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Regulation of *CYP26A1* expression by selective RAR and RXR agonists in human NB4 promyelocytic leukemia cells

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Abstract

All-*trans* retinoic acid (ATRA) can induce complete remission in acute promyelocytic leukemia (APL), but resistance to this treatment develops rapidly partly due to increased ATRA metabolism. Among the cytochrome P450s (CYPs) involved in ATRA metabolism, the ATRA-inducible cytochrome P450 26A1 (CYP26A1) is particularly active although the molecular mechanisms involved in its regulation are not well defined in the target leukemia cells. To study *CYP26A1* expression and regulation in APL cells, we used the NB4 promyelocytic leukemia cell line. *CYP26A1* constitutive expression was barely detectable in NB4 cells, but ATRA could induce high levels of *CYP26A1* expression, which reached a maximum at 72 h. To further define *CYP26A1* induction mechanisms in the NB4 leukemia cells, we used RARs and RXR selective agonists. The RARα agonist BMS753 could elicit maturation, as expected, but not *CYP26A1* expression. Treatment with the RARβ agonist BMS641, or the RARβ/γ agonist BMS961, could not elicit maturation, as expected, nor induce *CYP26A1* expression. Because *CYP26A1* expression could not be induced by RAR ligands alone, NB4 cells were then co-treated with the RXR agonist BMS649. The RXR agonist alone could not induce *CYP26A1* expression, nor in combination with either the RARβ agonist or the RARβ/γ agonist. However, the combination of the RXR agonist and the RARα agonist could elicit a marked induction of *CYP26A1* expression. In conclusion, we have shown that *CYP26A1* induction is not essential for the granulocytic maturation of NB4 leukemia cells, and that *CYP26A1* induction requires the activation of both RARα and RXR in these cells.

Keywords: *CYP26A1* regulation; NB4 leukemia cells; RAR; RXR; Maturation

1. Introduction

Vitamin A and its derivatives (retinoids) are involved in important physiological processes such as reproduction, cell proliferation, differentiation, apoptosis, and embryonic development [1]. Retinoids also exhibit several pharmacological properties useful in the prevention and treatment of several cancers [2], including acute promyelocytic leukaemia (APL), which is particularly responsive to all-*trans*-retinoic acid (ATRA) [3]. APL is a subtype of myeloid leukemia (M3), characterized by the accumulation of cells blocked at the promyelocytic stage. This leukemia exhibits a specific chromosomal translocation t(15;17) involving the PML locus on chromosome 15 and the RARα locus on chromosome 17, thus generating a chimeric gene PML-RARα, translated into a chimeric nuclear receptor PML-RARα which functions as an aberrant receptor [4–9].

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid receptor (RAR) family and the retinoid X receptor (RXR) family. These two families comprise three subtypes (α, β, and γ) and include several isoforms [10]. These receptors belong to the superfamily of nuclear hormone receptors and act as ligand-activated transcription factors [10,11]. RARs function as heterodimer together with RXR in vitro and in vivo. The ligand–receptor complexes act as inducible transcription regulators of several genes by binding to specific retinoic acid response elements (RARE). The natural ligands for the RARs are ATRA, its oxidized metabolites, and also its

Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; CYP, cytochrome P450; CYP26A1, cytochrome P450 26A1; RAR, retinoic acid receptor; RXR, retinoid X receptor

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stereoisomers 9-*cis*-RA and 13-*cis*-RA, whereas RXRs are activated by 9-*cis*-RA [10,12]. The RXR can also act as homodimer on transcription activation in vitro via the retinoid X response element (RXRE), and also as heterodimer with other nuclear receptors, e.g., the thyroid hormone receptor (TR), the peroxisome-proliferator activated receptor (PPAR), and the Vitamin D receptor (VDR), on their specific response elements.

