included to ensure that the complex is binding the kappaB site in a sequence specific manner.

Chromatin immunoprecipitation assays (ChIP)

Cells were fixed with 1% formaldehyde in media for 10 min at 37°C to cross-link DNA with proteins. Then, the cells were washed twice with PBS1X containing proteases inhibitors. Cells were resuspended in lysis buffer and sonicated (Bioruptor®, Diagenode) to obtain DNA fragments to 200-1000 bp. After pre-clearing with salmon sperm DNA/protein G agarose beads (Upstate, Millipore), the samples underwent immunoprecipitation with antibodies specific against p65, p300, acetylated histone H3, acetylated histone H4 (Upstate, Millipore) or rabbit/mouse IgG at 4°C overnight. DNA-protein complexes were sequentially washed, eluted (1% SDS, 50 mM NaHCO3) and cross-link reversed by heating at 65°C overnight. DNA was recovered by proteinase K digestion, phenol-chloroforme purification and ethanol precipitation. DNA was amplified by real-time PCR using the Power master mix SYBR green® on a ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). Primers sets were designed to overlap the NF-kappaB binding sites of the CYR61 promoter and the RARE site of the RARB2 promoter. Specificity of the assay was tested by amplification of an intergenic region (between the GAPDH gene and the chromosome condensation-related SMC-associated protein gene) not bound by transcription factors (negative controls). The primers sequences are provided as supplementary information (Table 1). Data for each immunoprecipitated sample were normalized to its corresponding input (total chromatin fraction) and expressed as fold enrichment above background (IgG controls) or as fold change compared to untreated sample using the comparative Ct method.
**Statistical methods**

All data were obtained from at least three independent experiments performed in triplicate, and the results are presented as mean and standard error of the mean. To demonstrate statistical significance a two tailed Student's t-test was used (* denotes a p-value of at least < 0.05).

**Clinical study**

In parallel to these in vitro studies, 17 patients (8 male and 9 female, median age at diagnostic 50 yrs) with progressive metastatic DTC and decreased or absent RI uptake were included in a prospective open clinical trial (CRIC99340). These patients (9 PTC and 8 FTC including 2 Hurthle and 1 insular tumour) had previously received multiple treatments: high cumulative RI dose (median 13.7 GBq), surgery of metastatic sites (#8), External Beam Therapy (#3), cimentoplasty (#1), and bisphosphonate (#1). Group 1 (n=13) had pulmonary, bone and/or mediastinal macrometastases and group 2 (n=4) had lung and/or mediastinal infra-centimetric metastases. After informed consent they all received 13-cis RA for 8 weeks (1 mg/kg/day), followed by RI treatment (3.7 GBq) after 4 weeks of LT4 withdrawal. Treatment was repeated when re-induction of RI uptake on post-therapy scan was obtained. In the absence of RI uptake, a further regimen with bexarotene (300 mg/m²/day) for 8 weeks was undertaken, then followed by RI treatment (3.7 GBq) after rhTSH stimulation to avoid associated central hypothyroidism (24). To become eligible to bexarotene, patients were required to have acceptable organ function defined as follows: adequate hematologic function, defined as WBC ≥ 3,000/µL, absolute neutrophil count ≥ 1,500/µL, and platelet count ≥ 100,000/µL; normal coagulation parameters; bilirubin ≤ 1.5 times the upper limit of normal (ULN); AST/ALT ≤ 2.5 x ULN; and serum creatinine ≤ 2.5 x ULN (or creatinine clearance > 40 mL/min). In addition, only patients who had a normal baseline fasting triglyceride level were allowed to enter this study; triglycerides could be normalized before study entry with use of an anti-lipemc agent. Response was immediately judged on RI re-induction on post-therapy scan, and 4-6 months later on TG levels and tumour size by conventional imaging techniques according to RECIST criteria (25).
RESULTS

Distinct differentiation potential by ATRA, 13-cis RA and bexarotene in Follicular Thyroid Cancer (FTC) cell lines

