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REVIEW

Retinoic acid resistance in acute promyelocytic leukemia

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Primary resistance of PML-RARα-positive acute promyelocytic leukemia (APL) to the induction of clinical remission (CR) by all-*trans* retinoic acid (ATRA) is rare but markedly increases in frequency after ≥2 relapses from chemotherapy-induced CRs. Nevertheless, even in *de novo* cases, the primary response of ATRA-naive cases is variable by several measures, suggesting involvement of heterogeneous molecular elements. Secondary, acquired ATRA resistance occurs in most patients treated with ATRA alone and in many patients who relapse from combination ATRA chemotherapy regimens despite limited ATRA exposure. Although early studies suggested that an adaptive hypercatabolic response to pharmacological ATRA levels is the principal mechanism of ATRA resistance, recent studies suggest that molecular disturbances in APL cells have a predominant role, particularly if disease relapse occurs a few months after discontinuing ATRA therapy. This review summarizes the systemic and APL cellular elements that have been linked to clinical ATRA resistance with emphasis on identifying areas of deficient information and important topics for further investigation. Overall, the subject review strongly supports the hypothesis that, although APL is an infrequent and nearly cured disease, much can be gained by understanding the complex relationship of ATRA resistance to the progression and relapse of APL, which has important implications for other leukemias and malignancies.

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Introduction

This review presupposes familiarity with the essential clinical aspects of acute promyelocytic leukemia (APL) and with basic molecular mechanisms involved in the maturation response of APL cells to all-*trans* retinoic acid (ATRA). For readers seeking more background information, many reviews are available.[1–6] Although five different chromosome translocations have been associated with APL, all involving the retinoic acid receptor-alpha (RARα) gene, this review is restricted to the t(15;17) that generates the PML-RARα oncoprotein. About 99% of APL cases are PML-RARα-positive, and a substantial body of clinical, pharmacological and cell biological correlative information has now accrued from clinical trials employing ATRA therapy. Some of the most interesting information relates to ATRA resistance, much of which has been developed since this subject was last comprehensively reviewed.[7]

In this review, clinical ATRA resistance shall be defined as the inability to achieve or sustain complete hematological remission (CR) of APL on ATRA therapy. By this criterion, primary ATRA resistance is very uncommon, since CR can be achieved in all but a few reported ATRA naive cases, excluding early death from disease complications or the early development of toxic complications. On the other hand, the development of secondary, acquired ATRA resistance is very common in patients treated continuously with oral ATRA as a single agent. As induction therapy has improved by using ATRA in combination with chemotherapeutic agents, the incidence of ATRA resistance has declined, but it remains a significant deterrent to the maximum application of this highly specific agent, especially in the 10–30% of patients who relapse from CRs achieved and consolidated with ATRA-containing regimens. Increasingly, it is recognized that there is great heterogeneity in sensitivity to ATRA effects on APL cells *in vitro* and *in vivo*, and, although it is convenient for discussion to fix a definition of ATRA resistance, it, in reality, reflects a spectrum of aberrations with complex permutations. This review attempts to update and integrate information related to these complexities following the outline in Table 1.

Clinical observations

Primary ATRA resistance

Table 2 summarizes the results of major reported studies in which ATRA was utilized as a single agent in either oral or intravenous liposomal (L-ATRA) form for the induction of CR in ATRA-naive cases.[8–20] Among >375 cases of *de novo* disease with no previous anti-leukemia therapy, only two instances of remission induction failure due to ATRA-resistant disease were reported. In only one of these cases was the diagnosis of APL established by genetic criteria, ie the finding of

Table 1 Outline of topics related to ATRA-resistance in APL

Clinical observations
- Primary ATRA resistance
- Secondary, acquired ATRA resistance

Elements of acquired ATRA resistance
- Systemic elements
  - Increased catabolism
  - Sequestration by CRABP
  - Relation of alternative retinoids to hypercatabolism
- APL cellular elements
  - *In vitro* ATRA sensitivity testing
  - Mechanisms that may limit ATRA delivery to cell nucleus
    - Decreased effective APL cell uptake
    - Increased cytoplasmic sequestration
    - Increased cytoplasmic catabolism
    - Decreased nuclear transport
  - Aberrations of nuclear receptor-gene response mechanisms
    - Overall considerations
    - Focus on PML-RARα: *in vitro* studies
    - Focus on PML-RARα: clinical studies

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the PML-RARα transcript by RT-PCR assay.[16] Similarly, in other large clinical trials in which ATRA was used in combination with chemotherapy, no[18–21] or a very low[22–24] incidence of primary resistant disease was reported in *de novo* APL patients. Although most patients entered on these trials had cytogenetic or molecular studies to make the genetic diagnosis of APL, a significant fraction of patients did not have these studies. In almost all instances, these data were not reported specifically related to the ATRA induction failure cases, raising the possibility that some of these cases may not have had PML-RARα-positive APL. For example, in the North American trial INT0129 in which this was reported, 3/4 ATRA (single agent) induction failure cases tested by RT-PCR lacked PML-RARα mRNA.[16]

At first relapse from previous chemotherapy or in cases initially refractory to chemotherapy, two cases of primary ATRA resistance, both of which had documented genetic APL by the t(15;17) marker chromosome, have been reported among approximately 60 evaluable cases (Table 2). These results suggest that the incidence of primary ATRA resistance is either the same or slightly higher than in *de novo* disease, although long-term outcome is less favorable (reviewed in Ref. 25). Only a few reported cases of ATRA induction following two or more relapses from chemotherapy-induced CRs are available. However, in two studies with oral ATRA, 3/10 patients[9,13] and, in one study with L-ATRA,[20] 3/3 patients, respectively, failed to achieve CR. This high incidence (46%) of ATRA resistance in ATRA-naive patients with two or more relapses indicates the development either of cross-resistance to chemotherapeutic agents, as discussed in more detail below, or of non-specific molecular aberrations related to leukemic progression. Importantly, at least six of these cases were documented to be positive for the t(15;17) by repeat cytogenetic analysis just prior to retreatment with ATRA or L-ATRA.[9,20]

Cytogenetic or molecular confirmation of genetic APL in cases with primary clinical ATRA resistance is important, because several instances of leukemic chimeras have been reported in which APL cells are admixed with alternative leukemia cells, most frequently t(8;21)/AML1-ETO-positive cells.[26–30] For example, in the INT0129 trial, a case that was PML-RARα-positive but lacked the t(15;17) in the few analyzable metaphase cells (as occurs in ~15% of cases analyzed) was crossed over to the chemotherapy induction arm of the protocol because of ATRA toxicity (but also with a poor initial response) and, after initially achieving CR, the patient relapsed with PML-RARα-negative AML.[31] Similar instances have been cited in other studies involving ATRA induction as a single agent[32] or in combination with chemotherapy.[29,30] Most convincingly, in two cases, transcripts for both PML-RARα and AML1-ETO could be detected prior to therapy but after treatment with combined ATRA/chemotherapy, there was selective loss of the PML-RARα transcript or t(15;17) with retention and relapse with AML1-ETO-positive leukemia.[29,30] Since RT-PCR is such a sensitive method, positive results for PML-RARα might be observed even with a relatively minor subpopulation of APL cells in a leukemic chimera, which could then unfold as an apparent primary ATRA-resistant case.

Few other specific mechanisms of potential primary ATRA resistance have been identified. Based on few cases, evidence

Table 2 Reports of primary ATRA resistance in ATRA-naive APL cases

Previous chemoRx	*Evaluable cases*[a]	*ATRA form and dose*[b]	*No. resistant*	*Diagnostic criteria*	*Study/Ref.*
None/*De novo* disease	15	Oral 45	1	Cytologic	Huang *et al*[8]
	41	Oral 60–80	0		Chen *et al*[10]
	2	Oral 45	0		Castaigne *et al*[9]9
	10	Oral 25	0		Castaigne *et al*[11]
	54	Oral 45	0		Soignet *et al*[15]
	14	Oral 45	0		Fenaux *et al*[12]
	153	Oral 45	1[c]	Molecular	Tallman *et al*[16]
	59	Oral 45	0		Asou *et al*[18]
	27[d]	IV Lipo 90	0		Estey *et al*[19]
	20[d]	IV Lipo 90	0		Douer *et al*[20]
1st Relapse or 1st induction failure	8	Oral 45	0		Huang *et al*[8]
	6	Oral 60–80	0		Chen *et al*[10]
	10	Oral 45	1	Cytogenet	Castaigne *et al*[9]
	12	Oral 25	1	Cytogenet	Castaigne *et al*[11]
	11	Oral 45	0[e]		Ohno *et al*[13]
	7	Oral 50	0		Cortes *et al*[17]
	4	IV Lipo 90	0		Douer et al[20]
≥1 Relapse[f]	25	Oral 45	0		Soignet *et al*[15]
≥2 Relapses or 2nd refractory	5	Oral 45	3	Cytogenet	Castaigne *et al*[9]
	5	Oral 45	0[e]		Ohno *et al*[13]
	3	IV Lipo 90	3	Cytogenet	Douer *et al* [20]

[a]Early death cases and, in some trials, cases treated with chemotherapy in addition to ATRA have been subtracted from the total reported cases, because they are not evaluable for the effectiveness of ATRA as a single agent to induce CR.
[b]Dose in mg/m^2/day except liposomal ATRA is every other day.
[c]APL cells from three additional reported cases with ATRA resistance tested negative for the PML-RARα fusion gene.
[d]Cases are partially overlapping from both references.
[e]Data from 'First Study' cited in Ref. 13.
[f]Cases not segregated by relapse number but evidently a minority of cases had >1 relapse.