ATRA, which is one of the most active natural retinoid, is metabolized into several oxidized metabolites [13,14] by human cytochrome P450s (CYPs) among which the following ones appear as the most active: CYPs 3A7, 3A5, 2C18, 2C8, 3A4, 2C9, 1A1, and 4A11 [15]. In addition to these identified CYPs, the novel cytochrome P450 26 (CYP26), which is inducible by ATRA and exhibits specific 4-hydroxylase activity, has been identified in zebrafish, mouse and humans [16–18]. Since the discovery of the first human CYP26 (now named CYP26A1), two other CYP26 (CYP26B1 and CYP26C1) have been identified [19–21]. CYPs 26A1 and 26B1 are highly specific for the hydroxylation of ATRA, and are less effective for the hydroxylation of its isomers 9-*cis*-RA and 13-*cis*-RA [22,23], whereas CYP26C1 metabolizes both ATRA and 9-*cis*-RA [20].

Although most patients can achieve clinical remission upon treatment with ATRA in combination with chemotherapy, relapse is observed in about 30% of patients. Patients in relapse frequently become resistant to ATRA due to a selection of non-PML-RARα leukemic clones, to increase cellular levels of cellular retinoic acid-binding proteins (CRABPs), to mutation in the E domain of the mutated PML/RARα, or to increased metabolism and clearance of ATRA due to induction of cytochrome P450s [24–26].

Because *CYP26* induction is involved in ATRA resistance, the purpose of this work was therefore to investigate *CYP26A1* expression and regulation in the target human acute promyelocytic leukemia cells, because *CYP26A1* expression could modulate ATRA levels in cancer cells. To this purpose, we used the human NB4 cell line which is sensitive to ATRA induced-maturation and exhibits the characteristic t(15;17) translocation observed in APL [27]. In the present study, by employing selective RAR and RXR receptor agonists, we show that the induction of *CYP26A1* expression requires the activation of both RARα and RXR nuclear receptors in the NB4 cell line, and that *CYP26A1* induction is not essential for the granulocytic maturation of these leukemia cells.

2. Experimental procedures

2.1. Materials

BMS753, BMS641, BMS961, and BMS649 were kindly provided by Dr. Hinrich Gronemeyer (IGBMC, Illkirch, France). ATRA and TTNPB was purchased from Sigma–Aldrich. α-^{32}P-UTP was purchased from NEN Life Science Products. The rabbit polyclonal antibodies against RAR and RXR receptors, RARα [RPα(F)], RARβ [RPβ(F)2], RARγ [RPγ(mF)], RXRα [RPα(mF)], and RXRβ [RPβ(F)], were kindly provided by Dr. Cécile Rochette-Egly (IGBMC, Illkirch, France). Stock solutions of retinoids (10^{-2} M) were prepared in ethanol and stored protected from light at −80 °C.

2.2. Cell culture

The human NB4 cells [27] were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (PAA Laboratories), 2 mM L-glutamine, 50 units/ml penicillin G, 50 μg/ml streptomycin (Life Technologies, Inc.), and were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cell density (10^5 cells/ml) was assessed with an electronic particle counter and size analyzer (Coulter Electronics). Stock solutions of retinoids (at 10^{-2} M in ethanol) were further diluted in culture medium to the desired concentration indicated in each experiment. Final concentration of ethanol in culture medium did not exceed 0.01%. All experimental procedures were light protected.

2.3. RNA isolation

Total RNA was extracted from NB4 cells after the indicated treatment and exposure time, using the Trizol Reagent (Invitrogen) following the manufacturer's instructions, and stored at −80 °C.

2.4. RNAse protection assay

A 332 bp fragment of *CYP26A1* cDNA was generated by RT-PCR using mRNA from NB4 cells treated with ATRA for 48 h at 1 μM (for method description, see RT-PCR assay below). This fragment was cloned in the pGEM-T vector (Promega) and sequenced. This plasmid was used to generate anti-sense mRNA probe for CYP26A1 mRNA detection. The plasmid was linearized by the restriction enzyme SacII (Biolabs, Inc.). As the linearization generated 3′ overhang ends, the linearized plasmid was subjected to the DNA polymerase I large fragment (Klenow) (Promega) for 15 min at 22 °C. Transcription of anti-sense CYP26A1 probes was performed using the RiboProbe In Vitro Transcription System SP6/T7 Kit (Promega) and α-^{32}P-UTP (NEN, Life Science Product, Inc.) according to the manufacturer's instructions. hL32 and hGAPDH probes were also radiolabeled and used as controls. RNAse protection assays were performed using the RiboQuant Ribonuclease Protection Assay Kit (Pharmingen) as described by the manufacturer. Briefly, 20 μg of total RNA were hybridized to the radiolabeled anti-sense probes described above (10^4 cpm/μl for each probe) in 10 μl of