FTC133 and FTC238 cells provide a well established model for the study of differential thyroid cancer retinoid sensitivity based on different criteria, such as thyroid specific functions (DIO1 and NIS expression), cell-cell or cell-matrix interaction (intercellular adhesion molecule-1 and E-cadherin), differentiation markers (alkaline phosphatase, CD97 and TG), cell growth and tumorigenicity (26). In order to characterize and compare the cell lines used for this study, we quantitatively assessed the expression of genes associated with thyrocyte differentiation (PAX8, TPO, thyroid stimulating hormone receptor (TSHR), DIO1, NIS and TG) (5). Increased expression of these differentiation-associated genes was observed in FTC133 cells after 24 h treatment with ATRA $10^{-6}$ M. In contrast, no difference was observed in the FTC238 cell line (Fig. 1A). Thus, FTC238 remains refractory to ATRA differentiation induction contrary to the FTC133 cell line. After treatment of FTC238 cells with 13-cis RA or bexarotene, currently used retinoids in thyroid cancer patients, no up-regulation of thyroid differentiation markers was noted (Fig. 1A) correlating with absence of morphological aspects of differentiation as observed in FTC133 cells which harbour a more homogenous and spindle shaped aspects after ATRA or 13-cis RA treatment (Supplementary Fig. S1).

Up-regulation of RARB and RXRG expression correlates with achievement of ATRA differentiation in FTC cell lines

We have previously shown that FTC133 and FTC238 cell lines present respectively low and null expression of RARB mRNA levels (21). In this study, we examined the gene expression levels of the different RARs and RXRs by quantitative RT-PCR. RARA, RARG, RXRA and RXRB are expressed in both cells lines while RARB and RXRG are barely detectable. RARA mRNA is the major retinoic acid receptor mRNA detected in FTC133 cells whereas RXRA is predominant in FTC238 cells. Globally, compared to FTC133 cells, FTC238 cells have a lower expression of RARs and a higher expression of RXRs (Fig. 1B).

In cancer cells with altered RA receptors, differentiation induced by pharmacological concentrations of RA is associated with the up-regulation of receptor expression via activation of endogenous normal receptors by the retinoids (27). Up-regulation of RARs and RXRs gene expression was monitored in the two cells lines after incubation with ATRA $10^{-6}$ M. ATRA induced a significant RARB and RXRG mRNA
expression in only the RA-differentiation sensitive FTC133 cell line but not in RA-resistant FTC238 cells (Fig. 1C). Thus ATRA differentiation refractoriness of the FTC238 cell line may be related to absence of RARB and RXRG expression and its induction by ATRA.

**Bexarotene induces RARB and RXRG expression and inhibits cell growth and invasion in ATRA-resistant FTC238 cells**

RARs and RXRs gene expression was monitored in both cell lines after incubation with either 13-cis RA or bexarotene. These retinoids were not more effective than ATRA to induce RARs and RXRs expression in FTC133 cell line (data not shown). Interestingly, in the ATRA-resistant FTC238 cells both retinoids allowed a significant recovery of RARB and RXRG expression levels (Fig. 2A). This up-regulation of RA receptors by bexarotene could not be linked to induction of differentiation-associated genes (Fig. 1A) but to a decrease of cell growth in a dose-dependent manner (Fig. 2B). Inhibition of cell growth by bexarotene in FTC238 cells was not due to apoptosis as no increase in the percentage of Annexin V positive cells was noted after incubation with bexarotene (Fig. 2C) but rather to an inhibition of proliferation. Cell population in the G1 phase increased from $75.4 \pm 1.3\%$ to $89.8 \pm 5.5\%$ compared to control ($p=0.002$). This increase was accompanied by a decrease of cell population in the S/G2/M phase ($13.2 \pm 4.3\%$ compared to $23.4 \pm 4.2\%$, $p=0.01$) (Fig. 2D). The small increase in subG1 population was not significant confirming the low level of Annexin V positive cells after bexarotene. As the FTC238 cell line is derived from a pulmonary metastasis, we also investigated the invasive activity of these cells by their ability to migrate through a collagen layer. A 30% decrease of invasion was observed upon bexarotene treatment ($p=0.02$) (Fig. 2E). Thus bexarotene restores RARB and RXRG expression in FTC238 cell line and inhibits cell proliferation and invasion but does not increase differentiation.

