Rexinoid-Triggered Differentiation and Tumor-Selective Apoptosis of Acute Myeloid Leukemia by Protein Kinase A–Mediated Desubordination of Retinoid X Receptor

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Abstract

Apart from PML–retinoic acid receptor-α (RARα) acute promyelocytic leukemia all other acute myeloid leukemias (AML) are unresponsive to retinoid differentiation therapy. However, elevating the levels of cyclic AMP (cAMP) confers onto retinoid X receptor (RXR)–selective agonists (“rexinoids”) the ability to induce terminal granulocyte differentiation and apoptosis of all-trans retinoic acid–resistant and insensitive AML cells and patients’ blasts. Protein kinase A activation leads to corepressor release from the RAR subunit of the RAR-RXR heterodimer, resulting in “desubordination” of otherwise silent RXR, which acquires transcriptional competence in response to cognate ligands. Rexinoid-cAMP induction of endogenous RARβ is blunted in mouse embryo fibroblasts lacking RARs, but reintroduction of exogenous RARβ reestablishes responsiveness, thus confirming that the RARs-RXR heterodimer is the rexinoid mediator. The apoptogenic effect of this treatment involves enhanced expression of the death receptor DR5 and its cognate ligand, tumor necrosis factor–related apoptosis inducing ligand, both of which are known to induce apoptosis in a tumor cell–selective manner and lead to the activation of initiator caspases. Immunohistochemistry confirmed induction of tumor necrosis factor–related apoptosis inducing ligand and DR5 in AML patient blasts cultured ex vivo. AML patients’ blasts responded to rexinoid-cAMP combination treatment with induction of maturation and apoptosis, independent of karyotype, immunophenotype, and French-American-British classification status. Clonogenic assays revealed complete inhibition of blast clonogenicity in four out of five tested samples. Our results suggest that despite the genetic, morphologic, and clinical variability of this disease, the combination of rexinoids and cAMP-elevating drugs, such as phosphodiesterase inhibitors, might lead to a novel therapeutic option for AML patients by inducing a tumor-selective death pathway. (Cancer Res 2005; 65(19): 8754-65)

Introduction

Acute myeloid leukemia (AML; ref. 1) is characterized by an accumulation of granulocyte or monocyte precursors in bone marrow and blood. Despite improved therapies, the survival rate among patients who are <65 years old is only 40%. AML is a heterogeneous disease that originates from differentiation blocks at various stages of commitment and maturation within the myeloid lineage. This heterogeneity is reflected by morphologic differences of the blasts, which is at the origin of the French-American-British (FAB) classification into nine distinct subtypes that differ with respect to the myeloid lineage involved and the degree of cell maturation (2). Recent studies have supported the concept that aberrant expression or mutation of lineage–specific transcription factors, or the formation of abnormal transcription factor hybrids due to chromosome translocation cause this heterogeneous disease and correlate with specific FAB subtypes (3).

Acute promyelocytic leukemia (APL, classified as FAB M3/M3v) accounts for ∼5% to 10% of AML cases (1). APL has received particular attention not only as the prototype of successful cancer differentiation therapy which leads to overall survival rates of ∼70% at 5 years (4, 5), but also because the cause of this leukemia and the success of the all-trans retinoic acid (ATRA) therapies are understood in molecular detail. In 98% of cases, APL originates from a t(15;17)(q22;q21) chromosomal translocation, which generates a fusion protein (PML-RARα) that causes the disease. At the molecular level, the PML fusion with retinoic acid receptor-α (RARα) results in an enhanced recruitment of histone deacetylase complexes that epigenetically silence cognate gene programs; apparently, this silencing cannot be relieved at physiologic concentrations of ATRA (for reviews, see refs. 6, 7–11). In addition to the formation of heterochromatin over RAR target genes, the formation of the PML-RARα fusion protein results in a variety of signaling aberrations that also affect blast survival and self-renewal of stem cells (12–17). Supraphysiologic ATRA levels, however, induce dissociation of the silencing complex, activate the differentiation program and, furthermore, activate tumor-selective death signaling (18, 19).

Unfortunately, >90% of AML patients that do not present with the t(15;17) translocation are refractory to ATRA-based differentiation therapy. In addition, APL with the alternative translocation t(11;17)(q23;q21) leading to the formation of the PLZF-RARα fusion, which is the second most common molecular subgroup of...
the disease, is generally resistant to classical retinoid therapy (20). Moreover, relapse of APL patients is frequently associated with mutations in the ligand-binding domain of the PML-RARα fusion protein resulting in abrogation of ligand-binding capacity (21). Thus, there is serious need for additional therapeutic tools to treat patients with AML.

Retinoids are ligands for RAR that heterodimerize with retinoid X receptors (RXR); the corresponding RAR-RXR heterodimers are thought to mediate the retinoid signal. RXRs could, in addition to forming heterodimers with RARs, heterodimerize with a great number of other nuclear receptors (for a general review, see ref. 22). Whereas ATRA is wellaccepted to correspond with the endogenous ligand for RARs, the nature, origin, and signaling function of endogenous RXR ligand(s) is still a matter of debate (23). Several synthetic RXR selective ligands have been generated and their analysis revealed that these retinoids are transcriptionally silent when binding to RXR in heterodimers with nonliganded partners. However, together with retinoids, retinoids can act synergistically (24). Mechanistic analyses revealed that RXR-bound corepressors do not dissociate from the heterodimer in the absence of a RAR ligand, thus preventing retinoid-induced recruitment of coactivators—this phenomenon is usually referred to as “RXR subordination”—whereas the synergy between retinoid and retinoid agonists is due to the cooperative recruitment of a single coactivator to the heterodimer (23, 25).