hybridization buffer overnight at 56 °C. Then, the mixtures were submitted to an RNAse treatment to digest the remaining free probes and the single-strand RNA. The remaining 'RNAse-protected' probes were purified and electrophoresed on a denaturing 5% polyacrylamide gel. After drying the gel was exposed to a Kodak X-OMAT AR film. Gels were quantified by scanning densitometry using the Multi-Analyst software.

2.5. RT-PCR assay

cDNAs were synthesized by reverse transcription from 1 μg of mRNA from cells treated as indicated in the various experiments, using the Reverse Transcription system Kit (Promega) as described by the manufacturer. PCR was performed for 35 cycles on 2 μl of cDNA using the PCR Core System I Kit (PROMEGA, France) according to the manufacturer's instructions. The oligonucleotides used were: 5′-GAGACCCTTCGACTGAATCC-3′ (sense), and 5′-GGAGGTCCATTTAGAAGCTGC-3′ (anti-sense) for hCYP26A1, which generate a 332 bp fragment; 5′-CTCAGACACCATGGGGAAGGTGA-3′ (sense), and 5′-ATGATCTTGAGGCTGTTGTCATA-3′ (anti-sense) for hGAPDH that generate a 450 bp fragment.

2.6. May-Grünwald Giemsa staining

Cytospin preparations of 2×10^5 cells were allowed to air dry, and incubated in pure May-Grünwald solution for 5 min, then in 50% May-Grünwald/water for 10 min, washed in water and incubated in a 20% Giemsa/water solution for 20 min. The slides were then washed in water, air dried, and examined under immersion microscopy (Leica, magnification ×63).

2.7. Western blot analysis

Nuclear protein extracts were prepared from untreated NB4 cells as follows: the cells were harvested and washed once with cold PBS, centrifuged at 1300 rpm at 4 °C, and resuspended in buffer A (10 mM HEPES pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT) for 10 min at 4 °C. After centrifugation for 1 min at 5000 rpm at 4 °C, cells were lysed for 1 min on ice in buffer A containing 0.2% NP-40, 1.5 μg/ml protease inhibitor cocktail (PIC) and 0.2% PMSF and centrifuged 1 min at 5000 rpm. Supernatants corresponding to cytoplasmic proteins were collected, and the nuclear proteins contained in the pellets were extracted with buffer B (20 mM HEPES pH 7.9; 0.2 mM EDTA; 0.5 mM DTT; 0.42 M NaCl; 25% (v/v) glycerol; 1.5 μg/ml PIC; 0.2 mM PMSF) for 20 min on ice and centrifuged 1 min at 5000 rpm at 4 °C. Cytoplasmic and nuclear extracts were frozen at −80 °C. Nuclear protein extracts (10 μg) were boiled in presence of 5% β-mercaptoethanol and loaded onto 8% SDS-polyacrylamide gels, electrophoresed, and blotted onto PVDF membranes (Immobilon P, Millipore). The membranes were blocked with 4% non-fat milk in PBS for 3 h at room temperature, then incubated with primary rabbit antibody directed against RARα, RARβ, RARγ, and RXRα (1/4000), and RXRβ (1/1000) in PBS 0.1% Tween 20 (PBS-T), 0.5% milk for 18 h at 4 °C. Membranes were washed five times for 5 min with PBS-T, incubated with horseradish peroxidase-coupled anti-rabbit antibody (1/10,000) (Jackson Laboratories) for 30 min in PBS-T at room temperature, and washed five times for 5 min with PBS-T. Detection was performed as described in the ECL Kit (Amersham). Protein from HL60, HeLa, and COS-7 cells transfected with RARβ and RARγ were used as controls.