**Bexarotene effectiveness in DTC patients**

In a clinical proof of concept open clinical trial, 17 DTC patients with progressive metastatic DTC and decreased or absent RI uptake, were first treated by 13-cis RA for 8 weeks (1 mg/kg/day), followed by RI treatment (3.7 GBq) after 4 weeks of LT4 withdrawal. Patient’s characteristics and clinical response are presented in supplementary table S2. Clinical response was assessed on RI uptake and RECIST criteria (25). After one course of 13-cis RA, one patient had a partial response (PR), 7 patients a stable disease, and 9 patients a progressive disease. Treatment with 13-cis RA induced RI uptake in metastases of 6/17 (35%)
patients, irrespective of the clinical response, though the patient with a PR did have also induced RI uptake (patient n° 1). All responding patients had macroscopic metastatic tumours (group 1). Of these 6 patients, three received a second 13-cis RA treatment, also followed by a new RI re-induction. Responses remained the same. Of note two of these patients had a tumour size reduction of more than 50% (patients n°1 & 3).

Bexarotene treatment was undertaken in 5 patients who did not respond to the first course 13-cis RA and were eligible for bexarotene. Two patients (patients n° 8 & 9) presented an increase in RI uptake suggesting that resistance to 13-cis RA could be bypassed in some metastases by bexarotene as illustrated in Fig. 3. These 2 patients had macroscopic metastatic tumours (group 1). The RI uptake responses noted in Group 1 with macroscopic metastases may well be explained by detection limitations. Tumour regression or stabilisation was noted by conventional imaging techniques in 3 other patients treated by bexarotene but with no increased RI uptake (patients n° 10, 14, & 15). Thus, tumour response was not always correlated to RI uptake correlating with the in vitro data. Furthermore, metastasis heterogeneity led to differential metastases responses in a given patient.

**Bexarotene inhibits transactivation and expression of NF-kB target genes**

In order to further analyse the mechanisms by which bexarotene inhibits FTC238 cell growth and invasion, we focused our studies on the Nuclear Factor kappaB (NF-kappaB) pathway. NF-kappaB, a transcription factor known to be a key regulator of genes involved in the control of proliferation, apoptosis and invasion is constitutively activated in most of cancers (28). Moreover, members of the nuclear receptor family have been shown to interfere with NF-kappaB signalling (29). We thus hypothesized that the activation of the RXR pathway by bexarotene could interfere with NF-kappaB and analysed the expression of genes known to be downstream effectors of NF-kappaB (Fig. 4A). Bexarotene treatment decreased the expression of *MYC*, *CCND1*, *CYR61* and *CXCL1* genes involved in proliferation and invasion, but not the expression of *BCL2* and *XIAP* genes correlating with the inhibition of proliferation and invasion and absence of apoptosis observed upon bexarotene treatment (Fig. 4B). This mechanism requires the presence of *RXRG* expression restored by bexarotene, as the inhibition of NF-kappaB target genes expression by bexarotene is not observed following down-regulation of *RXRG* by siRNA (Fig. 4C). To further analyse NF-kappaB activity in FTC238 cells we transiently transfected FTC238 cells with a luciferase reporter plasmid under the control of a synthetic promoter that contains direct repeats of the transcription recognition sequences for NF-kappaB. Bexarotene repressed both constitutive basal and phorbol myristate acetate
(PMA)-induced NF-kappaB transactivation activity (Fig. 4D). Thus bexarotene through induction of RXRG represses transcriptional activity and target gene expression of NF-kappaB and results in loss of cell growth and invasion. A decrease of proliferation may also be obtained on these cells by inhibiting NF-kappa pathway by a known chemical inhibitor JSH-23 confirming the biological effect of activated NF-kappaB pathway in these cells (Fig. 4E).