SPOTLIGHT

was presented that APL cells harboring the uncommon V-form of PML-RARα with relatively long deletions of the carboxy-terminus of PML exon 6 have reduced sensitivity to ATRA-induced maturation.[33] Although some supportive evidence for an adverse clinical impact of the V-form has emerged, there have been insufficient cases to establish this, and it may have a greater impact on disease-free and overall survival than on CR.[34,35] More specifically, in one reported V-form case (case 1, Ref. 33, case 16 Ref. 31) with a long PML exon 6 deletion and an over-compensatory insertion of sequence from RARα intron 2,[34,36] two novel molecular findings related to ATRA resistance have been made by independent investigative groups: (1) a small deletion mutation in the normal PML allele[37] and (2) a sequence motif in the RARα intronic insert associated with increased binding of corepressor protein.[36] The APL cells of this patient, who discontinued ATRA induction therapy because of the development of cerebral aspergillosis,[33] had a defect in superoxide generation, as assessed by the nitroblue tetrazolium dye reduction test, which was disproportionate to the loss of cytological response. This defect became more severe after relapse from a brief chemotherapy-achieved CR[31] without, however, apparent change in either of the two molecular defects.[36,37] These results illustrate how more than one molecular defect may contribute to reduction of the primary maturation response of APL cells to ATRA.

Also consistent with the notion that ATRA-sensitivity – which is just the obverse of ATRA resistance – is related to multiple factors, is a recent study of the *in vitro* induction of APL cell maturation under non-saturating ATRA conditions.[38] When tested at 0.1 μM ATRA for only 3 days, there was great heterogeneity in the level of APL cell maturation achieved, which contrasts with the more uniform high-level of terminal differentiation of APL blasts from the great majority of cases after 5 or 6 days incubation.[33,38] This heterogeneity is difficult to explain by any common molecular mechanism that decreases sensitivity and, reciprocally, increases relative resistance. As noted,[38] a variety of factors may contribute to this heterogeneity, including inherent differences in intracellular molecules mediating the ATRA response, in effective APL cellular uptake of ATRA, or in modulation by cytokines. Extending this logic to the *in vivo* situation, inherent differences in systemic ATRA metabolism[39,40] might further contribute to an individual patient's position on the ATRA sensitivity/resistance spectrum. However, these heterogeneity elements appear to be insufficient to produce primary ATRA resistance, as defined, since virtually all *de novo* APL patients achieve CR, although in some cases this may be quite short-lived. In the few documented primary ATRA resistant cases, mostly after relapse from chemotherapy Table 2, the partial or transient response of some of these cases could reflect a quantitative change in the heterogeneity elements and/or could reflect their interaction with drug-resistant mechanisms induced by prior chemotherapy. Notably, patients from the APL93 clinical trial with >50% APL blast maturation under the non-saturating ATRA conditions had significantly better disease-free survival (DFS) by both univariate and multivariate analysis, than those with less maturation.[38] Further studies are needed to determine if pretreatment heterogeneity elements are related to secondary, acquired ATRA resistance that so frequently attends post-ATRA therapy clinical relapse.

Secondary, acquired ATRA resistance

Table 3 summarizes studies in which attempts were made to induce another CR after relapse from ATRA-containing therapy, using ATRA or an alternative retinoid as a single agent. The data are presented as the proportion of cases that failed to achieve CR on retinoid retreatment and include only cases in which sufficient retreatment was administered to document that CR could not be reachieved because of resistant disease rather than failure for an alternative reason, such as early death or toxicity. Studies in which this could not be clearly assessed were excluded from this analysis, eg 12/15 cases that failed to achieve a second CR on ATRA as a single agent after relapse from an ATRA-induced CR.[41,42] Similarly, some cases were excluded because the achievement of CR apparently involved supplementation with chemotherapy, eg in 13/13 CRs after first relapse from prior treatment with ATRA + chemotherapy.[43]

Discounting differences in effectiveness of the different agents, the data indicate that the incidence of ATRA resistance is inversely related to the time since last ATRA exposure: 18/18 (100%) patients who relapsed while taking ATRA treatment; 19/25 (76%) patients who relapsed within 1 year of ATRA treatment; and 15/44 (34%) patients who relapsed after 1 year of ATRA treatment. Such observations led to the hypothesis that a time-reversible mechanism is the primary basis of ATRA resistance in APL.[44] Although this hypothesis is likely true to a certain extent, there are reasons, including molecular pharmacological and genetic considerations (*vide infra*), for suggesting that other mechanisms may be predominant in many ATRA-resistant cases. Other general considerations that tend to attenuate this hypothesis are that a non-specific property of leukemic relapse is that short CR durations are associated with more aggressive, less treatment-responsive disease and that there have been no reported instances of reversal of documented clinical ATRA resistance in any individual case with time. The observation that the incidence of resistance to alternative retinoids, including liposomal ATRA, 9-*cis* RA and Am80, are associated with a higher incidence of cross-resistance in cases that relapsed within a few months of stopping oral ATRA treatment (Table 3) also seems counter to the reversibility concept, as discussed in more detail below. Briefly, if ATRA resistance is primarily related to an adaptive, reversible metabolic response to ATRA, these alternative retinoids that are able to bypass elements of this response would be expected to be more effective than ATRA at inducing CR in the early off-ATRA period. But, as summarized in Table 3, the data do not appear to support this concept.

Another potentially important recent observation related to the timing of ATRA resistance derives from a study of minimal residual disease (MRD) monitoring to detect molecular, as opposed to clinical, relapse. In this study, 7/12 patients (58%) who had a molecular relapse following CR induced by ATRA + idarubicin (AIDA protocol) achieved a second molecular remission following treatment with oral ATRA (45 mg/m^2/day) as a single agent.[45] All of the ATRA-responsive molecular relapses, documented by two successive positive RT-PCR assays for PML-RARα mRNA, occurred between 2 and 12 months of initially achieving molecular remission, a time-frame strongly overlapping with the five cases that did not reachieve molecular remission on ATRA alone.[45] Since the data in Table 3 indicate that retreatment with oral ATRA during this time-frame, even at increased doses, was effective in achieving second clinical CRs in only 2/11 (18%) patients, the molecular data raise the additional possibility that acquisition of ATRA resistance may be associated with the expansion of the APL cell clone from a molecularly detectable to a clinical relapse level.

Another factor to consider is the number of previous

Table 3 Reports of acquired ATRA resistance: incidence of failure to achieve second, single-retinoid-induced remission

Agent and dose[a]	*Evaluable cases*[b]	*No. previous relapses*	*No. of failures/Total cases Time off ATRA*			*Study/Ref.*
			0–1 mo	*2–12 mos*	*>12 mos*	
ATRA 100–120	9	1	6/6	1/1	1/2	Chen *et al*[10]
ATRA 45	6	1	1/1	5/5		Delva *et al*[91]
ATRA 90[c]	17	1–2	10/10	3/5	1/2	Warrell *et al*[14]
9-*cis* RA 30–230	6	1–5	1/1	1/1	3/4	Miller *et al*[46]
Am80	24	1		3/3	7/21	Tobita *et al*[100]
Lipo ATRA 30–110	6	1–4		2/3	1/3	Estey *et al*[47]
Lipo ATRA 90	18	1		3/6	2/12	Douer *et al*[20]

[a]Dose in mg/m^2/day or, for liposomal ATRA, every other day.
[b]Early deaths or withdrawals and, in some trials, cases treated with chemotherapy in addition to ATRA have been subtracted from total reported cases.
[c]Relapse cases with ≥2 months may have received standard 45 mg/m^2/day dose of ATRA.

relapses. In the study by Warrell *et al*,[14] it can be inferred from the trial design that all 10 unsuccessful '0 time-off ATRA' cases were in second relapse, while three successful reinductions at later times were most likely first relapse cases. Similarly, the single successful reinduction with 9-*cis* RA reported by Miller *et al*,[46] and the three successful reinductions with L-ATRA reported by Estey, *et al*,[47] were first relapse cases. All of the cases with two or more relapses had been treated with prior chemotherapy, and, as apparent from Table 2, non-ATRA-specific cross-resistance becomes a factor with two or more relapses.

Despite the apparent establishment of ATRA resistance after two or more relapses, it was employed after multiple relapses in five cases in combination with sodium phenylbutyrate, a histone deacetylase inhibitor (HDI).[48,49] In one case in which progressive disease was noted with ATRA (45 mg/m^2) as a single agent after a third relapse, a CR of several months duration was achieved after the addition of phenylbutyrate and increasing ATRA to 90 mg/m^2.[48] Although it is unclear if the ATRA was contributory to this response, it is notable that the *in vitro* maturation of the ATRA-resistant APL cells from this patient was augmented by the combination of ATRA with sodium butyrate[50] and that in a murine APL model of progressive disease on ATRA as a single agent, the response to HDI *in vivo* required combination with ATRA.[51] These observations suggest that, as noted for ATRA-naive APL, the genesis of ATRA resistance is likely heterogeneous and multifactorial such that some elements of the APL cellular response to ATRA may persist even after multiple relapses.