3. Results

3.1. CYP26A1 expression and induction by ATRA in NB4 cells

CYP26A1 expression and inducibility by ATRA (1 μM) was assessed in the NB4 leukemia cells between 0 and 96 h. *CYP26A1* constitutive expression was barely detectable at time 0 h (Fig. 1). Following ATRA treatment, *CYP26A1* expression increased as a function of exposure time, and was induced 2-fold at 24 h, 7-fold at 48 h, 22-fold at 72 h, and 18-fold at 96 h (Fig. 1B). These results were also confirmed by semi-quantitative RT-PCR, which showed a 3-fold increase of *CYP26A1* expression at 24 h and a 7-fold increase at 48 h (data not shown). A dose-dependence study ranging from 0.01 to 1 μM of ATRA for 72 h showed that only pharmacological concentrations of ATRA (1 μM) could significantly induce *CYP26A1* expression in the NB4 cells (data not shown).

3.2. Effect of selective RAR agonists on CYP26A1 expression in NB4 cells

Because ATRA is a specific ligand for the RARs, we next examined the possible involvement of RARα, RARβ, and RARγ in the induction of *CYP26A1* expression in NB4 cells by employing selective RAR agonists. Fig. 2 shows that none of the selective agonists tested could induce *CYP26A1* expression (BMS753, RARα agonist; BMS641, RARβ agonist; BMS961, RARβ/γ agonist). As expected, the ATRA positive control markedly induced *CYP26A1* expression in these conditions. These results were confirmed by the RT-PCR method, which showed that BMS753, BMS641, BMS961, or TTNPB (a RAR pan-agonist) could not significantly induce *CYP26A1* expression (data not shown). These data suggested that the activation of RARs alone is not sufficient to induce *CYP26A1* expression, and that other signaling pathways could be involved.

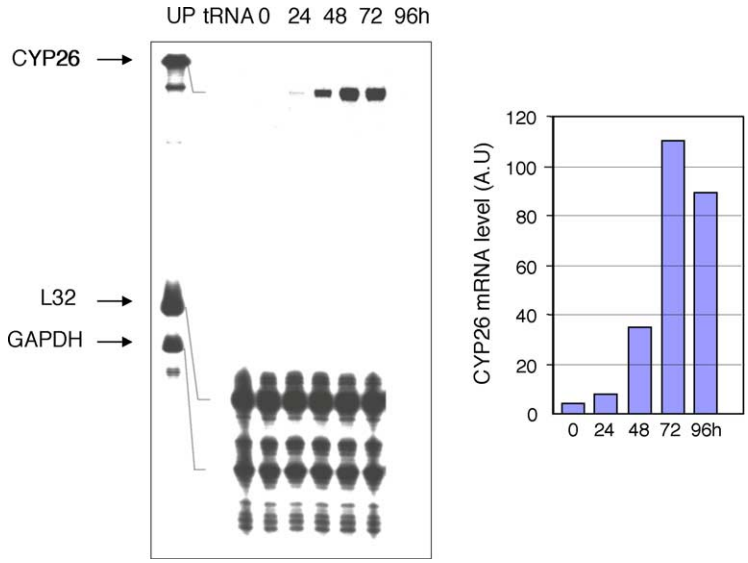

Fig. 1. *CYP26A1* induction by ATRA in NB4 human promyelocytic leukemia cells. The human NB4 cells were exposed to all-*trans*-retinoic acid (ATRA) at a concentration of 1 μM for the indicated times. Panel A: RNAse protection assay was performed as described in Section 2. Panel B: densitometric evaluation of *CYP26A1* expression obtained by scanning densitometry of the gel shown in Panel A. The data are normalized to GAPDH and L32 expression. The Multi-Analyst software was used. AU, arbitrary units.