Interestingly, bexarotene targeting of the NF-kappaB pathway may also be observed in other thyroid cancer cells (FTC133) and in acute myeloid cells (NB4) albeit in different degrees but not in breast cancer cells (MCF7). Inhibition of cell proliferation was essentially achieved in the NB4 cell line (Supplementary Fig. S2A) while a decrease of invasion capacity was observed in FTC133 cell line (Supplementary Fig. S2B). Reduced expression of NF-kappaB proliferation and invasion target genes was noted in a cell-context manner (Supplementary Fig. S2C).

**Bexarotene represses NF-kB pathway at the transcriptional level**

To explore the mechanism(s) whereby bexarotene induces NF-kappaB repression in FTC238 cells, we examined the effect of bexarotene on different steps of the NF-kappaB pathway (Fig. 5A). First, we analyzed the translocation of the active p65 subunit of NF-kappaB to the nucleus compartment. No significant change in p65 levels in nuclear extracts was noted after treatment suggesting that bexarotene mediated its effect on NF-kappaB repression at the transcriptional level (Fig. 5B). However, p65 binding on a synthetic NF-kappaB responsive element was not modified in nuclear extracts of treated cells in the presence or absence of both PMA (Fig. 5C). Furthermore, chromatin immunoprecipitation (ChIP) assay using anti-p65 antibody on NF-kappaB binding sites of CYR61 promoter gene confirmed these results (Fig. 5D). Thus these data suggest that bexarotene represses transactivation of NF-kappaB genes without interfering with either the translocation or the binding of p65 to NF-kappaB responsive elements.

Interestingly, by ChIP assay, the co-activator p300 was detected on the p65 binding site of CYR61 promoter gene (Fig. 5E). As expected, bexarotene induced p300 recruitment at the RARE with increased level of histones H3 and H4 acetylation (Fig. 5F). However, at p65 binding sites, bexarotene released p300 (Fig. 5E) and decreased levels of histone acetylation (Fig. 5F) resulting in reduced promoter permissiveness. Thus, in thyroid cancer cells, bexarotene represses NF-KappaB target gene expression through downregulation of the transactivation potential of p65 on its binding site via the bexarotene-RXR dependent release of the nuclear coactivator p300 and resulting histone deacetylation.
DISCUSSION

RA therapy has been attempted in thyroid cancer patients essentially to restore iodine uptake for treatment of recurrent metastases by radioactive iodine. We confirmed similar response rate on RI uptake (35%) obtained by others groups (30 to 40%) (14-16). Increased RI uptake in thyroid cancer may be explained by NIS expression. Induction of NIS expression by RA in breast cancer cells was reported to be mediated by genomic and/or non genomic mechanism requiring RARs and RXRs expression (30, 31). Since bexarotene increases RARB and RXRG expression in FTC238 cells without induction of NIS, it appears that thyroid cancer cells could have other regulation mechanisms of NIS expression.

Using follicular thyroid cancer cell lines with differential response to retinoids we have previously identified that one of the mechanism inherent to RA resistance may lie in the non permissive status of the RARB gene promoter, relieved by HDAC inhibitors (21). The present study suggests that along with RARβ, RXRγ may equally be involved in thyroid oncogenesis, as previously suggested (32) and represents a novel biomarker of RA therapy. We identified, bexarotene, the first highly selective RXR ligand to be currently studied in clinical (33-35) as a growth inhibitor of the RA-differentiation resistant FTC238 cell line, to an extent not achieved with ATRA. In cutaneous lymphoma cell lines, bexarotene induces arrest of cell cycle in G1 phase and apoptosis with activation of caspase-3 and PARP cleavage as well as down-regulation of survivin (36). A proof of principle clinical trial with bexarotene in patients with non-small cell lung cancer showed regression of biomarkers such as cyclin D1, cyclin D3 and EGFR (37). In this study we show that bexarotene down-regulates MYC and CCND1 expression in the ATRA resistant FTC238 cells, extending the notion that rexinoids can by-pass absence of RARβ expression. Interestingly, in these cells, bexarotene up-regulates RARB and RXRG which ATRA treatment fails to do. It has previously been reported, albeit in another histological type, anaplastic thyroid cancers, that the presence of RARβ and RXRγ was a prerequisite for growth inhibition by RXR ligands (38). Thus in parallel with ATRA differentiation efficacy which requires up-regulation or restoration of RARβ and RXRγ levels, bexarotene growth inhibition may also require the up-regulation of both receptors. This growth inhibition could not be attributed to enhanced apoptosis as annexin V positivity was not detected but rather to a G1 phase arrest of the cycle in accordance with the down-regulation of CCND1 expression. In line with these in vitro studies, our clinical results show that bexarotene treatment of thyroid cancer patients who failed to respond to 13-cis RA therapy leads, in
some patients, not only to radioiodine uptake but also to a reduction in size and number of metastases without apparent differentiation and radioiodine uptake.