Systemic elements in acquired ATRA resistance

Increased catabolism

An early assessment of acquired ATRA resistance in APL was that it develops mostly, if not entirely, as an adaptive metabolic response to the sudden increase in ATRA to pharmacological levels ($>10^{-7}$ M) from normal physiological levels ($\leq 10^{-8}$ M).[7] It was plausibly argued that the near-universal development of ATRA resistance in patients continuously treated until relapse is more likely due to a common regulatory change(s) than to the acquisition of a fixed genetic alteration(s). The theoretical argument for an adaptive role is enhanced by the consideration that an inducible protective systemic mechanism might reasonably have been evolutionarily selected to prevent toxicity from high ATRA concentrations due to disruption of regulatory pathways required for its natural function as an embryonic morphogen and as a co-factor in tissue homeostasis throughout life.[52,53]

Beyond plausibility, pharmacokinetic (PK) studies demonstrated that ATRA treatment induces a hypercatabolic response that reduces achievable peak ATRA concentrations and area under the curve (AUC) values by up to 80% compared to first-treatment values. This was initially demonstrated in an evaluation of relapse cases[54] but was subsequently found to occur in virtually all cases within a few days of initiating ATRA therapy.[55–58] Subsequent studies demonstrated that this hypercatabolic state is reversible on an intermittent, every-other-week schedule up to 11 weeks of treatment.[59] A subsequent study suggested that after 2 weeks of ATRA therapy 1 week off drug was insufficient for reversal of the hypercatabolic state.[58] No PK studies have been reported after prolonged continuous ATRA therapy to test the time needed to reverse the hypercatabolic state.

Several factors likely contribute to the systemic hypercatabolic response, all of which have been associated with increased oxidation metabolites, principally 4-oxo-RA-glucuronide in urine (10-fold) and 4-oxo-RA in plasma (<2-fold).[55] The oxidative catabolism of ATRA is mediated by microsomal membrane-bound cytochrome P450 (CYP) enzymes, principally in the liver,[60] encoded by a supergene family that catalyzes the oxidation of a wide variety of endogenous and exogenous substrates.[61] Although vitamin A and retinoic acid had been noted to upregulate some CYP species,[62,63] only recently has a CYP enzyme family been identified that is specifically dedicated to auto-regulation by ATRA,[64,65] of which at least two different forms with different tissue distributions have been described, CYP26A1 and CYP26B1.[53] It has been suggested that CYP26 is an important element in the hypercatabolic response following the administration of ATRA to APL patients, but this has not been specifically reported.[66]

A study prior to the identification of CYP26 with hepatic microsomal fractions containing mixed CYP enzymes suggested that the predominant effect of ATRA might be mediated by upregulation of lipid hydroperoxides, which are co-factors for CYP activity via the so-called 'peroxide shunt', rather than by direct upregulation of CYP enzymes *per se*.[67] Lipid hydroperoxides are derived from prostaglandins, a synthetic pathway initiated from arachidonic acid involving lipoxygenases, which can be upregulated by ATRA.[68] The principal clinical evidence that increased CYP activity contributes to the hypercatabolic response was that pre-treatment with anti-fungal imidazole inhibitors of P450 enzymes, including ketocona-

SPOTLIGHT

zole,[40] liarozole[69] and fluconazole,[70] could inhibit the early development of the hypercatabolic response to ATRA administration. Although the initial study indicated that ketoconazole was effective up to 1 month of continuous ATRA therapy,[40] subsequent studies demonstrated that the effect of these agents is only transitory.[58,69] One study with hepatic microsomes demonstrated that both ketoconazole and fluconazole can inhibit direct, NADPH-mediated CYP oxidation of ATRA but not lipid hydroperoxide-dependent oxidation.[70] Alternatively, the imidazole inhibitors could affect lipid hydroperoxide activity by inhibiting their synthesis from prostaglandins.[71] These agents can inhibit auto-induced oxidative metabolism of ATRA in a variety of cell types *in vitro*, including tumor cells (see below), which has been linked to inhibition of CYP26,[65,66] but the exact mechanism has not been determined nor, as noted above, has the relationship to the systemic hypercatabolic response to pharmacological doses of orally administered ATRA been determined.

Sequestration by CRABP

Another molecular factor that has been related to the hypercatabolic response to ATRA therapy is upregulation of small (15–18 kDa) cellular RA-binding proteins encoded by two different genes, CRABP-I and CRABP-II. CRABP-I is ubiquitously expressed, predominantly in the cytoplasm of many cell types, where it may protect against the teratogenic effects of excess RA during embryonic development.[52,72] Based on much experimental data, the primary function of CRABP-I appears to be regulation by cytoplasmic sequestration and catabolism of the amount of free RA that gains access to the cell nucleus.[73–75] The molecular mechanisms controlling regulation of CRABP-I levels in different cell types are poorly understood, but they appear to involve complex transcriptional regulation of the gene promoter,[76] since there is no indication that mRNA and protein expression are dyscoordinate.[77]

CRABP-II has commonly been considered to have the same essential functions as CRABP-I, since it is 73% homologous to CRABP-I and since these proteins are highly conserved evolutionarily.[74–76] The possibility that CRABP-II might have an even more direct protective effect against increased ATRA levels was enhanced by the finding that this gene, in contrast to CRABP-I, has an ATRA response element (RARE) in its gene promoter.[78,79] However, a negative effect of CRABP-II expression on ATRA-mediated nuclear activity has never been reported,[75] and increasing recent evidence suggests that its primary function is instead a positive, stimulatory effect on ATRA-regulated gene activity.[80–85] CRABP-II has a distinct tissue distribution from CRABP-I[72] and is visualized in the nucleus as well as the cytoplasm, suggesting an ATRA shuttle function between these cellular compartments.[82,86] Additionally, CRABP-II has been associated with increased intracellular synthesis of ATRA.[87] At the molecular level, CRABP-II has been demonstrated to directly interact with nuclear receptors RARα and RXRα in an ATRA-independent manner, acting to coactivate RAR:RXR activity in the presence of ATRA[82,85] and to serve as a targeted carrier of bound ATRA from the cytoplasm to nuclear RA receptors.[83,84] Thus, although the information about the CRABPs remains incomplete, CRABP-I appears to regulate access of free ATRA with primary negative effects in tissues enriched with catabolic enzyme machinery, while CRABP-II appears to be primarily geared to enhance ATRA activity in cells with high demand for ATRA-regulated gene activity.

Following the short-term administration of oral ATRA, a rapid, reversible increase in CRABP (generic designation if CRABP type is uncertain or unknown) was noted in the skin of monkeys. From analysis of the kinetics of the plasma ATRA PK values vis-à-vis skin CRABP levels, it was concluded that this might be a component but probably not the major factor in the hypercatabolic response to ATRA administration.[7,56] No discrimination was made between CRABP-I or -II, but it seems reasonable to suppose that CRABP-II was involved to some extent, since skin is the major site of its expression and since it is selectively induced after topical ATRA application in humans. Attention was focused on a likely role of CRABP-II in the hypercatabolic ATRA response by the demonstration of a marked increase in its expression in bone marrow cells and in APL cells following ATRA therapy,[44,90,91] however, these findings have not been confirmed[92] (*vide infra*). Also of note, no studies of CRABPs have been performed in liver tissue following ATRA administration in primates, the principal site of oral ATRA metabolism.[56] In summary, a modest increase in sequestration of ATRA by CRABP is likley contributory to the systemic hypercatabolic response during oral ATRA therapy, and increased metabolism secondary to quantitative CRABP changes may also be contributory but there is no experimental evidence to support this possibility.

Relationship of alternative retinoids to hypercatabolism

If systemic hypercatabolism of ATRA is the primary factor in the development of resistance to ATRA therapy, then treatment approaches that circumvent this response should be effective in overcoming ATRA resistance. Three such treatment approaches have been reported. The first of these utilized 9-*cis* RA (9cRA) in 11 patients who had relapsed from previous ATRA therapy.[46,93] In contrast to ATRA, 9cRA can activate RXRα in addition to RARα.[94,95] Despite this bifunctional activity, 9cRA has approximately the same potency as ATRA in inducing differentiation of NB4 cells and fresh APL cells.[46] Pharmacokinetic data from both this study and an earlier study in monkeys[96] demonstrated that 9cRA induces its autocatabolism to a substantially lesser degree than ATRA – although a recent phase I study in children minimized the difference between 9cRA and ATRA.[97] Other studies support the concept that 9cRA is less subject to self-induced autocatabolism, including the observation that it may be primarily metabolized by non-oxidative pathways[66] and that it has considerably reduced binding to CRABPs.[98,99] In any event, 9cRA produced CR in 3/10 (30%) evaluable ATRA relapse patients,[93] only six of whom were presented in sufficient detail to include in Table 3[46]. The investigators' conclusion that 9cRA is incapable of overcoming ATRA resistance was buttressed by PK information illustrating that, even at the lowest administered dose, it reached levels comparable to ATRA *in vivo* and that 9cRA was no more effective than ATRA in inducing maturation of ATRA-resistant APL cells *in vitro*.[46]

A second treatment approach utilized the synthetic RARα-specific retinoid Am80, which induces *in vitro* differentiation of NB4 cells with 10-fold greater potency than ATRA, binds to CRABPs with low affinity and is resistant to oxidation.[100] Of 24 evaluable patients who relapsed from ATRA therapy 3–58 months (median, 22 months) after the last ATRA treatment, 14 (58%) achieved CR. There was no difference in the interval off ATRA in cases that failed *vs* those that achieved CR on Am80[100], although 3/3 cases treated within 3 months of the

last ATRA treatment failed to achieve CR (Table 3). Notably, achievement of CR correlated most closely with sensitivity of the APL cells to Am80-induced terminal maturation *in vitro*. Four of eight (50%) of these CR patients who did not receive an allogeneic bone marrow transplant during the second CR were in continuing CR after consolidation chemotherapy with a median follow-up of 49 months.[101]

A third treatment approach utilized L-ATRA,[102] which is approximately equipotent to free ATRA in inducing maturation of APL cells *in vitro*.[103] At a therapeutic dose of 90 mg/m^2, L-ATRA yields peak blood concentrations about 10-fold higher than peak plasma levels achieved with standard dose oral ATRA (45 mg/m^2).[47] Peak concentrations and AUC values were maintained for at least 15 days of continuous treatment and in one case up to 85 days of treatment. It was suggested that this might be related to less activation of hepatic microsomes and reduced delivery to catabolic liver and skin sites. In this initial phase I study, 3/6 patients[47] and, in a successor study, 5/18 patients,[20] ie 8/24 (33%) overall evaluable ATRA relapse patients, were resistant to re-induction of CR with L-ATRA (Table 3). As for Am80, the incidence of CR was higher in cases in which the hiatus from previous ATRA exposure exceeded 1 year: 4/9 (44%) CRs at <1 year *vs* 12/15 (75%) CRs at >1 year (Table 3). Although all CRs in the phase I study occurred in first relapse cases,[47] differences in relapse history probably do not account for the difference in time-stratified CR rates, since all of the L-ATRA reinduction failures at <1 year in the successor trial at constant dose L-ATRA occurred in first relapse cases.