There is ample evidence that retinoids are chemotherapeutic and chemopreventive drugs that are also active in non-APL malignancies (6, 26–28). Also, retinoids on their own, despite their subordination in some heterodimers, can exert cancer-preventive activity, although the mechanistic basis has remained elusive (for review, see refs. 29, 30). We have previously shown a further paradigm by which pure retinoids can spring into action when combined with drugs that elevate cyclic AMP (cAMP) levels. Importantly, such a drug combination exerts antileukemic action even in APL cells that have become resistant to retinoic acid treatment (31), revealing that the signaling is clearly distinct from that induced by retinoic acid. Here, we reveal the mechanistic basis of the so-called “cAMP-retinoid crosstalk”, show that it is mechanistically distinct from the synergy observed between protein kinase A (PKA) agonists and RXR ligands, and report that pure retinoids, when used together with agents that increase intracellular cAMP levels, induce postdifferentiation apoptosis in pure rexinoids, when used together with agents that increase protein kinase A (PKA) agonists and RAR ligands, and report that mechanistically distinct from the synergy observed between multiple blast cultures reveals transfection of an expression vector encoding SV40 Tag. Immortalized cells were infected with CRE-expressing adenovirus (a kind gift from M. Giovannini) at a multiplicity of 100 PFU/cell and cloned by limiting dilution. Excision was verified by both PCR on the three RAR loci and failure to induce the transcription of two retinoic acid target genes, RARβ1 and cytochrome P450 26A1 (CYP26A1). RARα cDNA was reintroduced into excised cells by retroviral transfer followed by G418 selection. Quantitative PCR on the engineered MEFs or U937 hematopoietic cells was done using TaqMan assays for mouse or human RARα and CYP26A1. β2-Microglobulin and TBP were used as internal controls. Cells were treated with the indicated compounds at a concentration of 10−6 mol/L for 12 hours. Induction was calculated over the preinduction level of expression, assuming a 100% yield of the PCR assay. Details on the verification of the triple RAR knock-outs, reintroduction of the RARα expression cassette, and primers used for RARβ1 expression analysis are available on request.

Colony-forming assays. Ficoll-purified blasts (1 × 106) were resuspended in 0.3 mL of RPMI 1640, added to 3 mL MethoCult GF (Stem Cell Laboratories, Vancouver, Canada) and vehicle or ligands (1 μmol/L BMS retinoids or SR11237, 200 μmol/L 8CPT-cAMP, 1 μmol/L theophylline, 100 μmol/L rolipram, 500 μmol/L IBMX) were added. After complete resuspension, 1.1 mL of the mixture was plated into the wells of a six-well plate (Falcon, BD, Franklin Lakes, NJ); at least two wells of the same plate were filled with sterile water. Cultures were placed in a 37°C humidified incubator at 5% CO2.

Differentiation analysis. Cells were harvested and resuspended in 10 μL phycocerythrin-conjugated murine anti-human CD11c (CD11c-PE; IgG1) and 10 μL FITC-coupled murine anti-human CD14 (CD14-FITC; IgG2a; PharMingen, San Diego, CA). As isotypic controls, FITC-conjugated mouse IgG2a and PE-conjugated mouse IgG1 were used. Ex vivo cultured blasts of patient no. 7 (Table 1) were exposed to PE-conjugated murine anti-human CD56 and anti-human CD11b FITC-conjugated mouse monoclonal antibodies (DAKO Corp., Carpenteria, CA), using murine IgG1 antibody (DAKO Corp.) as a negative control. Samples were incubated for 30 minutes at 4°C in the dark, washed in PBS, and resuspended in 500 μL PBS containing propidium iodide (PI; 0.25 μg/mL). Differentiation was determined exclusively for viable PI-negative cells. Cell surface expression of DR5 was measured after retinoid-PKA agonist exposure (1 μmol/L SR11237; 200 μmol/L 8CPT-cAMP) for 4 days using monoclonal anti-DR5 antibodies (IgG; Alexis, San Diego, CA) and FITC-coupled secondary antibody. Mouse IgG1 was used as isotypic control.

Nitroblue tetrazolium staining. Cells (1 × 106) resuspended in 500 μL of culture medium were mixed with 500 μL of solution containing 0.2% nitroblue tetrazolium (NBT) and 200 ng 12-0-tetradecanoylphorbol-13-acetate (Sigma). After incubation for 30 minutes at 37°C, the formazan deposits were dissolved by 500 μL lysis buffer (50% dimethylformamide, 20% SDS, pH 7.4). The degree of NBT reduction was quantified by measuring OD510.

Apoptosis and cell cycle analyses. Sensitization of PLB985 cells to near sublethal doses of TRAIL by retinoid-PKA pretreatment was as follows. PLB985 cells were treated for 4 days with 1 μmol/L SR11237 and 200 μmol/L 8CPT-cAMP. Cells were spun down and resuspended in fresh medium, before TRAIL, SR11237, and 8CPT-cAMP were added to final remaining AML samples (Table 1), peripheral blood containing 80% to 90% leukemic blasts was purified over Ficoll and processed as described (34). This study was approved by the Ethical Committee of the Second University of Naples. Cell morphology was analyzed after May-Grünwald staining. At least 300 cells were analyzed for each treated culture. Each treatment was repeated at least three times, with each experiment done in triplicates. Transient transfections for transactivation assays were done according to routine procedures using luciferase or CAT reporter genes, the corresponding constructs have been described previously (25). For two-hybrid assays, HeLa cells were transiently transfected with 20 ng of RARαVP16 or RARβ2αaVP16, 40 ng of Gal-SMRT and 200 ng PRKAα per well of a 24-well plate using the calcium phosphate method. Primary MEFs were generated from triple RAR “floxed” (RARαlox12/12;RARβ12/12;RARγ12/12) mouse embryos (35–37), generously provided by N. Gyselinken, immortalized by transfection of an expression vector encoding SV40 Tag. In non-APL malignancies (6, 26–28). Also, rexinoids on their own, despite their subordination in some heterodimers, can exert cancer-preventive activity, although the mechanistic basis has remained elusive (for review, see refs. 29, 30).
permeabilized for 20 minutes with 100 μg/mL PI and exposed to 1 μM ATRA or 1 μM SR1237 plus 200 μM 8CPT-cAMP. Numbers in the "Ctrl", "ATRA", and "RXR-PKA" columns refer to the percentage of apoptosis determined by Annexin-V staining and FACS analysis; the number in parentheses are the days of analysis and correspond to the length of ex vivo treatment [apoptosis was not quantitated in case no. 7 but was obvious in the RXR-PKA case ("YES") from morphologic inspection]. In general, blasts that responded to SR1237-8CPT-cAMP also responded to LG1069–phosphodiesterase inhibitors (theophylline, rolipram, or IBMX). Blasts also tested in colony-forming assays with SR11237 and 8CPT-cAMP.