Past and current concept of metastases initiating tumour cells highlights the importance of cell adhesion proteins, proteases and angiogenesis. Retinoids and bexarotene have been shown to inhibit angiogenesis and metastasis in lung and thyroid cancer murine models (39, 40). RA was shown to decrease secretion of VEGF in thyroid tumour cells in vitro (39). In our study, CYR61 and CXCL1, proteins known to be involved in the invasion process, were down-regulated by bexarotene in FTC238 cells and may explain the observed decrease of cell invasion through a collagen layer. CXCL1 is a small cytokine belonging to the CXC chemokine family involved in angiogenesis, inflammation and tumorigenesis. Overexpression of CXCL1 in melanocytes is associated with constitutive activation of NF-kappaB leading to tumour progression (41). CYR61, a member of a family of secreted matrix-associated proteins, is described as a key regulator of breast cancer invasion (42). The observed rapid down-regulation of CYR61 gene expression induced by bexarotene in FTC238 cells favours a direct transcriptional control. Response elements for both retinoic acid receptors dimers and NF-kappaB are present in the promoter region of the CYR61 gene. As CYR61 is involved also in pathways leading to enhance NF-kappaB activation via integrins/PI3K/Akt (43), we may also surmise that in these cells an autocrine loop between CYR61 and NF-kappaB maintains tumorigenicity. NF-kappaB is known to be constitutively activated in thyroid cancer cell lines and patient samples and linked to resistance to apoptosis (44, 45). We found NF-kappaB constitutively activated in FTC238 cells with high levels of p65 detected in the nucleus and in protein complexes bound to NF-kappaB sites. Interestingly, incubation with bexarotene results in decreased transactivation of an NF-kappaB reporter plasmid strongly suggesting that the inhibition of cell growth observed with bexarotene in FTC238 cells is achieved via repression of NF-kappaB and down-regulation of genes such as MYC and CCND1. Though repression of NF-kappaB may results from various mechanisms at the cytoplasmic and nuclear level, in FTC238 cells, bexarotene did not inhibit p65 translocation to the nucleus nor its binding to NF-kappaB responsive elements, contrary to results reported with fenretinide, a synthetic retinoid (46). Members of the nuclear receptor family, including glucocorticoid, estrogen, progesterone and androgen receptors and more recently PPARγ have been shown to inhibit NF-kappaB activity and to physically interact with p65 in vitro (29). Furthermore, competition for DNA NF-kappaB response elements between NF-kappaB and other transcription factors may occur. This was shown for the same sequence in the promoter of the human GnRH II gene for p65 and RARα/RXRα (47). However in FTC cells, neither could we detect RXR in the p65
transcriptional complex by ChIP nor RXR/p65 interaction by co-immunoprecipitation. These results may well be explained by the low expression of endogenous RXR in these cells. However, other mechanisms may be present in FTC cells. Indeed, our data suggest that bexarotene induces CYR61 promoter repression at the p65 binding site by releasing the p300 coactivator and inducing histone deacetylation. In LPS-activated macrophages, Na et al. have also demonstrated that retinoid-mediated suppression of the IL-12 production may involve both inhibition of NF-kappaB-DNA interactions and competitive recruitment of p300 and SRC-1 between NF-kappaB and RXR (48). Thus we can surmise that in FTC cells, the activation of RXR by its ligand, bexarotene, leads to the release of p300 whether by competitive recruitment or by conformational change in the p65 transcriptional complex resulting in a histone deacetylation, promoter repression and a down regulation of NF-kappaB target genes.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).
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FIGURE LEGENDS