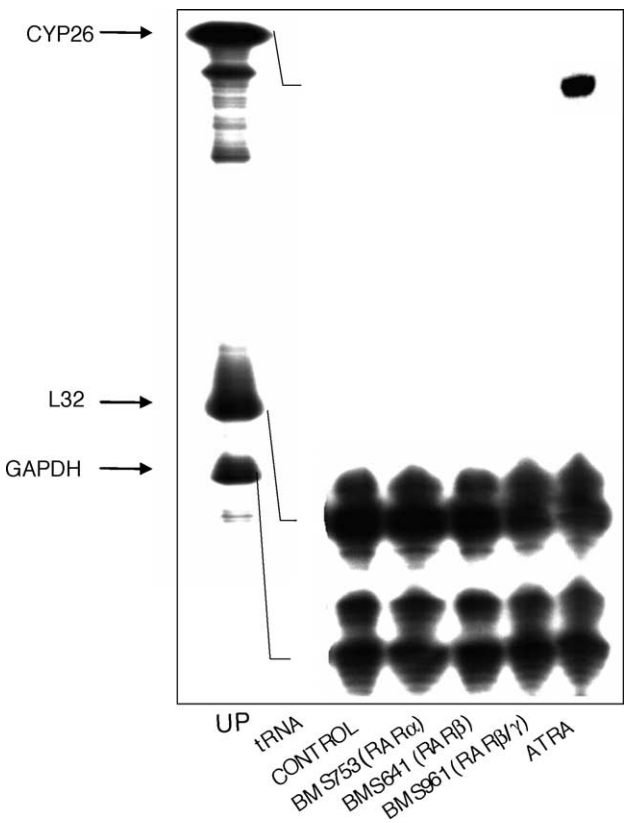

Fig. 2. *CYP26A1* expression after selective RAR agonists exposure. NB4 cells were exposed for 72 h at 1 μM of the selective agonists RARα (BMS753), RARβ (BMS641), RARβ/γ (BMS961), or to ATRA. CYP26, human *CYP26A1* cDNA; C, untreated control cells. RNAse protection assay was performed as described in Section 2.

3.3. Role of CYP26A1 expression in NB4 leukemia cells maturation

Concomitantly to the induction of the *CYP26A1* expression in NB4 cells, granulocytic maturation (as assessed by the May Grünwald Giemsa staining) was also observed in this maturation-sensitive line exposed to ATRA (1 μM, 72 h) (Fig. 3). As expected, the RARα agonist (BMS753) could also induce granulocytic maturation of NB4 cells, whereas the RARβ agonist (BMS641) and the RARβ/γ agonist (BMS961) did not induce maturation [28]. These data indicated that the *CYP26A1* induction does not appear to be essential to the leukemia cell maturation process because none of the RARs selective ligands could induce *CYP26A1*, whereas the selective RARα agonist (BMS753) could induce cell maturation (compare Figs. 2 and 3). In addition, when the ATRA resistant NB4-LR1 cell line was co-treated with ATRA and a cAMP analogue (8-CPT), cell maturation was observed in 72 h, as expected with this dual signaling condition [27,29], but *CYP26A1* expression was not induced in these conditions (data not shown).

3.4. Effect of RXR and RAR co-activation on CYP26A1 expression in NB4 cells

Because CYP26 gene expression was not inducible by RAR selective ligands alone, we next exposed NB4 cells to selective RAR agonists in combination with a RXR pan agonist (BMS649). When the RXR pan-agonist was used alone it did not induce the *CYP26A1* expression (Fig. 4),