The above three study approaches strongly suggest that both Am80 and L-ATRA are able to produce second CRs in a fraction of post-ATRA relapse patients in whom this would not be possible by re-treatment with oral ATRA. Although the number of evaluable cases is small, only 4/15 (27%) ATRA-retreated *vs* 14/24 (58%) Am80-treated and 16/24 (67%) L-ATRA treated ATRA-relapse cases achieved CR. Whether or not this difference is due to 'overcoming' persistent ATRA hypercatabolism, as opposed, simply, to greater efficacy of the these compounds, is, however, not clear. With the exception of the ineffective 9cRA trial for relapse APL,[46] PK data are quite meager but suggest that insufficient L-ATRA levels were not the reason for failure in two cases.[47] Although it seems likely that hypercatabolism of ATRA is involved in early ATRA resistance, particularly in cases that relapse while taking ATRA (no data available for other agents; Table 3), the data with the alternative retinoids, which also have a higher incidence of failure in cases that relapse in the 2–12 month post-ATRA time-frame despite their markedly reduced susceptibility to ATRA-induced catabolic mechanisms, strongly imply that other mechanisms are primarily involved in these treatment failures.

APL cellular elements in acquired ATRA resistance

In vitro ATRA sensitivity testing

Although fresh APL cells do not replicate effectively during short-term culture, their response to ATRA, nevertheless, follows a concentration- and time-dependent maturation sequence similar to that of exponentially proliferating, ATRA-responsive cells from leukemia cell lines, such as HL-60 and NB4.[33,38,104–106] Also, although the *in vitro* culture environment, typically including 10–20% fetal bovine serum and an enriched source of culture medium such as RPMI 1640 or Iscove's modified Dulbecco's medium (IMDM), presumably markedly differs from the environment of APL cells *in vivo*, the *in vitro* maturation response, properly studied, usually correlates well with clinical response to ATRA therapy. As described above, variations in the sensitivity of ATRA-naive APL cells under non-saturating ATRA conditions (0.1 μM for 3 days) is positively correlated with DFS but not with the incidence of near-universal CR.[38]

At the time of first relapse from previous ATRA therapy, whether or not given in combination with chemotherapy, there was a variable loss of APL cell sensitivity to ATRA-induced APL cell maturation *in vitro* in the great majority of cases.[10,31,46,91,92] In some of these cases, the loss of ATRA sensitivity was detectable as reduced APL cell terminal differentiation even at high ATRA exposure conditions, eg 1 μM ATRA $\times \geq 5$ days, while in other cases it was apparent only under more discriminating non-saturating ATRA exposure conditions. After two or more relapses, APL cell sensitivity to ATRA-induced maturation *in vitro* is typically reduced and is associated with clinical ATRA resistance (Table 2).[46,91] These observations re-emphasize that ATRA sensitivity/resistance of APL cells is relative, that it reflects a continuum of intrinsic APL cellular variations with a bearing on the efficacy of ATRA therapy and that at some level cellular ATRA resistance shares cross-resistance with chemotherapeutic agents and/or with intrinsic cellular alterations involved with progression of the leukemic process.

Mechanisms that may limit ATRA access to the cell nucleus

The terminal differentiation response of APL cells depends primarily on the delivery of sufficient ATRA to nuclear receptors (PML-RARα and RARα) to modulate the transcriptional activity of requisite genes.[1,4,107] Therefore, relative ATRA resistance can develop in two fundamental ways: by limiting the access of ATRA to nuclear receptor sites or by aberrations in the nuclear transcription machinery, including RA receptors and downstream co-factors required to produce activation of appropriate target genes. ATRA access to nuclear transcription sites may be impeded by essentially four different mechanisms, as detailed below.

Decreased effective APL cell ATRA uptake: Information about the net uptake of ATRA by fresh APL cells is available from two investigative groups. In a study of ATRA-naive APL cells from nine patients, the first group found a nearly 10-fold difference in the uptake of ATRA between low ($n = 3$; median 20 pmol/10^6 cells, range 17–60 pmol/10^6) and high ($n = 6$; median 195 pmol/10^6 cells, range 140–230 pmol/10^6) ATRA uptake cases.[108–109] This corresponded to differences in *in vitro* sensitivity of APL cells from the low or high uptake subgroups to ATRA-induced maturation, $\leq 45\%$ *vs* $\geq 55\%$, respectively.[108,109] However, these subgroups could not be tested for potential differences in clinical response, since all three of the low uptake cases experienced early death, while all of five high uptake cases treated with combined ATRA + chemotherapy achieved CR.[109] The second investigative group found no significant difference in ATRA uptake between five *de novo* and five ATRA relapse patients who had difficulty achieving a second CR with ATRA-containing therapy,[110] and this was apparently associated with decreased *in vitro* ATRA-induced maturation of fresh relapse APL cells.[111]

SPOTLIGHT

SPOTLIGHT

The second investigative group additionally reported that an ATRA-resistant sub-line of the APL cell line NB4 showed no difference in ATRA accumulation compared to the parental cell line, despite major differences in sensitivity to ATRA-induced maturation.[110,111] These results are similar to a study of ATRA-resistant HL-60 cells in which no differences were found in ATRA uptake compared to parental ATRA-sensitive HL-60 cells in either serum-containing, as in the fresh APL and NB4 cell reports, or in serum-free culture medium.[112] In the HL-60 system, ATRA uptake was demonstrated to occur very rapidly (in <1 min) and increased 10-fold in serum-free medium, which was associated with a ~10-fold decrease in the ATRA concentration required to induce terminal differentiation. Although the relevance of such *in vitro* observations to ATRA resistance that develops under clinical conditions is uncertain, they are a reminder that it is not known whether the major site of ATRA activity *in vivo* is on APL cells in the blood or in the bone marrow and extramedullary sites where the cells may be bathed in low-protein interstitial fluid that could have a marked effect on ATRA potency.

Effective cellular uptake of pharmacological agents is determined by the balance of influx and efflux rates. There is no precedent for increased extrusion of unconjugated lipophilic ligands for nuclear receptors, like ATRA, that enter cells by passive diffusion across the cell membrane.[113] Nevertheless, it has been queried whether increased expression of members of the ATP-binding transmembrane transporter gene superfamily that can produce multidrug resistance (MDR) might have an effect on ATRA uptake. The relevance of this query is enhanced by the fact that most clinical protocols involve the co-administration of ATRA with an anthracycline and/or cytarabine, both of which can induce MDR in AML.[114,115] It is well-recognized that APL cells express no or much lower levels of P-glycoprotein (PGP), the prototype MDR gene, than other myeloid leukemia cells.[116,117] However, the data in relapse APL are somewhat controversial, based on small case numbers. In an early study, 2/2 relapse ATRA-resistant cases were reported to express low levels of PGP, and the PGP inhibitor verapamil significantly restored the ability of ATRA to induce terminal differentiation.[118] However, in two subsequent reports with five[110] and seven[119] relapse cases, only one relapse patient had a small increase (three-fold) in PGP. Two other members of the MDR family lung resistance-related protein (LRP) and multidrug resistance-associated protein (MRP1) were also not increased after relapse from combined ATRA/anthracycline therapy.[119] After ≥ 2 relapses, however, 3/4 cases had a two to three-fold increase in PGP,[119] which is obviously of interest related to the clinical observation that APL cases with multiple relapses from chemotherapy but without prior ATRA exposure are frequently ATRA resistant (Table 2).