Figure 1: ATRA-induced differentiation is correlated with RARB and RXRG expression levels in FTC cells lines. (A) Distinct thyroid differentiation marker induction by retinoids in FTC cells lines after 24 hours treatment with ATRA 10^{-6} M, 13-cis RA 10^{-6} M, bexarotene 10^{-6} M or vehicle. (B) RARs and RXRs mRNA expression levels under basal conditions in FTC cell lines. mRNA levels were measured by quantitative RT-PCR normalized for the PBGD gene as reference. Results are expressed as relative mRNA expression using comparative Ct method. (C) RARs and RXRs mRNA induction after 2 hours treatment with ATRA 10^{-6} M in FTC133 and FTC238 cells. mRNA levels were measured by quantitative RT-PCR normalized for the PBGD gene as reference. Results are expressed as fold increase based on the expression observed in the absence of ATRA (arbitrarily set at 1) using comparative Ct method.

Figure 2: Bexarotene restores RARB and RXRG expression and reduces cell proliferation and invasion in ATRA-resistant FTC238 cells. (A) RARs and RXRs mRNA expression after 2 hours treatment with bexarotene 10^{-6} M were calculated by quantitative RT-PCR via comparative Ct method, using PBGD as reference gene. Results are expressed as fold induction based on the basal expression observed in the absence of retinoid (arbitrarily set at 1). (B) Cell viability at day 6 under indicated concentrations of retinoid was measured by a MTS assay. Results are expressed as percentage of cell viability compared with the absence of retinoids. (C) Cells were treated for 6 days with bexarotene 10^{-6} M. Cells were incubated with annexin V and PI and then analyzed by flow cytometry. For the positive control we incubate cells in 3% formaldehyde during 30 min. Values represent apoptotic cells defined as annexin V + / PI – cells. (D) Cell cycle was analyzed by flow cytometry with PI after 6 days treatment with bexarotene 10^{-6} M. (E) Cells were plated in serum-free medium in a Transwell and treated for 3 days with bexarotene 10^{-6} M. Invasion capacity was measured by colorimetric reaction. Optical density (DO) at 540 nm was correlated to the number of cells invading through the collagen layer in transwell.

Figure 3: Post-therapy scan. (A) Before treatment. (B) After 8 weeks of 13-cis RA treatment: no RI uptake at expected metastatic sites. (C) After 8 weeks of bexarotene treatment: RI uptake at cervical metastatic lesion subsequently confirmed by positive cytology.
Figure 4: Bexarotene represses transactivation and expression of NF-kappaB target genes. (A) Genes regulated by NF-kappaB and involved in the control of proliferation, apoptosis and invasion. (B) mRNA expression of NF-kappaB regulated genes after 2 hours treatment with bexarotene 10^{-6} M measured by quantitative RT-PCR. Results are expressed as fold change based on the basal expression observed in the absence of bexarotene (arbitrarily set at 1) via comparative Ct method, using PBGD as reference gene. (C) After 48 h of transfection with a RXRG siRNA, cells were treated with bexarotene for 2 hours and RT-qPCR for NF-kappaB target genes performed. (D) Cells transiently transfected with a NF-kappaB binding sites-luciferase construct were exposed to bexarotene 10^{-6} M or/and PMA 10 ng/mL for 24h. Luciferase activity was normalized with β-galactosidase activity. (E) Cell viability at day 6 under indicated concentrations of JSH-23 was measured by a MTS assay. Results are expressed as percentage of cell viability compared with the absence of drug.