### Table 1. Comparison between ATRA and rexinoid-PKA responsiveness of AML patients' blasts

<table>
<thead>
<tr>
<th>No.</th>
<th>FAB</th>
<th>Ctrl</th>
<th>ATRA</th>
<th>RXR-PKA</th>
<th>Karyotype</th>
<th>Immunophenotype *</th>
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<tbody>
<tr>
<td>1</td>
<td>M1</td>
<td>13 (5)</td>
<td>14 (5)</td>
<td>61 (5)</td>
<td>46, XX</td>
<td>CD11b+/−, CD1c+, CD13+/−, CD14−, CD33+, CD34−, CD56+/−</td>
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<td>2</td>
<td>M1</td>
<td>10 (3)</td>
<td>8 (3)</td>
<td>31 (3)</td>
<td>46, XX</td>
<td>CD11b+, CD1c+, CD13+, CD14+, CD33−, CD34−, CD56−, CD117−, DR+, MPO+</td>
</tr>
<tr>
<td>3</td>
<td>M1</td>
<td>19 (4)</td>
<td>18 (4)</td>
<td>49 (4)</td>
<td>complex</td>
<td>CD11b+, CD1c+, CD13+, CD33+, CD34−, CD56+, CD117+, DR−, MPO+</td>
</tr>
<tr>
<td>4</td>
<td>M2</td>
<td>13 (4)</td>
<td>12 (4)</td>
<td>35 (4)</td>
<td>46, XX</td>
<td>CD11b+, CD1c+, CD13+, CD33+, CD34−, CD56−, DR+, MPO+</td>
</tr>
<tr>
<td>5</td>
<td>M2</td>
<td>11 (5)</td>
<td>9 (5)</td>
<td>60 (5)</td>
<td>46, XX</td>
<td>CD10+, CD11b+, CD1c+, CD13+, CD14−, CD33+, CD34+, CD56−, CD117−, DR+, MPO+</td>
</tr>
<tr>
<td>6</td>
<td>M3 (PML-RARe positive)</td>
<td>13 (5)</td>
<td>49 (5)</td>
<td>58 (5)</td>
<td>46, XX, t(15;17)</td>
<td>CD2+, CD11b−, CD1c−, CD13−, CD14+, CD33+, CD34+, CD56−, CD117+, DR−, MPO+</td>
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<tr>
<td>7</td>
<td>M3 (PLZF-RARe/RARe-PLZF–positive)</td>
<td>NO</td>
<td>YES</td>
<td>46, XX, t(11;17)</td>
<td>(q23q12-21) [12]/46, XY[3]</td>
<td>CD2−, CD3−, CD5−, CD7−, CD9+, CD4+</td>
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<td>8</td>
<td>M4</td>
<td>16 (5)</td>
<td>15 (5)</td>
<td>40 (5)</td>
<td>46, XX, t(9;11)</td>
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<tr>
<td>9</td>
<td>M5</td>
<td>10 (6)</td>
<td>7 (6)</td>
<td>39 (6)</td>
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<td>CD11c+, CD13+, CD33+, CD117+, DR+, MPO+</td>
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<tr>
<td>10</td>
<td>M4</td>
<td>12 (3)</td>
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<td>46, XX</td>
<td>CD11c+, CD13+, CD33+, CD117+, DR−, MPO+</td>
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<td>49 (4)</td>
<td>46, XX</td>
<td>CD11c+, CD13+, CD33+, CD117+, DR+, MPO+</td>
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</tbody>
</table>

NOTE: The blasts of 13 AML patients were cultured ex vivo and exposed to 1 μM/L ATRA or 1 μM/L SR1237 plus 200 μM 8CPT-cAMP. numbers in the "Ctrl", "ATRA", and "RXR-PKA" columns refer to the percentage of apoptosis determined by Annexin-V staining and FACS analysis; the number in parentheses are the days of analysis and correspond to the length of ex vivo treatment [apoptosis was not quantitated in case no. 7 but was obvious in the RXR-PKA case ("YES") from morphologic inspection]. In general, blasts that responded to SR1237-8CPT-cAMP also responded to LG1069–phosphodiesterase inhibitors and, when tested, induction of differentiation/apoptosis corresponded to reduction of clonogenicity. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay. Blasts of patient 12 (who died the same day) responded in ex vivo cultures only partially. Case no. 8 corresponds to a secondary clonogenic assay.

*Surface antigen positivity was defined on the basis of expression in <20% (−−), ≥20% to 40% (++−) and >40% (+++) of the gated blast cell population.†Blasts also tested in colony-forming assays with SR11237 and 8CPT-cAMP.

†Clonogenic assays done in the presence of LG1069 and phosphodiesterase inhibitors (theophylline, rolipram, or IBMX).
casp-8 and AEVD-pNA for casp-10), samples were incubated for 1 hour at 37°C and the OD405 was determined on a microplate reader. To block caspase activity after rexinoid-cAMP treatment, pan-caspase (Z-VAD-fmk; R&D, San Diego, CA) inhibitor was added to the culture medium to a final concentration of 100 μM/L.

RPA assays. To check the expression of rexinoid-cAMP induced mRNAs, multiplex RNAse mapping was done. Total RNA was extracted with Trizol (Invitrogen-Life Technologies, Carlsbad, CA) and RNAse protection assays were done according to the supplier’s instructions (PharMingen). Briefly, 4 μg of total RNA and 6 to 8 × 105 cpm of α-32PUTP-labeled template sets were used; after RNAse treatment, protected probes were resolved on 5% urea-polyacrylamide-bis-acrylamide gels.

Immunohistochemistry. To analyze the expression of DR5, immunohistochemistry was used. Pellets from 5 × 107 NB4 cells were lysed in radioimmunoprecipitation assay buffer and proteins were separated on 12% acrylamide gels. After blotting, nitrocellulose filters were probed with anti-DR5 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). For normalization, blots were reprobed with anti-actin antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA).