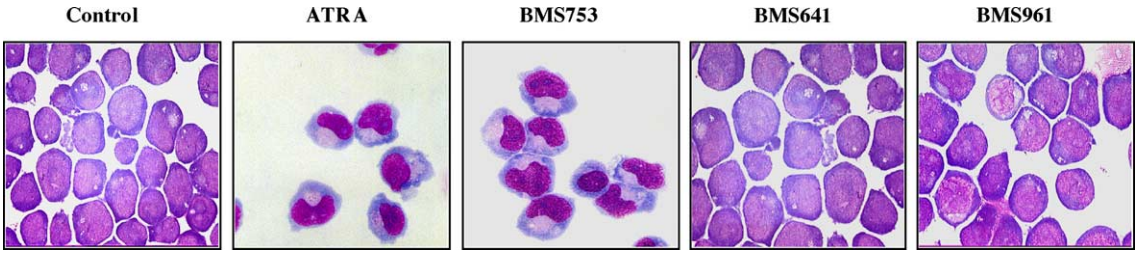

Fig. 3. Maturation of NB4 cells treated with various RAR agonists. May Grünwald Giemsa staining of NB4 acute promyelocytic leukemia cells exposed for 72 h at 1 μM to ATRA or to the indicated RAR agonists (BMS753, RARα; BMS641, RARβ; BMS961, RARβ/γ).

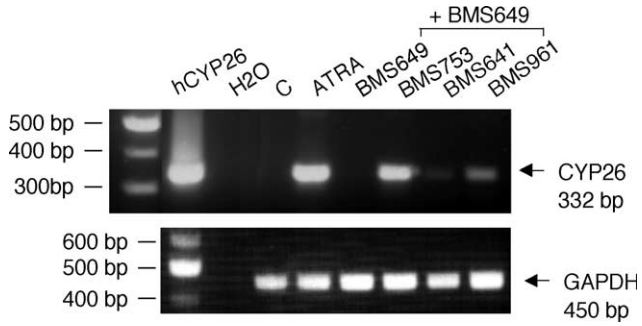

Fig. 4. *CYP26A1* expression after selective RAR and RXR agonists exposure. RT-PCR assay on mRNA from NB4 cells exposed to the selective RXR agonist BMS649 (0.8 μM, 72 h) used alone, or in combination with the RAR selective agonists BMS753 (RARα), BMS641 (RARβ), and BMS961 (RARβ/γ) (1 μM, 72 h). hCYP26, human *CYP26A1* cDNA; H_2O, negative control; C, untreated control cells; ATRA, positive control (1 μM, 72 h). RT-PCR was performed as described in Section 2.

whereas the combination of the selective RARα agonist (BMS753) with the RXR pan-agonist (BMS649) caused a marked induction of the *CYP26A1* expression. No induction of *CYP26A1* was observed with the combination of the selective RARβ agonist (BMS641) with the RXR pan-agonist (BMS649), and a low induction was observed with the combination of the RARβ/γ agonist (BMS961) and the RXR pan-agonist (BMS649) (Fig. 4). Taken together, these data indicated that the optimal induction of the *CYP26A1* required the co-activation of both RARα and RXR nuclear receptors.

3.5. Retinoid receptor status of NB4 cells

Because the lack of induction of *CYP26* in NB4 cells treated with either the RXR or the RAR agonists alone could have been due to an absence of nuclear receptors expression, we examined the status of RAR and RXR receptors in these cells. As shown in Fig. 5, NB4 cells expressed the 3 RAR isotypes and also the RXRα and RXRβ isotypes.

4. Discussion

Acute promyelocytic leukemia is particularly sensitive to ATRA-induced differentiation and is therefore considered as a model of differentiation therapy of cancer. Although clinical resistance to ATRA develops rapidly, in part due to increased systemic metabolism, little information is available concerning ATRA metabolism in the target leukemic cells. Although several CYPs are involved in the metabolism of ATRA [15], the novel CYP26A1 has attracted attention because it is induced by ATRA and metabolizes this molecule in a specific manner into four oxidized metabolites [16–18]. Because *CYP26A1* expression in leukemic cells could influence their response to ATRA therapy, the purpose of this study was therefore to evaluate *CYP26A1* expression in human APL cells, and to study the mechanisms involved in its regulation. To this aim, we used the human APL NB4 cell line which