Figure 5: Bexarotene represses NF-kappaB pathway at the transcriptional level. (A) Schematic representation of NF-kappaB pathway. (B) Cells were treated with bexarotene 10^{-6} M for the indicated times. Nuclear extracts were prepared, fractioned on SDS-PAGE and electrotransferred to nitrocellulose membranes. Immunoblots were performed with an anti-p65 antibody. Anti-actin antibody was used to control equivalent protein loading. (C) Nuclear extracts of 2 h bexarotene and/or PMA-treated cells were incubated with an oligonucleotide containing a NF-kappaB consensus binding site. Bound NF-kappaB was detected by incubation of samples with an anti-p65 antibody and detected by colorimetric reaction using an HRP-conjugated secondary antibody. (D) Cells were exposed to bexarotene 10^{-6} M or/and PMA 10 ng/mL for 2 hours. Cross-linked protein-DNA complexes were immunoprecipitated with anti-p65 antibody. Primers flanking the NF-kappaB binding site of the CYR61 promoter were used in quantitative PCR amplification. Samples were normalized to their respective input and values expressed relative to untreated counterparts. (E) Cross-linked protein-DNA complexes were immunoprecipitated with anti-p300 antibody. Primers flanking the NF-kappaB binding site of the CYR61 promoter and the RARE of RARB2 promoter were used in quantitative PCR amplification. Samples were normalized to their respective input and expressed as fold enrichment above background (IgG controls). (F) Cross-linked protein-DNA complexes were immunoprecipitated with anti-acetylated histone H3 and anti-acetylated histone H4 antibodies. Samples were normalized to their respective input and values expressed relative to untreated counterparts.
Figure 1

A

Figure 1A shows the mRNA expression (fold increase) of various genes under different treatments. The x-axis represents different treatments: ATRA, 13-cis RA, and Bexarotene. The y-axis represents mRNA expression as fold increase. The treatments are divided into two groups: FTC133 and FTC238. The treatments include NIS, DIO1, TG, TPO, TSHR, and PAX8.

B

Figure 1B displays the relative mRNA expression of RARA, RARB, RARG, RXRA, RXRB, and RXRG. The relative mRNA expression is shown for FTC133 and FTC238.

C

Figure 1C illustrates the mRNA expression (fold increase) for ATRA under FTC133 and FTC238 conditions. The genes analyzed include RARA, RARB, RARG, RXRA, RXRB, and RXRG. The data indicates some significant changes in mRNA expression.
Figure 2

A

![Bar chart showing mRNA expression (fold increase) for RARA, RARB, RARG, RXRA, RXRB, and RXRG.

B

![Viability graph for ATRA and Bexarotene at 0.1μM, 1μM, and 10μM.

C

![Annexin V assay for Untreated, Bexarotene (2.9 ± 1.8%), and Positive control (12.6 ± 2.7%).

D

![Flow cytometry histograms for G1 and S/G2/M phase distributions in Untreated and Bexarotene conditions.

E

![Invasion assay for Untreated and Bexarotene conditions.]
Figure 3

A
Before Treatment

B
After 13-cis RA

C
After Bexarotene
Figure 4

A

NF-κB

Proliferation

↑ CCND1

C-MYC

Invasion

↑ CYR61

CXCL1

Apoptosis

↑ BCL2

XIAP

B

mRNA expression (fold change)

CCND1

CMYC

CYR61

CXCL1

Bcl-2

XIAP

C

mRNA expression (fold change)

CCND1

CMYC

CYR61

CXCL1

siCRT

siCRT

siRXRG

Untreated

Bexarotene

D

NF-κB site

Luciferase

p50

p65

Luciferase activity

Bexarotene

PMA

E

Viability / untreated (%)

0,1 µM

1 µM

10 µM
Figure 5

A

PMA → IkBα → proteasome

IkBα

p50 p65

cytoplasm

nucleus

DNA NF-κB site

B

Bexarotene

p65

actin

ratio

0 1 1.01 0.95 0.99 0.93 0.95 1.4

0 1 2 3 4 5 6

p65 binding (fold change)

C

Bexarotene - + - + +
PMA - - + + +

D

Bexarotene - + - + +
PMA - - + + +

E

Untreated

Bexarotene

p300 fold enrichment above background

F

Untreated

Bexarotene

Fold change

CYR61 RARB2 CYR61 RARB2

H3 acetylation H4 acetylation
Bexarotene via CBP/p300 induces suppression of NF-kappaB-dependent cell growth and invasion in thyroid cancer

Audrey Cras, Beatrice Politis, Nicole Balitrand, et al.

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