The possible role of multidrug resistance in ATRA resistance has been studied further in ATRA-responsive cell line models. ATRA-resistant HL-60 were reported to express low levels of PGP mRNA and to have increased maturation in response to ATRA when used in combination with the PGP inhibitor verapamil.[118] Furthermore, targeted destruction of these PGP (MDR1) transcripts, using a specific ribozyme, partially restored ATRA sensitivity.[120] In contrast to these findings, however, it was reported that stable transduction of the MDR1 gene into NB4 cells did not affect either the effective uptake of ATRA in the presence or absence of the specific PGP inhibitor PSC833 or the bioresponse (maturation or apoptosis) of these cells to ATRA.[110,111]

In sum, the results suggest that variations in ATRA uptake are a minor element in ATRA resistance, although, given the observation that relatively minor variations in APL cell ATRA sensitivity can be associated with differences in long-term outcome,[38] it cannot be discounted as a subtle contributor in *de novo* disease or as factor in ATRA resistance after multiple relapses from chemotherapy, although the most pertinent cell line model does not provide support for this possibility.[110,111]

Increased cytoplasmic sequestration: Cytoplasmic sequestration of ATRA was proposed as a major contributor to acquired ATRA resistance in APL related to the finding of markedly increased CRABP expression in the APL cells of four relapse cases with decreased sensitivity to ATRA-induced differentiation.[44,91] Similar increases were observed in predominantly normal bone marrow cells after ATRA treatment, and it was established by a gel electrophoretic method that the cytoplasmic ATRA binding activity was CRABP-II, not CRABP-I.[90] As previously mentioned, this hypothesis seemed logical, since the CRABP-II gene promoter contains a RARE, which, in response to pharmacological ATRA concentrations, could lead to increased CRABP-II production, providing a protective feed-back loop by which the incoming ATRA could be increasingly sequestered in the cytoplasm. However, in a larger screening study of 36 APL patients designed to assess the clinical associations of CRABP-II levels, it was unexpectedly found that CRABP-II was constitutively expressed in ATRA-naive cells, that its level did not significantly differ in bone marrow aspirate cells from patients following ATRA or chemotherapy induction treatment, that there was no increase in CRABP-II in relapse APL cells, and that there was no correlation between CRABP-II level and sensitivity to ATRA-induced maturation *in vitro*.[92] As also previously mentioned, these results seem more consistent with the subsequent findings that CRABP-II functions as a positive rather than a negative regulator of ATRA activity by acting as a shuttle for the delivery of ATRA to nuclear transcription sites and as a co-activator of RARα. These developments raise the possibility, contrary to prior thinking, that *decreased* rather than increased expression or activity of CRABP-II may be associated with ATRA resistance in APL cells, which requires investigation.

No alternative molecule to supplant CRABP-II as a vehicle of cytoplasmic sequestration has been suggested. Worth mentioning, perhaps, is that HL-60 cells selected *in vitro* for ATRA resistance develop marked alterations of cell membrane glycoproteins and glycolipids that, if also present in ATRA-resistant APL cells, might possibly affect the cellular disposition and activity of ATRA (reviewed in Ref. 121).

Increased cytoplasmic ATRA catabolism: Intracellular ATRA catabolism occurs primarily by oxidation, involving microsomal membrane-bound P450 enzymes.[60,122] The hypothesis that increased APL cellular metabolism is likely a factor in acquired ATRA resistance, apparently rests entirely on observations that ATRA can upregulate P450 enzymes in other cell systems, since there are no reported studies of ATRA catabolism in ATRA-resistant APL cells. In two studies of ATRA catabolism in ATRA-sensitive NB4 cells, rather different results were reported. In one, ATRA was slowly metabolized over several days without the observation of oxidative polar metabolites in either the cells or culture medium.[108] In the other, the rate of ATRA catabolism is not stated but was associated with the generation of several polar metabolites in mixed extracts of cells and medium.[123] Possibly, these differ-

ences could be related to serum differences between the two studies, a suggestion based on a study of HL-60 cells.[112] In the presence of serum, ATRA catabolism in HL-60 cells occurred slowly (days) without observable polar metabolites, while under serum-free conditions ATRA was rapidly catabolized ($t_{1/2} < 4$ h), associated with conversion to polar catabolites in the culture medium but relative preservation of intracellular ATRA. Compared to the parental line, an ATRA-resistant subline showed no difference in the presence of serum but approximately four-fold more rapid catabolism in serum-free conditions. Whether this increased catabolism is a cause of the ATRA resistance, however, is questionable, since the parental and ATRA-resistant subline were each ≥10-fold more sensitive to ATRA-induced differentiation in serum-free medium than in serum-containing medium.[112] These HL-60 cell observations are consistent with those in other types of cultured cells (breast cancer cells) in which ATRA sensitivity to growth inhibition is positively correlated with catabolic rate.[124,125]

Another consideration regarding the role of ATRA catabolism as a potential mechanism of ATRA resistance is that all identifiable ATRA oxidative catabolites were shown to be only slightly less effective than ATRA in inducing NB4 cell terminal differentiation.[123] As noted, it is possible that the exogenous application of these compounds might have a different action than their intracellular generation following assimilation of pharmacological ATRA doses. Nevertheless, these observations, like those on the effects of catabolic rate, do not support a role of increased catabolism in ATRA resistance. Some reported data do, however, favor this possibility, namely, the observation that the ATRA sensitivity of the aforementioned ATRA-resistant HL-60 subline was augmented by co-treatment with the P450 enzyme inhibitor clotrimazol.[118] Clearly, more research is required, particularly considering the recent identification of ATRA-dedicated P450 enzymes.[65,66]

Decreased nuclear transport: There has been no previous consideration that alterations in the delivery of ATRA to the nucleus could be involved in the development of resistance to ATRA. This is because ATRA was believed to enter the nucleus solely by passive diffusion, which is entirely a function of the concentration gradient of free ATRA from the cell cytoplasm to the nucleus.[74,122] Passive diffusion may, in fact, be the major default pathway in many cell types, since mice deficient in either CRABP-II or in both CRABP-I and -II are essentially normal.[126] Although this default mechanism may also apply in APL cells, the question must be asked as to why APL cells are among the very limited cell types, albeit malignant, in adults that constitutively express CRABP-II.[92] The recent finding that CRABP-II acts to specifically channel the transfer of ATRA to RARα in the nucleus[83,84] necessarily raises the consideration that this facilitated carrier system may be subject to regulations for nuclear transport proteins.[127,128] Thus, investigations are needed to determine whether or not CRABP-II expression is required for the ATRA response of APL cells and, if so, to determine if defects in this system might contribute to ATRA resistance.

Aberrations of ATRA nuclear receptor-gene response mechanisms

Overall considerations: Once ATRA is delivered to PML-RARα/RARα in the APL cell nucleus, whether by passive diffusion or facilitated transport, the gene transcriptional response pathway is extremely complex. In addition, to the nuclear receptor oligomeric complexes of PML-RARα/RARα and RXRα,[129–131] at least four other classes of multi-protein complexes are involved in regulating transmission of the ATRA receptor signal to the basal transcriptional apparatus containing RNA polymerase II.[132,133] An important function of two of these classes, co-repressor and p160 co-activator complexes, is to regulate the level of acetylation of nucleosomal histones. Co-repressors decrease acetylation by recruiting members of the histone deacetylase (HDAC) enzyme family, which function to maintain nuclear chromatin in a condensed, inactive state. Conversely, p160 co-activators have endogenous histone acetylase (HAT) enzyme activity or recruit auxiliary proteins with this activity and maintain chromatin in an open, active state.[134,135] Information is rapidly accruing about the detailed molecular mechanisms by which these opposing enzyme activities dynamically control gene transcriptional activity by regulating the acetylation status of specific lysine residues in nucleosomal histone tails[136] and by interacting with alternative transcriptional control mechanisms involving methylation and phosphorylation of specific histone residues.[137,138] A third transcription complex is the so-called DRIP/TRAP complex, which is involved in transcriptional activation of RARE-regulated promoters.[139] This complex, like the HAT p160 coactivators, interacts directly with the AF-2 region of nuclear receptors in a ligand-dependent manner and is also directly linked to RNA pol II. Although required for the transcriptional activation response, the relationship of the DRIP/TRAP complex to the HAT coactivator complex is unclear. A fourth class of complexes is a family of so-called ATP-dependent 'chromatin remodelling machines', the most familiar member of which is the Swi/Snf (acronyms from yeast genetic terminology) co-activators, which are also involved in ligand-regulated transcriptional activation.[140,141]

Any of the four complex classes cited above could, theoretically, be involved in the development of ATRA-resistant APL cells. However, relatively little information is so far available. An exception is a study of the DRIP/TRAP complex in wild-type and three different ATRA-resistant sublines of NB4 cells, as well as a variety of other hematopoietic cells, including HL-60 cells.[142] It was found that this complex is expressed in an invariant and ubiquitous fashion in all cell types studied, suggesting that it does not have a role in ATRA resistance in these cells.[142] Although abnormalities of both co-repressors[143] and p160 co-activators[144,145] have been associated with other hematopoietic malignancies, there have been no reports of intrinsic abnormalities of these proteins in APL. Evidence has, however, been presented in a murine myeloid culture model indicating that variations in co-repressor and p160 co-activator activity can occur as a function of the stage of myeloid cell differentiation associated with variations in sensitivity to ATRA-mediated bioactivity.[146] Additional studies in this system indicated that the variations in these co-factor activities are not due to different levels of expression of these proteins, leading to the suggestion that they may be related to changes in post-translational modification.[147] Although these intriguing observations may be related to ATRA resistance in future investigations, the focus of this review will be on increasing evidence that abnormalities of PML-RARα function are key elements in many instances of acquired ATRA resistance both *in vitro* and *in vivo*.