To detect DR5 and TRAIL, by immunohistochemistry, AML patients’ blasts were spun onto glass slides, fixed for 10 minutes in 4% formalin, washed in PBS and incubated in 1% H2O2 to inactivate endogenous peroxidase. The slides were washed thrice in PBS, incubated for 1 hour in 4% BSA-2% FCS, washed in PBS, and incubated overnight in PBS-0.2% BSA containing goat anti-DR5 or anti-TNFR (R&D, Alexis) antibodies. After three washes in PBS, the slides were incubated for 20 minutes with the appropriate biotinylated secondary antibody [anti-goat polyvalent biotinylated IgG (Lab Vision Corp., Fremont, CA)] washed in PBS, incubated for 20 minutes with streptavidine peroxidase (Lab Vision), washed in PBS and processed with 3,3′-diaminobenzidine (Lab Vision). After washing in double-distilled water, nuclei were stained with hematoxylin (30 minutes). Finally, the slides were washed for 5 minutes each with H2O, 50%, 70%, 80%, 90%, 100% ethanol, and xylene.

Results

Initial experiments with established AML cell lines (HL60, PLB985, U937, NB4, and NB4R2) showed that the combination of RXR and PKA agonists efficiently induced differentiation, growth arrest, and apoptosis in all tested myeloid cells, whereas it was without effect on several other cell types, such as epithelial HeLa cells. In contrast ATRA or other synthetic retinoids alone or in combination with retinoids resulted in differentiation and/or apoptosis of only some but not all cell lines (data not shown).

Maturation of PLZF–retinoic acid receptor-α acute promyelocytic leukemia blasts. To extend these observations to the blasts of ATRA-insensitive APL patients, we assessed the efficacy of the combined treatment to induce maturation of PLZF-RARα-positive blasts carrying the t(11;17) chromosomal translocation. We used the novel rexinoid BMS749 (38), a potent RXR agonist and simultaneous antagonist of RAR, to exclude the possible effects of serum-borne traces of retinoic acid. Notably, PLZF-RARα blasts responded with differentiation after 3 days of the treatment as shown by nuclear morphology (Fig. 1A), NBT reduction, and CD11b expression (Fig. 1B and C), as well as by down-regulation of CD56 (Fig. 1C), which is expressed in PLZF-RARα positive APL (39).

Figure 1. Induction of maturation and apoptosis of AML patients’ blasts. A–C, ATRA-resistant t(11;17) APL blasts differentiate in response to a 3-day treatment with rexinoid (BMS749) and PKA (BCT-cAMP) agonists as revealed by nuclear morphology (A), NBT reduction assays (B), and expression of CD56 and CD11b cell surface markers (C). D, morphology of blasts of patient no. 5 (FAB-M2; Table 1) cultured ex vivo for 7 days in vehicle (“untreated”) or 1 μmol/L ATRA (top), or treated for 4 days with 1 μmol/L SR11237 plus 200 μmol/L BCT-cAMP (bottom; three different views illustrate cell maturation and apoptosis). Similar results were obtained in ex vivo cultures of blasts of all other patients described in Table 1. E, synergistic induction of apoptosis with rexinoid and PKA agonist in AML blasts. Cells were treated for 6 days with 1 μmol/L SR11237, 200 μmol/L BCT-cAMP, or both and submitted to FACS analysis (cell cycle and PI staining).
of differentiation and apoptosis in established AML cell lines prompted us to investigate whether rexinoid-cAMP treatment would also be effective for the blasts of AML patients. Indeed, in ex vivo cultures, acute myeloid leukemia blasts of a patient (no. 5 in Table 1) classified as FAB-M2 with no apparent karyotype abnormality, differentiated and displayed morphologic changes reminiscent of cell death following 4 days of rexinoid-cAMP treatment. Notably, various stages of induced myeloid maturation could be identified (metamyelocyte, band, and polymorphonuclear neutrophil) and nuclear fragmentation indicated apoptosis (Fig. 1D). FACS analysis of Annexin V-PI–stained cells confirmed severe apoptosis (data not shown; for percentage of apoptosis, see Table 1). In contrast, morphologic analysis or Annexin V-PI staining after exposing the blasts to ATRA for 7 days did not reveal any sign of differentiation or apoptosis (top). FACS analyses of another blast sample clearly revealed the synergistic effects of cAMP and SR11237; only a marginal increase of apoptosis was seen after 6 days ex vivo culture with cAMP alone and no effect for the rexinoid. However, nearly 40% of the blasts died upon treating them with the cAMP and rexinoid together (Fig. 1E).

To assess the clonogenic potential of AML blasts upon treatment, we used fresh blasts and measured colony formation in semisolid medium. Although colonies were readily seen in the nontreated condition, the presence of rexinoid and cAMP occluded colony formation completely (Table 1; see section below), indicating that these compounds affected the actively proliferating fraction of blasts. Similar results have been obtained with the blasts of 11 other non-APL AML patients, irrespective of FAB subtype, karyotype, or immunophenotype. Generally, rexinoid and cAMP induced maturation and subsequent apoptosis (Table 1).

Mechanism of death induction. We have previously observed that in APL cells, ATRA induces the expression of the tumor-selective death ligand, TRAIL (18), which is the cause of retinoid-dependent apoptosis, and defined the molecular basis of TRAIL induction (19). To study the possible implication of this tumor-cell-selective death-signaling pathway in rexinoid-induced apoptosis in the presence of elevated cAMP levels, we used initially multi-probe RNase mapping to compare in established NB4 APL cells death signaling programs induced by ATRA with those induced by rexinoid-cAMP treatment. Both treatments enhanced TRAIL mRNA levels concomitantly with the onset of apoptosis albeit ATRA was more efficient (Fig. 2A, lanes 6 and 13). Conversely, there was a very strong induction of the TRAIL receptor DR5 mRNA upon rexinoid-cAMP treatment; in fact, the induction was more than one magnitude higher than that seen with ATRA. The same stimulation was also observed at the protein level by immunoblot analysis, which indicated a major stimulation of both DR5 receptor and its splice variant ("DR5 sv") relative to the actin control (Fig. 2B). Increased expression of TRAIL and DR5 was also seen with HL60 and U937 cells (data not shown).

FACS analysis with anti-DR5 antibodies confirmed that the newly produced TRAIL receptor protein is correctly sorted, as it leads to enhanced cell surface expression of DR5 in both NB4 and PLB985 cells (Fig. 3A). Importantly, the overexpressed DR5 is functional, as rexinoid-cAMP pretreated cells became highly susceptible to low doses of exogenous TRAIL, which on their own were hardly apoptogenic (Fig. 3B, compare the extent of death in lanes 2 and 4 with that in lane 5). Moreover, the rexinoid-cAMP treatment led to activation of the initiator caspases 8 and 10, which are known downstream mediators of the TRAIL-DR5 complex (refs. 40, 41; Fig. 3C). Consistent with these results, the pan-caspase inhibitor Z-VAD abrogated rexinoid-cAMP induced death (Fig. 3D). These results show that rexinoid-cAMP–induced expression of DR5 sensitizes myeloid leukemia cells to TRAIL-induced apoptosis and indicates that the amount of endogenous TRAIL induced by this program is limiting.