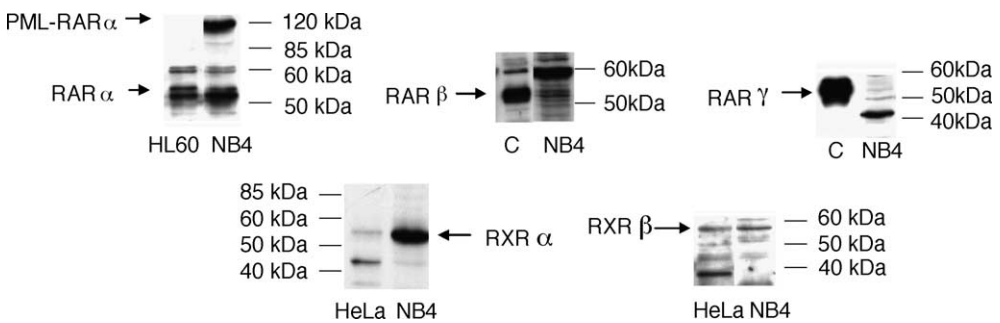

Fig. 5. Western blot analysis of RARs and RXRs expression in NB4 cells. Nuclear protein extracts from untreated NB4 cells were analyzed by Western blot as described in Section 2. Positive controls for the various nuclear receptors were HL60 cells, HeLa cells, and COS-7 cells (labeled C) transfected with RARβ and RARγ cDNA.

expresses the t(15;17) translocation and is sensitive to ATRA-induced maturation.

We have found that the constitutive expression of the *CYP26A1* was very low in the NB4 cell line. This gene expression was however strongly induced by ATRA (22-fold at 72 h compared to untreated cells) and this induction occurred in parallel to cell differentiation. These findings are in agreement with other human cancer cell lines in which *CYP26A1* is also inducible in ATRA-sensitive cells [22]. Although *CYP26A1* induction and maturation were both observed with ATRA treatment, the use of the specific RARα agonist BMS753 has shown that *CYP26A1* induction was not required for granulocytic maturation, since BMS753 could elicit maturation but could not induce *CYP26A1*. Similar results were reported in ES cells where it was observed that although the *CYP26* induction was parallel to neuronal differentiation, these two events were independent [18]. Considering the ATRA concentration required, our data show that a pharmacological concentration of ATRA (1 μM) was required for *CYP26* induction, as it is observed in most studies [30].

To further investigate the mechanisms involved in *CYP26A1* regulation, we used RAR and RXR selective agonists. Our results indicate that the activation of a single RAR receptor, or of a RXR receptor, could not induce *CYP26A1* expression. Indeed, the activation of both RARα and RXR receptors by their selective ligands was required to elicit a significant induction of *CYP26A1* in NB4 cells. The involvement of the RARs in the *CYP26A1* regulation has already been suggested by the use of a RARα-selective antagonist which could block the ATRA-induced *CYP26A1* expression in NB4 cells [31], and also by the use of various RARs agonists which could induce *CYP26A1* when used alone in various cell lines [32]. Our findings indicate that *CYP26A1* induction could not significantly be induced in NB4 cells by RAR selective agonists when used alone, but could markedly be induced by the combination RARα and RXR agonists. In line with this observation, the RAR-mediated *CYP26A1* induction can also be synergized in several cell lines by the use of RXR selective agonists which alone are ineffective [23,32,33].

Although the presence of the nuclear receptors has been assessed by Western blot, their presence is not a proof of their functionality. For example, Sonneveld et al. [33] have shown that in P19-RAC65 cells that express dominant negative RARα, the *CYP26* is not inducible in spite of the receptor expression. It is therefore important that functional tests are conducted in parallel, and this is precisely what was done in the present work: in addition to show the expression of the 3 RAR (α, β, and γ) isotypes and the 2 RXR (α and β) isotypes in NB4 cells (Fig. 5), we have also shown that RARα is functional in terms of differentiation (Fig. 3), that the RARα, RARγ, and RXRs are functional in terms of *CYP26A1* induction (Figs. 2 and 4).

In conclusion, we have shown that *CYP26A1* induction is not essential for the granulocytic maturation of NB4 leukemia cells, and that the induction of this gene requires the activation of both RARα and RXR nuclear receptors by their selective ligands. These results may be relevant to define better strategies aimed at improving retinoid-based therapy of acute promyelocytic leukemia and other cancers.

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