Focus on PML-RARα: in vitro studies: ATRA resistance

and PML-RARα have been most extensively studied in the first-established APL cell line NB4.[148] Although derived from a patient who failed to achieve remission on ATRA therapy, the established, hypotetraploid, t(15;17)-positive (two copies) NB4 cells had similar sensitivity to ATRA-induced maturation to freshly explanted leukemic cells from ATRA-naive APL cases.[148,149] It was noted, however, that a small fraction of NB4 cells failed to mature in response to ATRA, suggesting some level of intrinsic ATRA resistance, possibly derived from the ATRA-treated donor.[150] This ATRA-resistant subpopulation did not provide a strong competitive advantage, since 15 months of continuous exposure to 1 μM ATRA were required to derive a stably resistant subline (NB4-R1),[106,150] recently redesignated NB4-LR1 (Table 4).[151] NB4-LR1 cells were found to be only partially resistant to ATRA, since ATRA induced several molecular events characteristic of PML-RARα/RARα, RARE-mediated responses to ATRA in NB4 cells, including downregulation of RXRα and proteolytic degradation of PML-RARα. However, 'triggering' of cytological maturation required treatment of 'ATRA-primed' LR1 cells with agents that increased intracellular levels of cAMP and activated cAMP-dependent protein kinase (PKA), most effectively the stable cAMP analog, 8-chloroadenosine cyclic 3′,5′-phosphorothioate (8-CPT-cAMP).[106] The LR1 subline has additional properties of being unusually sensitive to ATRA-induced apoptosis, despite failure to downregulate Bcl-2 in contrast to the parental line,[153] and of undergoing telomeric loss leading to growth arrest and cell death on exposure to ATRA for >2 weeks[154] (surprisingly, in view of the history of continuous ATRA selection needed to select the subline[150]). Notably, both of the latter ATRA effects could be subverted by administering 8-CPT-cAMP to stimulate LR1 cell maturation. These observations, with additional supportive data, have been interpreted to indicate that the LR1 type of ATRA resistance, a phenotype that apparently can be repeatedly generated from NB4 cells, is due to uncoupling of the classical ATRA/RARE response pathway from an essential cAMP component and that the cell maturation response is dominant over alternative default response pathways for ATRA and other retinoids, including RXR-specific 'rexinoids' in APL cells.[155,156] This notion raises the important consideration that ATRA resistance might arise from defects in the cAMP response pathway rather than in the RAR/RARE response system *per se*. Some experiments with PKA inhibitors provide support for this notion,[106,156] but further experiments are needed to document the absolute requirement of the cAMP response system for ATRA/RARE activity and to better understand the molecular interactions between these two transcriptional regulatory response systems.

Table 4 ATRA-resistant NB4 sublines: redesignation as recently recommended[a]

Original designation	*New designation*	*PML-RARα abnormality*[b]	*Ref.*
NB4-R1	NB4-LR1	None reported	Duprez *et al*[150] Ruchaud *et al*[106]
NB4-R1	NB4-DR1	ΔPhe286	Nason-Burchenal *et al*[159]
NB4-R4	NB4-MR4	Leu398Pro	Shao *et al*[157]
NB4/RA	NB4-NR1	Pro407Leu	Kitamura *et al*[158]
NB4-R2	NB4-LR2	Gln411STOP	Duprez et al[160]

[a]Following the recommendation of Roussel *et al*,[151] each ATRA-resistant subline includes the initial of the last name of the corresponding author of the paper that first-described the derivation of the subline.
[b]PML-RARα mutations resulting in amino acid changes are numbered by reference to normal RARα1.[161]

In contrast to the NB4-LR1 cells that express wild-type (WT) PML-RARα, variable alterations of PML-RARα have been observed in other NB4 sublines selected for ATRA resistance. Most frequently, this has involved mutations, all of which have been localized in the ligand-binding domain (LBD) of the RARα portion of the molecule.[157,–160] One of these mutants (NB4-DR1; Table 4 designation), a three nucleotide deletion (ΔPhe286 with reference to normal RARα1[161]) that preserved the open reading frame (ORF), was derived by direct clonal isolation, ie without ATRA selection, from parental NB4 cells.[159] Interestingly, this intrinsic mutation has not been reported in any other ATRA-selected NB4 subline, despite being associated with a very high level of ATRA resistance in the DR1 subline (no growth inhibition at 10 μM ATRA).[159] This suggests either that the mutation had been very recently acquired *in vitro* in the parental NB4 cells or that the mutation has little selective advantage in the absence or presence of ATRA *vs* the mass of NB4 cells bearing WT PML-RARα.

In two other laboratories, ATRA-resistant NB4 sublines harboring unique missense mutations were derived after several months of selection in ATRA followed by sub-selection either by cloning or limiting dilution.[157,158,162] In one of these studies, at least two out of nine alternative clones derived from the same ATRA-selected and -resistant subculture did not contain mutations, although the PML-RARαs from these clones had aberrant ATRA binding patterns compared to WT PML-RARα, as assessed by high-performance liquid chromatograpy (HPLC).[157,162] The mutant clone in this study, NB4-MR4 (Table 4 designation), contained a Leu398Pro mutation that severely compromised ATRA binding, minimally transactivated transcription from RARE-containing reporter gene promoters at high ATRA concentrations, and produced strong dominant-negative activity *vs* normal RARα.[157,163] The defective transcriptional activity of the Leu398Pro mutant was associated with excessively tight binding of corepressor complex, which could be partially overcome by treatment with a histone deacetylase inhibitor (HDI), trichostatin A (TSA), in the presence of ATRA.[163] Despite the severe defects engendered by the Pro398Leu mutation, the NB4-MR4 cell subline, could activate a subset of ATRA-responsive genes, including myeloblastin and CD18.[157,162] Unlike NB4-LR1 cells, the NB4-MR4 cells did not downregulate RXRα or degrade PML-RARα after ATRA exposure.[162,164]

The other missense mutation, Pro407Leu, was also associated with a markedly blunted ATRA response of the NB4-NR1 subline (Table 4 designation), harboring the mutation,[158] although no functional analyses of the mutant PML-RARα were reported. Notably, similar to non-mutant NB4-LR1 cells, the NB4-NR1 cells could be induced to partially differentiate by combined ATRA + 8-CPT-cAMP treatment. However, in this case, ATRA priming was not required and the differentiation response was not attended by degradation of PML-RARα or reorganization of microspeckled nuclear bodies.[158] The lack of the latter features was previously associated with induction of NB4 cell maturation by a default rexinoid pathway in combination with 8-CPT-cAMP.[155] This possibility seems unlikely to be related to the differentiation response of NB4-NR1 cells, however, since the RARα-specific synthetic retinoid Am80, which cannot be metabolically converted to an RXR ligand, induced maturation as effectively as ATRA or

SPOTLIGHT

9cRA in combination with 8-CPT-cAMP.[158] Thus, as suggested by the authors, the differentiation response of the NB4-NR1 cells to the combined agents most likely occurred through some modification of the PML-RARα/RARE response pathway or through relief of dominant-negative repression of normal RARα. These considerations emphasize the need for further studies to better understand the relationship of the various retinoid/rexinoid response pathways to the cAMP/PKA response pathway in APL cells.[156]

A final reported mutation in ATRA-resistant NB4 cells, a nonsense mutation corresponding to amino acid 411 of RARα1 (Gln411STOP), was detected in a secondary subline, NB4-LR2 (Table 4 designation), derived from the NB4-LR1 subline by continuous treatment with both 1 μM ATRA and 100 μM 8-CTP-cAMP.[106,160] Only one of the two PML-RARα genes was mutated, and neither of the proteins were detectable by Western blot analysis with specific antisera.[160] The mutant PML-RARα protein, truncated by 52 amino acids, lacked direct transcriptional transactivation capacity and was a potent dominant-negative inhibitor of RARα and WT PML-RARα activity on a DR5 RARE reporter in transient transfection assays.[160] The dual findings of potent dominant-negative inhibition and lack of evidence of stable expression did not permit a judgment as to which mechanism is the basis for the inability of ATRA to induce maturation of the NB4-LR2 cells via the RARE response pathway,[160] although there are precedents for both (*vide infra*). The latter defect in LR2 cells has been of value in documenting the existence of alternative retinoid ± cAMP maturation and cell death pathways in APL cells that are obscured in the presence of an intact, dominant ATRA/RARE response system under usual cell culture conditions.[155,165,166] Although it is unclear whether these back-up, default pathways have any role in the response of ATRA-sensitive APL cells, they may become active or provide therapeutic alternatives in ATRA-resistant disease.[156] In fact, a preliminary report indicated that combination treatment with a synthetic RXR agonist and RARα antagonist (BMS749), in combination with 8-CPT-cAMP, was able to partially differentiate APL cells harboring the PLZF-RARα oncoprotein that blocks the classical ATRA response by activating the ATRA/RARE-independent, rexinoid pathway.[167]

Notably, the Gln411STOP mutation recently found in NB4-LR2 cells had previously been detected in two independently selected sublines of ATRA-resistant HL-60 cells,[168,169] suggesting that this is a mutational hotspot for ATRA resistance in myeloid leukemia cells. Although not specifically demonstrated, in one subline (HL-60R) the 411 mutation was evidently heterozygous, since the inferred resistance mechanism was dominant-negative inhibition of the normal RARα allele. This inference was supported by demonstrated dominant-negative activity of the mutant RARα *vs* RARα in transient transfection assays[168] and by evidence that an RARα protein of aberrant size with reduced ATRA binding affinity was expressed in HL-60R cells.[170] Proof of endogenous dominant-negative activity is difficult to establish with certainty, however, since it depends on the level of expression of the mutant *vs* WT allele, as discussed related to another ATRA-resistant HL-60 subline with a different heterozygous carboxy-terminus truncation mutation of RARα.[171] The other ATRA-resistant HL-60 subline with a Gln411STOP mutation (HL-60/RA-res or, simply, RA-res) was homozygous, and, lacking a normal RARα allele, cannot have produced resistance by a dominant-negative mechanism. Although *in vitro* transcription and translation of mRNA from the RA-res cells indicated that the truncated protein can be exogenously stable,[171] an RA-binding protein of corresponding size was not detected in these cells.[172] In a comparative study, RA-res cells responded to alternative inducers of terminal neutrophilic differentiation much better than HL-60R cells.[173] This might be related to the homozygous loss of RARα expression in RA-res cells, since expression of unligated RARα has been demonstrated to be a specific and potent inhibitor of neutrophil maturation.[174,175]