We studied several other pro- and antiapoptogenic signaling pathways but no major differences were observed between ATRA and rexinoid-cAMP treatment for programs that are mediated by IAPs, TRAFs, and nuclear factor κB, and only minor effects were
noted for bcl2 family members, such as bcl2, bfl1/bcl2A1, mcl1, bik, bax, and bak. With respect to cyclin-dependent kinase inhibitors, the rexinoid-PKA crosstalk resulted in a stimulation of p21 in all cell lines tested, whereas ATRA stimulated predominantly p19 (particularly in PLB985) and to a lesser extent p21 gene expression (ref. 18; data not shown). Thus, as far as the induction of proapoptotic factors is concerned, there is a consistent superinduction of DR5 concomitant with a moderate increase of TRAIL mRNA levels in all cell lines that undergo postmaturation apoptosis in response to the rexinoid-cAMP treatment.

Tumor necrosis factor–related apoptosis inducing ligand–DR5 activation in acute myeloid leukemia–blasts. As the above data were derived from experiments with established cell lines, we investigated if the expression of TRAIL and its cognate receptor would also be enhanced in AML blasts. Several non–t(15;17) AML blasts were cultured ex vivo and exposed to 8CPT-cAMP and SR11237. In all cases, a strong induction of DR5 expression was apparent from immunohistochemical analysis (Fig. 2C, left; DR5 staining appears in brown). The nuclear morphology of the positively staining cells is indicative of terminal differentiation. Rexinoid-cAMP treatment induced also TRAIL expression in the differentiated blasts (Fig. 2C, right). Remarkably, no TRAIL or DR5 expression was seen in cells exhibiting a nondifferentiated phenotype (Fig. 2C, arrows). We conclude from this data that differentiation is a requisite for apoptosis.

Rexinoid crosstalk with phosphodiesterase inhibitors suppresses clonogenic growth of leukemic blasts. Although PKA agonists are not available for clinical use, phosphodiesterase inhibitors, which increase intracellular cAMP by blocking its degradation, are available for therapeutic indications other than leukemia. Theophylline, a pan-phosphodiesterase inhibitor, is used as a bronchodilator, and rolipram, a PDE4-selective inhibitor, is currently being studied in a phase II clinical trial for the treatment of multiple sclerosis (NIH Clinical Research Studies, protocol no. 01-N-0089). As far as rexinoids are concerned, Targretin (bexarotene, LG1069) has been approved for treating cutaneous T cell lymphoma in patients that are refractory to prior systemic therapy.

To investigate whether the phosphodiesterase inhibitors could be as efficient as CAMP, we first exposed PLB985 cells to LG1069 or SR11237 together with one of two pan-phosphodiesterase inhibitors, theophylline or IBMX, and, or together with the PDE4-selective inhibitor rolipram. All three drug combinations induced differentiation of PLB985 cells, as revealed by CD11c expression (Fig. 4A) and NBT reduction assay, which indicates the production of

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**Figure 3.** Rexinoid-PKA induces cell surface expression of TRAIL receptor DR5 and caspase-dependent cell death, which is superinduced by TRAIL. A, FACS analysis of DR5 expression on NB4 and PLB985 cell surfaces in the absence and presence of rexinoid and PKA agonists (4 days exposure) as depicted. B, PLB985 cells were pretreated for 4 days (lanes 3-5) or left untreated (lanes 1 and 2) with rexinoid and PKA agonists and subsequently exposed for another 4 days to exogenous TRAIL, SR11237, or 8CPT-cAMP. Apoptosis was determined by Annexin V-PI staining. C, the initiator caspases, casp-8 and casp-10, are activated by the crosstalk (lane 4) and, less pronounced, by cAMP alone (lane 2), whereas no significant induction is seen by the rexinoid alone (lane 3). D, rexinoid-cAMP (“SR + cAMP”)–induced apoptosis (lane 2) is nearly completely blocked by a pan-caspase inhibitor (“Z-VAD”, Z-VAD-FMK; lane 4).
superoxide, a functional marker for granulocyte differentiation (Fig. 4B). At equal retinoid/rexinoid concentrations, the rexinoid-phosphodiesterase inhibitor–induced differentiation of PLB985 cells was even superior to that seen by 100 nmol/L TTNPB alone and comparable to the TTNPB–phosphodiesterase inhibitor cross-talk (Fig. 4C). Apoptosis was induced with similar efficacies by either rexinoid or the retinoid, but required the presence of the phosphodiesterase inhibitor in both cases (Fig. 4D). Note the very strong synergy between the phosphodiesterase inhibitor and rexinoid for the induction of differentiation/apoptosis of PLB985 (Fig. 4) and other AML cells (data not shown), as no differentiation was seen with the phosphodiesterase inhibitors alone, and LG1069 alone induced only a low level of differentiation.

To study if the combination of these drugs could have the potential to be active on patients’ blasts, we treated primary blasts from several AML patients ex vivo with combinations of phosphodiesterase inhibitors and LG1069 and focused on the proliferative fraction of the blasts. For this, we used the particularly aggressively growing blasts of one patient (no. 9; Table 1) for clonogenic assays. Strong inhibition of colony cell growth was observed in the presence of LG1069 and either of the three phosphodiesterase inhibitors (Fig. 4E). Similar results were obtained in clonogenic assays with the blasts of several other AML patients (Table 1). These data suggest that a combination of rexinoids and phosphodiesterase inhibitors could limit blast growth in AML patients.