In the NB4 line, loss of PML-RARα protein expression has been demonstrated to be another mechanism of ATRA resistance. At least two independent sublines have been established by step-wise ATRA selection, with (NB4.306)[176] or without (NB4/007.6)[177] prior low-dose irradiation, that express comparable levels of PML-RARα mRNA as parental NB4 cells but which express no detectable PML-RARα protein.[176,178] In both sublines normal RARα protein is present at typical NB4 cell levels. The lack of PML-RARα protein expression was demonstrated to be at least partly due to degradation by a constitutively active proteosome in the NB4.006/7 cells but not the NB4.306 cells.[178] Caspase activation was not a factor in either subline, suggesting involvement of a third, unknown mechanism of proteolysis, which is likely dependent on post-translational modification, since *in vitro*-translated PML-RARα was not degraded by NB4.006/7 cell lysates. ATRA sensitivity was partially restored to the NB4.006/7 cells by transduction of PML-RARα, much more effectively so than by transduction of RARα. The overall results strongly support the conclusion that PML-RARα rather than RARα is positively involved in mediating at least the initial stages of the ATRA-induced maturation response in APL cells.[178]

In addition to the ATRA-resistant NB4 cell sublines, two other APL cell lines have been established associated with reduced ATRA sensitivity and with PML-RARα missense mutations in the LBD of the RARα region: line UF-1[179,180] and line AP-1060[181,182] (Sun *et al*, manuscript in preparation). In both lines, the karyotype is near-diploid with a single t(15;17), and these mutations were also detected in a specimen from the patient donor, indicating that they originated *in vivo*. Such naturally occurring mutations, associated with the unusual capacity for sustained *in vitro* growth, have the potential of providing novel insights into the selection pressures that lead to the clinical emergence of the APL cell subclones harboring PML-RARα mutations (*vide infra*).

The UF-1 line was derived from a patient, who, like the NB4 cell line donor,[148] had relapsed from two previous chemotherapy-induced remissions and then had failed to achieve remission with intensive ATRA therapy.[179] The clinical specimen containing the mutation was obtained when the patient was already resistant to ATRA therapy,[180] and it is therefore uncertain whether it was present prior to beginning or emerged during ATRA therapy.[179] The Arg276Trp mutation in this case was associated with a marked decrease in ATRA binding affinity[180] and with only partial maturation of UF-1 cells, even at 1 μM ATRA.[179]

The AP-1060 line was derived from a patient in third relapse after previous therapy with four different chemotherapeutic agents, three in combination with ATRA, and after a transient partial response to arsenic trioxide (case No. 1, Ref. 50 and Sun *et al*, manuscript in preparation). The missense mutation in this case occurred at the same site as in the NB4-NR1 subline but with a different amino acid change, Pro407Ser instead of Pro407Leu.[158] In contrast to NB4-NR1 cells, the AP-1060 cells were not strongly resistant to ATRA-induced maturation. Rather the ATRA-induced maturation dose–response curve was slightly right-shifted but markedly sharpened with no cytologic response observed at 0.01 μM

SPOTLIGHT

ATRA and a complete response at 0.1 μM ATRA. This profile was virtually superimposable on the ATRA transcriptional transactivation dose–response of the mutant PML-RARα on a DR5 reporter gene in transient transfection assays[181] (Sun *et al*, manuscript in preparation). Further studies are in progress to try to determine how or whether a mutation with such weak functional consequences might have contributed to clinical ATRA resistance and/or clinical relapse.

Focus on PML-RARα: clinical studies: Studies of potentially aberrant nuclear response mechanisms in fresh patient specimens, as representative of *in vivo* acquired ATRA resistance, have so far primarily focused on a search for mutations in the PML-RARα oncoprotein. As part of these investigations, it has been additionally established, however, that possible alternative aberrations of PML-RARα mRNA, such as gross rearrangements, deletions or alternative or abnormal splicing variants (detectable at very low levels by sensitive RT-PCR amplification in many APL cases; Ding and Gallagher, unpublished results) either are not or are very infrequently involved in the development of ATRA resistance.[31] Also, there have been no reported studies of PML-RARα proteins in fresh APL specimens, so it is unknown whether proteolytic degradation in the absence of PML-RARα mRNA alterations, as described in some ATRA-resistant NB4 sublines,[176,178] might be involved in some instances of clinical ATRA resistance.

Table 5 summarizes and references information about 15 APL relapse cases with reported mutations of PML-RARα, all of which are missense mutations. All mutations were derived from patients who had received ATRA treatment. Conversely, there have been no reports of PML-RARα mutations in ATRA-naive APL cases. The latter include studies of more than 50 *de novo* cases[31,183,184] but only eight cases after chemotherapy alone, all first relapse cases.[31] Thus, although the data imply that ATRA therapy is required for the selection of APL cell subclones harboring mutant PML-RARα at relapse, insufficient chemotherapy-only relapse cases, particularly after two or more relapses, have been studied to be certain of this conclusion. Further support for an ATRA requirement is added by the consideration of the nature of the mutations, which are confined to the region of PML-RARα related to direct ATRA effects (*vide infra*). Nevertheless, examination of the data in Table 5 raises questions about the relationship of ATRA therapy to the emergence of PML-RARα mutant subclones at relapse.

In some cases, the PML-RARα mutant subclone was detected after relapse while on prolonged ATRA therapy, eg cases 2, 5, 6[180] and 15. In case 5, it was further reported that bone marrow samples taken 3 and 6 months prior to relapse were PML-RARα positive but lacked the mutation, indicating that the subclone harboring the mutation was late-emerging at relapse.[185] This suggests that continuous exposure to pharmacological ATRA concentrations selected the mutant subclone over a several month period, as noted to occur in the selection of ATRA-resistant subclones of NB4 culture cells with missense mutations of PML-RARα.[157,158] However, in other cases, the relationship to ATRA selection pressure is less clear. For example, after 1 year of continuous ATRA maintenance therapy, case 12 remained PML-RARα-positive but lacked the mutation that emerged in the relapse subclone 1.5 months after the termination of ATRA therapy.[31,186] Moreover, in cases 8, 9 and 13, relapse subclones harboring PML-RARα mutations emerged at relapse many months after the termination of ATRA therapy and with interim negative analyses for mutations in PML-RARα-positive samples.[31,186] These observations indicate that, although prior ATRA therapy may have initially spawned the mutations, the forceful selection of the mutant subclones leading to relapse occurred at endogenous, physiological ATRA concentrations. This suggests that a secondary defect(s) develops and interacts in some alternative way with the PML-RARα mutation to provide the mutant sub-

Table 5 Relationship of naturally occurring PML-RARα mutations to clinical relapse from ATRA-containing therapy

Case No.	*PML-RARα mutation[a]*	*No. reported*	*Relapse No.*	*Days of ATRA Rx*	*Days off ATRA Rx*	*Mutations in pre-relapse samples[b]*	*Ref.*
1	Lys207Asn	1	4	>180	0	nx1(~500)	Zhou *et al*[50]
2	Arg272Gln	3	1	~210	0	N	Imaizumi *et al*[184]
3			1	~75	~270	N	Marasca *et al*[185]
4			2	>100	~60	N	Zhou *et al*[50]
5	Arg276Trp	2	1	~240	0	N,nx1(~90)	Marasca *et al*[184]
6			2		0		Takayama *et al*[180]
7	Gly289Arg	2	1	59	1437	N,nx2(600)	Gallagher *et al*[186]
8			3	>150	~100		Zhou, et al[50]
9	Leu290Val	1	1	52	174	N,nx2(90)	Ding *et al*;[31] Gallagher *et al*[186]
10	Arg294Trp	1	3	>200	<120		Zhou *et al*[50]
11	Met297Leu	1	1	~100	<60	N	Imaizumi *et al*[184]
12	Arg394Trp	2	1	415	49	N,nx4(49)	Ding *et al*;[31] Gallagher *et al*[186]
13			1	365	223	N,nx3(223)	Gallagher *et al*[186]
14	Pro407Leu	1	4	>120	~150		Zhou *et al*[50]
15	Met413Thr	1	1	142	0	N	Ding *et al*[31]

[a]PML-RARα mutations are numbered by codon of normal RARα1.
[b]Capital letters indicate tests for mutation done at disease presentation before any anti-leukemia treatment (N, negative); the small 'nxnumber' indicates the number of samples tested for a mutation with negative results between the time of complete remission and detection of the mutation at relapse; the numbers in parentheses indicate the number of days between the last interim test for a mutation prior to positive mutation detection at relapse.

clone with a competitive advantage over the predominant PML-RARα WT APL cells, leading to relapse.