Figure 4. Phosphodiesterase inhibitors functionally substitute PKA agonists in the crosstalk with rexinoid agonists. A, PLB985 cells were exposed for 7 days to two rexinoids (1 μmol/L SR11237 and 1 μmol/L LG1069), the pan-phosphodiesterase inhibitors theophylline (2 mmol/L) or IBMX (500 μmol/L), or the PDE4-selective phosphodiesterase inhibitor rolipram (100 μmol/L) alone or to combinations of rexinoid and phosphodiesterase inhibitor as indicated. Differentiation was assessed by FACS analysis of CD11c cell surface marker expression. B, similar treatment of PLB985 cells as in (A). As a functional marker for differentiation, superoxide production was determined using the NBT reduction method. C and D, PLB985 were treated for the indicated times with 2 mmol/L theophylline, 100 nmol/L LG1069, or 100 nmol/L of the RAR-selective pan-agonist TTNPB. The cells were assayed for NBT reduction (C) or apoptosis (D). E, inhibition of the clonogenic growth of AML blasts in the presence of phosphodiesterase inhibitors and the rexinoid LG1069. Colony formation after 18 days in presence of vehicle (left); no colonies form under identical conditions in the presence of the indicated combinations of LG1069 and phosphodiesterase inhibitors (right). Similar results were obtained with ex vivo cultures of blasts of three other AML patients.
Desubordination of retinoid X receptor in retinoic acid receptor–retinoid X receptor heterodimers by protein kinase A.

To assess the molecular basis of the rexinoid-cAMP crosstalk we first studied whether RAR-RXR or other RXR heterodimers would mediate this signaling pathway. We reasoned that strong antagonists selectively targeting the RAR subunit in the RAR-RXR heterodimer should have no effect if RXR heterodimers with other nuclear receptors were the signaling species. We took advantage of well-characterized RAR antagonists, BMS493 and BMS614 (25), which do not at all interact with RXRs, and tested if these antagonists would affect PLB985 differentiation induced by LG1069 in the presence of the phosphodiesterase inhibitor theophylline (Fig. 5A). As expected, a strong induction of differentiation, which was inhibited by an excess of the RXR-selective antagonist UV3003 (ref. 32; lane 7), was observed with the rexinoid-theophylline combination, whereas no effect was seen with either of the two agents alone (lanes 2–4). Importantly, however, the pure RARs antagonist, BMS614, as well as BMS493, a pan-RAR inverse agonist that enforces RAR-corepressor interaction (25), fully blocked the differentiation induced by the rexinoid–phosphodiesterase inhibitor combination (lanes 5 and 6), thus indicating that it is indeed the RAR-RXR heterodimer that responds to the rexinoid in the presence of elevated cAMP levels. In keeping with previous reports (ref. 42 and references therein), RAR antagonists also synergize efficiently with agents that increase intracellular CAMP levels, as the combination of ATRA and theophylline at concentrations which alone do not yield significant effects, strongly induce differentiation of PLB985 cells (lanes 2, 8, and 9). This synergy can be blocked by RAR antagonists (lanes 10 and 11) but not by the RXR antagonist UV13003 (lane 12; note that a weak inhibition is due to contamination of ATRA by 9-cis retinoic acid, no such inhibition was seen with pure RAR ligands), demonstrating that it operates exclusively through the RAR subunit of the heterodimer.

To study if the observed differentiation effect reflected gene regulation by the RAR-RXR heterodimer, a DR5 reporter gene was transfected into U937 cells and the response of the endogenous receptors to the various compounds was determined (Fig. 5B). Importantly, in the presence (but not absence) of theophylline or a synthetic CAMP analogue (8CPT-cAMP) the rexinoid LG1069 strongly induced transactivation of the DR5 reporter (lanes 8 and 12), which was blocked by the RAR inverse agonist BMS493 (lanes 10 and 14). As expected, the RAR-selective agonist TTNPB activated reporter gene transcription on its own (lane 2) and synergized with 8CPT-CAMP (lane 7), and less pronounced with theophylline (lane 11), in a BMS493 antagonized manner (lanes 9 and 13).

Our previous work indicated that RXR subordination results from the inability of RXR ligands to displace the corepressor from the heterodimer (25) and evidence has been presented suggesting that synergy between RXR and CAMP might also result from RXR desubordination (43). To assess if corepressor release from aporetinoids could be induced or facilitated by increased CAMP levels we used two-hybrid experiments in HeLa cells (Fig. 5C, left). Importantly, whereas RAR-VP16 interacted strongly with Gal-SMRT (lane 7), coexpression of the cDNA of the catalytic subunit of the human CAMP-dependent protein kinase gene PKA catalyzed this interaction (lane 15) nearly as efficiently as the RAR agonist TTNPB (lane 8). Thus, with respect to corepressor dissociation from the RAR-RXR heterodimer, activation of PKA is an apparent functional equivalent of RAR agonist binding. In parallel experiments in identical experimental conditions, NCoR also dissociated from RAR, albeit less efficiently than in the case of SMRT (data not shown). Two-hybrid experiments with the heterodimer composed of RARs and RXR-VP16 confirmed that, in principle, the heterodimer recapitulates the results obtained with RAR-VP16 (Fig. 5C, right, compare lanes 9 and 19). Thus, PKA activation also destabilizes the interaction between the RARo-RXR heterodimer and cognate corepressors.

We predicted that RXR desubordination should result in activation of endogenous retinoic acid target genes. Indeed, RARβ expression was 53-fold induced by rexinoids in the presence of CAMP (Fig. 5D, lane 4), whereas no significant induction was observed with each agent alone (lanes 2 and 3). Moreover, induction of RARβ by either rexinoid-cAMP or ATRA is blunted (Fig. 5E, lanes 3 and 5) in MEFs in which all three RARs have been deleted by using the Cre-lox technology (see Materials and Methods; refs. 35–37). This is in perfect agreement with the antagonistic affect of the inverse agonists BMS493 (25) on ATRA and rexinoid-cAMP inductions of RARβ (lanes 4 and 6). Most importantly, reintroduction of the RARs cDNA reestablished RARβ responsiveness to both rexinoid-cAMP and ATRA (Fig. 5F, lanes 4 and 5). Note that relative to its induction by ATRA, the induction of RARβ by rexinoid-cAMP exposure is significantly enhanced upon RARα reexpression, indicating that the RARo-RXR heterodimer is particularly prone to RXR desubordination.

Upstream activation of the protein kinase A signaling pathway leads to rexinoid-dependent myeloid cell differentiation and apoptosis. Given that upon the addition of exogenous CAMP analogues or blocking PDE-mediated CAMP degradation rexinoids could induce differentiation and apoptosis, we wondered if upstream activation of the signaling cascade would replicate the observed events. Indeed, whereas activation of adenylyl cyclase by the diterpene forskolin did not significantly affect PLB985 cell differentiation or apoptosis, addition of the rexinoids SR11237 or LG1069, which alone were without effect, dramatically induced cell differentiation and subsequent apoptosis in a dose- and time-dependent manner (Fig. 5G and H).

Discussion

The precise regulation of multiple signaling pathways that accurately control cellular decisions to proliferate, differentiate, arrest growth, or initiate programmed cell death (apoptosis) is essential for normal life. Cancer arises when cells escape this balance and proliferate inappropriately without compensatory apoptosis. As differentiation generally limits the proliferative capacity and life span of a cell, agents able to restore normal differentiation pathways in cancer cells and/or induce postmaturational apoptosis are promising tools for cancer therapy (6, 8, 19, 44). Here we show that the recently identified novel APL differentiation pathway that relies on rexinoid-PCA crosstalk (31) is a powerful inducer of maturation and death of several established AML cell lines in vitro. Most importantly, primary AML blasts studied in ex vivo cultures responded to this treatment by induction of maturation, apoptosis, and loss of clonogenic potential in all cases tested, irrespective of FAB subtype, karyotype, and immunophenotype. In contrast, no response was observed with ATRA, suggesting that in the presence of elevated levels of CAMP,
Figure 5. The rexinoid-cAMP crosstalk involves signaling through the RARα-RXR heterodimer. A, PLB985 cells were exposed for 4 days to pan-phosphodiesterase inhibitors theophylline (2 mmol/L), the rexinoid LG1069 (30 nmol/L), the RAR pan agonist ATRA (30 nmol/L), in combination with the selective RARα antagonist BMS614 (1 μmol/L), the pan-RAR inverse agonist BMS493 (1 μmol/L), and the RXR antagonist UVI3003 (5 μmol/L) as indicated. Differentiation was checked by NBT reduction assay. B, luciferase assay on electroporated U937 submitted to 100 nmol/L TTNPB, 1 μmol/L BMS493, 1 μmol/L LG1069, 200 μmol/L 8CPT-cAMP, 2 mmol/L theophylline alone, or in combination as indicated. C, two-hybrid experiment to assess RARα-VP16-corepressor (left) or RXRα-RARα-corepressor (right) interaction, as schematically depicted. Cells were cotransfected with a luciferase reporter gene and the indicated vectors. PRKACA encodes the catalytic subunit of PKA. D, in the presence of elevated cAMP levels, rexinoids induce expression of the endogenous RARβ gene in U937 cells. Cells were exposed for 12 hours to 200 μmol/L 8CPT-cAMP and/or 1 μmol/L SR11237 as indicated, and RARβ expression was quantitated by real-time PCR. Similar results were obtained with CYP26A1 (data not shown). Note that the inverse agonist BMS493 (5 μmol/L) is a good pharmacologic mimic of RAR deletion. ATRA, 1 μmol/L. E, rexinoid-cAMP and ATRA responsiveness is reestablished in the triple RAR knock-out MEFs by expressing retrovirally transduced RARα cDNA. Cells were treated and analyzed as described in (E). G and H, the adenylate cyclase activating agent forskolin synergizes with rexinoids to induce cell differentiation and death. The PLB985 cells were treated with the indicated concentration of forskolin in combination with 1 μmol/L SR11237 or 100 nmol/L LG1069. After the indicated time, differentiation (G) and apoptosis (H) were assessed.
rexinoid agonists might extend the repertoire for differentiative and/or apoptotic treatment of AML.

Our mechanistic analysis shows that it is the RARα-RXR heterodimer, which mediates the differentiative and apoptogenic effect of the rexinoid-cAMP crosstalk. This is most obvious from the observations that (a) the endogenous retinoic acid target gene, RARβ, is activated by rexinoid-cAMP as by ATRA, albeit less efficiently, (b) pure RARα antagonists or pan-RAR inverse agonists blocked this crosstalk, (c) knock-out of all three RARs blunted both the ATRA and rexinoid-cAMP response, which (d) could be restored by reexpression of RARα. Thus, we show that the RAR-RXR heterodimer has two distinct options to activate its target program, either through the classical route using RAR agonists or through the combination of rexinoids and agents that increase intracellular cAMP levels (cAMP analogues or phosphodiesterase inhibitors). Note also that in keeping with previous observations (42, 45, 46), the signaling induced by RAR agonists (ATRA or TTNPB) is significantly enhanced in the presence of increased cAMP levels. However, in contrast to the rexinoid-cAMP crosstalk, the rexinoid-cAMP crosstalk is insensitive to RAR antagonists, clearly demonstrating that the two mechanisms are fundamentally distinct (see also below).

How to explain the ability of RXR to signal in view of the well-documented RXR-subordination in heterodimers with apo-RAR? Having previously identified persistent corepressor binding to the heterodimer as the cause of RXR subordination (25), using two-hybrid experiments, we assessed the fate of SMRT and NCoR in the presence of elevated cAMP levels. Importantly, RARα, the signaling receptor in myeloid cells, dissociates from the corepressor upon activation of PKA. Thus, PKA is a functional mimic of an RAR agonist with respect to coregulator binding. We asked if the known phosphorylation of RARα at Ser369 by PKA (47) could be responsible for this dissociation. However, our preliminary data reveal that activation of PKA still induced the dissociation of corepressors from RARαS369A (data not shown). Together, our results suggest a mechanism (Fig. 6) wherein signaling events increase cAMP levels, thereby inducing activation of PKA and entry of its catalytic subunit ("c") into the nuclear compartment leading to dissociation of the corepressor complex from RARα. This results in a "desubordination of RXR allowing RXR agonists to induce coactivator complex recruitment (for mechanistic details, see ref. 25), thus initiating the RARα-RXR heterodimer-dependent gene programs. Whether phosphorylation of RARαS369 contributes at all to corepressor dissociation (option 1 in Fig. 6) remains to be established, but other targets are certainly involved. This could be phosphorylation of the corepressor itself (option 2) or of presently unidentified factors (option 3) that acquire the ability to interfere with RARα-corepressor interaction upon phosphorylation.