Another consideration in evaluating the relationship of PML-RARα mutations to ATRA resistance and to disease relapse is the dysfunction imparted by the mutation. In this respect, Table 6 summarizes several functional features of nine unique missense mutations. All of these mutations are located in the LBD of the RARα-region of PML-RARα, and all had diminished ATRA binding compared to WT PML-RARα, which was quite variable in magnitude. Although the binding constants were not precisely quantified, several of the estimates from HPLC analyses were confirmed by a protease protection method[187] and seem reliable at a semi-quantitative level. For all tested mutants, there was a reasonably direct relationship between the degree to which ATRA binding was decreased and the ability to transactivate transcription from a reporter vector containing a DR5 RARE in transient transfection assays, recognizing that the dense association of corepressor proteins with PML-RARα results in at least a 10-fold higher ATRA concentration for transactivation compared to ATRA binding.[130,131] This suggests that defective ATRA binding is the major cause of ATRA resistance related to mutant PML-RARαs, although more detailed analyses indicate that in some instances the mutations additionally modify transcriptional transactivation by effects on direct interactions between PML-RARα and co-repressor and/or co-activator proteins.[163,187] Notably, several of the mutations produce relatively weak defects, activating the RARE-regulated reporter gene at ≤100 nM ATRA, an easily achieved pharmacological level. These assays are performed with monolayer cell cultures, eg COS-1 cells, that have been selected for low endogenous activity and strong support of exogenously transfected reporter vector activity, and therefore, they presumably contain a surfeit of the co-factors that are required to maximize transcriptional activity. This could decrease sensitivity for detecting adverse effects of the mutations, and, unfortunately, there have been very few studies of ATRA-induced terminal differentiation in the native relapse APL cells that harbor the PML-RARα mutations. In three cases studied (Table 6, Gly289Arg, Leu290Val and Pro407Ser), there was a close correlation between the ATRA concentrations required to induce APL cell differentiation *in vitro* and reporter vector transcriptional transactivation in COS-1 cells.[31,50] However, in two other cases (Table 6, Arg394Trp and Met413Leu), 10- or >100-fold higher ATRA concentrations were required to induce APL cell maturation,[31,50,187] suggesting either that alternative defects contributed to the APL cellular resistance or that quantitative differences in RARE-related transcription cofactors differentially affect the functional capacity of the mutant PML-RARα.

In the ATRA-resistant subline NB4-MR4, it was shown that both defective transcriptional transactivation on a DR5 reporter vector and cell maturation could be partially overcome by co-administering ATRA and a HDI, either sodium butyrate (NaB) or trichostatin A (TSA).[163] Such an interactive effect between ATRA and NaB on DR5-enhanced transcriptional transactivation has been shown, as well, for several of the naturally-occurring PML-RARα mutations (Table 6). Also, in one of only two studies of associated APL cell maturation, the combined effect of ATRA + NaB was greater than in the reporter transactivation assay (Table 6, Gly289Arg).[50] Since NaB alone was able to stimulate partial maturation of the APL cells harboring the Gly289Arg mutation,[50] these observations suggest that the ATRA-independent maturation-promoting activity of NaB alone was able to compensate for the strongly inactivating PML-RARα mutation and was likely positively involved in the clinical response of the patient to the combined agents.[48] In contrast, four other relapse APL cases failed to respond to combined ATRA + butyrate therapy,[49] despite harboring WT or less disabling PML-RARα mutations that could be strongly co-modulated by the two agents in the DR5 reporter transactivation assay (Table 6, mutants Lys207Asn, Arg272Gln and Arg294Trp).[50] These observations indicate that much remains to be learned about the relationship between PML-RARα mutant status and clinical ATRA resistance.

Until now, a principal model that has been cited for ATRA resistance in APL related to mutations in PML-RARα/RARα is the thyroid hormone resistance syndrome (RTHS) due to thyroid hormone receptor (THR) mutations.[50,184,187,188] THRs and RARs share close homology and mutations occur in similar locations with like effects on interactive co-repressor and co-activator proteins. Also, the THR may have a role in red blood cell maturation that is analogous to that of RARα in neutrophil maturation,[175,189] and the mutant THR erbA oncogene present in avian erythroblastosis[190] virus that causes erythroleukemia has a transcriptional repressor function analogous to unliganded RARα/PML-RARα.[191] However, the great majority of naturally occurring THR mutants, derived from cases of inherited RTHS, are strict loss-of-function mutations due to dominant-negative inhibition of the normal allele.[188,192] Although virtually all PML-RARα mutations also have loss-of-function regarding ATRA binding, it must be considered that these somatic mutations arise in a malignant cell population and might develop alternative effects. Such alternative effects have been recognized, for example, in the acquisition of androgen receptor mutations in prostate cancer, and they include promiscuous binding to alternative hormones and abnormal targeting by various cytokines.[193,194] Although the therapeutic selective pressure for the acquistion of androgen receptor mutations is typically the opposite from that for PML-RARα mutations in APL, ie ligand deprivation rather than ligand excess, it nevertheless seems likely that similar phenomena might occur under both circumstances. In this regard, it was recently reported that hematopoietic growth factors can activate RARs in an ATRA-independent, as well as an ATRA-dependent, manner.[147] Thus, the interplay of growth factor or other molecular pathways might differentially affect the activity of WT or mutant PML-RARα and, secondarily, influence the ability of the respective subclones to compete for growth and survival advantage in the host. This competitive relationship might vary during the course of disease as a function of changes in exogenous influences, eg treatment, or endogenous developments, eg additional APL cell mutations.

A further consideration related to endogenous APL cell alterations is the recent finding that PML-RARα can recruit DNA methyltransferase enzymes.[195] In this initial study, it was demonstrated that this recruitment is associated with hypermethylation and silencing of the possible tumor suppressor gene RARβ that contains a RARE in the promoter region. Since the PML-RARα–methyltransferase interaction was partly dependent on the RARα-component of PML-RARα,[195] a possibility is that mutations in this region might alter this activity on RARβ or other PML-RARα-targeted gene promoters. If so, such heritable epigenetic changes might amplify or broaden the effects of PML-RARα mutations and contribute to the enhanced survival and growth properties of APL subclones harboring these mutations that emerge at relapse.

Table 6 Naturally occurring PML-RARα mutations: ATRA concentrations (nM) required for PML-RARα receptor-binding and, in the presence or absence of NaB (1 mM), for ATRA activation of a RARE-regulated gene promoter or induction of maturation of fresh, mutation-harboring APL cells from patients[a]

PML-RARα	*PML-RARα*	*RARE reporter transactivation*		*APL cell maturation*	
Mutation	*ATRA-binding*	*ATRA*	*ATRA+NaB*	*ATRA*	*ATRA+NaB*
Wild-type	<1	<10	<10	10–100	~10
Lys207Asn	<10	10–100	~10	NT	NT
Arg272Gln	~10	10–100	~10	NT	NT
Arg276Trp	5–10	NT	NT	~1000	NT
Gly289Arg	$\gg$10	>1000	~1000	>1000	<1000
Leu290Val	$\gg$10	100–1000	~100	~1000	NT
Arg294Trp	>10	~100	<10	NT	NT
Arg394Trp	<10	~10	<10	~100	NT
Pro407Ser	>1<10	50–100	~10	50–100	50–100
Met413Thr	<10	~10	<10	>1000	NT

[a]For references see Table 5 and text.
ATRA, all-*trans* retinoic acid; NaB, sodium butyrate; RARE, retinoic acid response element; NT, not tested.

Summary and conclusions

Although clinical ATRA resistance in APL is defined as the inability to achieve or sustain clinical remission, there is, in reality, a spectrum of ATRA sensitivity/resistance in individual patients. Primary ATRA resistance is rare in *de novo* cases in the sense that CR can be achieved in almost all ATRA-treated patients with genetically verified PML-RARα-positive, non-chimeric APL. However, there is a range of heterogeneity elements that affect initial sensitivity to ATRA treatment, which has been linked to variations in longer term clinical outcome following combined ATRA + chemotherapy-induced clinical remissions. These heterogeneity elements are likely multi-factorial and with few exceptions have not been defined at the molecular level; it is unclear how they relate to relapse. Secondary, acquired ATRA resistance is near-universal in patients treated with continuous ATRA therapy, and, in these circumstances, an adaptive reaction to pharmacological ATRA administration is likely contributory to the ATRA resistance and to the selection of the APL subclone(s) that produce disease relapse. Although this reaction involves reversible ATRA hypercatabolism, the responsible molecular mechanisms remain poorly defined and may not provide a full explanation for on-ATRA relapses. Based on clinical observations with alternative retinoid formulations, such as 9cRA, Am80 and L-ATRA, that can by-pass this reaction, hypercatabolism does not appear to be a major element in ATRA resistance associated with relapse that follows discontinuance of ATRA by a few months. Relapse from treatment regimens with ATRA/chemotherapy combinations, usually after a several month interval from ATRA discontinuance, is nevertheless commonly associated with clinical ATRA resistance and with diminished APL cell sensitivity to ATRA-induced maturation *in vitro*. Early studies suggested that this is due to APL cellular alterations that limit access of ATRA to the cell nucleus by cytoplasmic sequestration and oxidative catabolism. However, subsequent studies have provided limited support for these hypotheses. Most dramatically, CRABP-II has been demonstrated to be constitutively expressed in APL cells and to have a positive rather than a negative role in delivering ATRA to nuclear regulatory sites. A caveat is that recently discovered P450 enzymes that have specificity for the oxidative catabolism of ATRA (CYP26) have not been thoroughly investigated for a possible role in ATRA resistance. In contrast, much evidence has accrued for an association of defects of PML-RARα, particularly mutations in the LBD of the RARα-region with ATRA resistance in clinical APL. To a considerable extent these findings are a confirmation of the clinical relevance of similar mutations and associated molecular alterations found in ATRA-resistant sublines of the APL cell line NB4 and the RA-sensitive AML cell line HL-60. Remarkably, however, the emergence of APL cell clones with these mutations at relapse following ATRA therapy is not necessarily related to ATRA selection pressure, and the quantitative defect in ATRA-regulated transcriptional activity does not consistently coincide with the loss of ATRA sensitivity of the mutation-harboring APL cells or seem of sufficient magnitude to explain selection of the associated relapse APL cell clone. These observations suggest that mutations of PML-RARα have complex effects on APL cell survival, growth and terminal differentiation, the detailed mechanisms of which remain to be elucidated. Finally, since PML-RARα mutations are not present in many ATRA-resistant relapse cases, other mechanisms, some likely involving perturbations of the multi-factorial nuclear complexes that regulate gene expression, remain to be discovered.

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