Importantly, there is a fundamental difference between the retinoid and rexinoid-cAMP induced signaling pathways, even though both similarly activate the RARα-RXR heterodimer. AML cells are not differentiated by retinoids but we show here that the differentiation and apoptosis program is fully activated by the rexinoid-PKA crosstalk. Thus, in addition to "RXR desubordination," further PKA-dependent gene programs facilitate RARα-RXR-mediated cell differentiation and apoptosis. Our results indicate that the crosstalk between retinoids and PKA should result in the activation of similar differentiation and apoptosis pathways. The precise "master switches" which lead to coordinated activation of the RARα-RXR heterodimer and PKA induced differentiation and TRAIL-DR5-dependent apoptosis remain to be identified.

In addition to revealing the mechanistic basis of RXR desubordination, our study provides important information about the apoptogenic program induced by rexinoid-cAMP synergy and the factors involved. We show that the combination of RXR and PKA agonists consistently leads to strongly enhanced expression of the TRAIL receptor DR5 in all myeloid cells tested. In contrast to retinoic acid treatment (18), TRAIL expression is only moderately increased activation of the RARα-RXR heterodimer and PKA induced differentiation and TRAIL-DR5-dependent apoptosis remain to be identified.

Figure 6. Mechanism of rexinoid-cAMP induced AML blast apoptosis. Top, subordination of RXR by apo-RAR is due to persistent corepressor (complex) interaction with RXR not allowing, possibly for sterical reasons, binding of the coactivator (complex) to agonist-bound RXR (for details see ref. 25). Bottom, signaling that leads to activation of PKA and nuclear compartmentalization of its catalytic subunit leads to dissociation of the corepressor complex from RARα due to three nonexclusive signaling options, (1) phosphorylation of RARαS369, which is insufficient on its own but might contribute to RXR desubordination, (2) phosphorylation of the corepressor, or (3) phosphorylation of an unknown factor that acquires the ability to interfere with RARα-corepressor interaction. For differentiation and apoptosis to occur in acute myeloid cells, additional cAMP-inducible gene programs need to be activated in addition to the RAR-RXR heterodimer-dependent gene regulation.

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enhanced and exogenous TRAIL can dramatically increase cell death in PKA-REW promoter-treated cells. Notably, the induction of DR5 and TRAIL was observed by immunohistochemical staining of several AML patient blast cultures treated ex vivo and staining correlated with a nuclear morphology indicative of cell differentiation. Therefore, it is likely that retinoid-cAMP signaling induces postmaturation apoptosis through TRAIL-DR5 ligand-receptor activation. However, we do not exclude TRAIL-independent cell death induction due to highly expressed DR5, because the death domains present in DR5 function as oligomerization interfaces and mere DR5 overexpression leads to TRAIL-independent apoptosis (48). Interestingly, DR5 activation in the absence of TRAIL also induces apoptosis in a tumor cell–selective manner as shown with anti-DR5 antibodies that induce receptor oligomerization (49). Thus, up-regulation of DR5 by retinoid-cAMP might be sufficient to induce AML blast apoptosis. Although our results provide compelling evidence for a contribution of the TRAIL-DR5 signaling to retinoid-cAMP–induced apoptosis, we do not exclude the additional implication of other death receptors (DR3, DR4), which we see induced in some, but not consistently in all, cell lines tested. The blockage of apoptosis by the pan-caspase inhibitor Z-VAD excludes a significant contribution of caspase-independent death signaling pathways.

We report here for the first time that in the presence of elevated cAMP levels, RRX-selective agonists differentiate and kill blasts derived from AML patients with retinoic acid–insensitive disease. Not only did PLZF-RARα–positive blasts fully differentiate upon this treatment, but non-APL AML blasts that are normally unresponsive to retinoids also underwent differentiation and apoptosis upon RRX-PKA treatment. The possible importance of the signaling option for AML therapy prompted us to look for alternative ways to increase cAMP levels, because to our knowledge, no synthetic cAMP analogue is presently in clinical use. Indeed, it is known that cAMP levels can be also elevated by treating cells with 3’,5’-cAMP phosphodiesterase inhibitors and the corresponding drugs are already in clinical use for the treatment of neurologic, cardiovascular, or inflammatory disorders (50). Moreover, phosphodiesterase inhibitors, such as the pan-specific theophylline or the PDE4-selective rolipram, can induce apoptosis in chronic lymphocytic leukemia concomitantly with elevated cAMP levels. These effects were reported to be cell type–dependent, as interleukin-2–cultured whole mononuclear cells and antiimmunoglobulin–stimulated CD19 (+) B cells were resistant to the induction of apoptosis by rolipram, whereas unstimulated CD19 (+) B cells were more sensitive (51). Furthermore, the PDE4-selective inhibitor rolipram suppressed the growth of acute lymphoblastic leukemia cells (52). As far as retinoids are concerned, we used LG1069 (53), a retinoid used for the treatment of cutaneous T cell lymphoma. Initially studying PLB985 cells, we saw a dramatic synergy between each of the three phosphodiesterase inhibitors, theophylline, IBMX, and rolipram, and LG1069 for induction of differentiation. Most importantly, the clonogenic growth potential of AML blasts in ex vivo cultures was completely suppressed by phosphodiesterase inhibitor–retinoid treatment.

Our observations might provide additional options for the treatment of ATRA-resistant or ATRA-insensitive AML. We note in this respect that in a retinoic acid and arsenic trioxide–resistant APL patient, the addition of theophylline to the therapeutic protocol yielded a complete clinical remission (46). In view of our results, it is possible that the RXR ligand 9-cis retinoic acid formed from, or present in, the ATRA given to the patient contributed to this response. In view of the low toxicity of retinoids (6), these observations and the clinical availability of the corresponding drugs provides a rationale for initiating clinical studies addressing the efficacy of combinatorial phosphodiesterase inhibitor–retinoid therapy in AML patients. Together with our recent finding that a very promising class of epigenetic anticancer drugs operates through activation of TRAIL expression (54, 55), the possibility to target both the ligand (TRAIL) by histone deacetylase inhibitors and the cognate receptors (DR4, DR5) by the above described retinoid crosstalk might present a promising therapeutic option to limit, or even substitute, chemotherapeutic drugs with non-genotoxic signaling compounds.

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**References**


Rexinoid-Triggered Differentiation and Tumor-Selective Apoptosis of Acute Myeloid Leukemia by Protein Kinase A–Mediated Desubordination of Retinoid X Receptor

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