All-Trans-Retinoic Acid Pharmacology and Its Impact on the Treatment of Acute Promyelocytic Leukemia

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ABSTRACT

The approach to the treatment of acute promyelocytic leukemia (APL) has changed dramatically over the past decade and, as a result, the long-term event-free survival for patients has improved significantly. The addition of the vitamin A derivative, all-trans-retinoic acid (ATRA), to treatment regimens has been responsible for this improvement in survival. Although ATRA is a potent remission induction agent in APL, continuous administration of ATRA as a single agent does not maintain patients in remission. Although lower plasma concentrations were initially noted at the time of relapse in patients with APL, subsequent studies have demonstrated that the decline in plasma drug concentrations occurs within one to two weeks of initiation of treatment, and possibly as early as three days. The inability to maintain adequate plasma concentrations of ATRA because of rapid upregulation of its catabolism is an attractive hypothesis to explain the inevitable recurrences in patients with initially responsive disease, but more recent data suggest that this mechanism alone is unlikely to be responsible for drug resistance. Cellular retinoic acid binding proteins (CRABPs) play a critical role in regulating the amount of free retinoic acid capable of reaching and activating nuclear receptors. Recent studies using leukemic blasts obtained at the time of relapse have demonstrated a shift in the ATRA dose-response curve in vitro. In addition, there is an upregulation in the expression of CRABP in leukemic blasts obtained at relapse. These observations suggest that ATRA resistance is not simply an inability to maintain therapeutic plasma concentrations of drug, but rather may be linked to the intracellular regulation of drug. The intricate nature of the homeostatic mechanisms that maintain tight control over retinoids, combined with the multiplicity of retinoid receptors and signaling pathways, leave open the possibility of a yet-to-be-defined mechanism of resistance that is independent of the clinical pharmacology of ATRA.

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Exposure to ATRA at a concentration of 1 µM resulted in 95% of cells differentiating, with a concomitant loss of their capacity to proliferate.

The first reports of the clinical use of ATRA in patients with APL came from Shanghai and were published in 1987 and 1988 [5, 6]. All 24 patients treated by Huang and colleagues attained a complete remission without experiencing marrow hypoplasia. These dramatic results were subsequently confirmed by investigators from Paris [7, 8] and New York [9]. Since then, more than 3,000 patients with APL worldwide have been treated with ATRA [10], and complete remission rates exceed 80% to 90% in most series [11]. In a randomized trial comparing daunorubicin plus cytarabine alone to ATRA plus the same chemotherapy regimen, an interim analysis revealed that the event-free survival at one year was significantly higher in the ATRA-plus-chemotherapy arm (76% ± 6%) compared with the chemotherapy-alone arm (50% ± 9%) [12]. Longer follow-up has confirmed these results [11] (Fig. 1).

Although ATRA is a potent remission induction agent in APL, continuous administration of ATRA as a single agent does not maintain patients in remission [8, 9]. The pharmacokinetic behavior of ATRA was initially thought to contribute to treatment failure because of the inability to maintain adequate plasma ATRA concentrations on chronic dosing schedules [13]. The clinical pharmacology of ATRA and its possible role in treatment failure will be discussed after reviewing mechanisms of action of retinoids.

Receptors and Binding Proteins

Retinoids play critical roles in growth, vision, reproduction, epithelial cell differentiation, and immune function [14]. The actions of retinoids are mediated through the nuclear retinoid receptors, which are members of the steroid/thyroid/retinoid hormone receptor family [15]. Retinoid receptors act as ligand-inducible transcription factors that enhance the transcription of target genes by binding to retinoic acid response elements (RAREs) in the promoter region of retinoid-responsive genes. The retinoid receptors can be divided into six regions, designated A through E (Fig. 2) [16, 17]. The C domain is a cysteine-rich DNA-binding domain which contains two zinc finger structures. The E domain contains the ligand (retinoid)-binding site and also has a region required for dimerization with other receptors. The amino acid sequences of C and E domains are highly conserved across the classes of retinoid receptors, whereas the A, B, and F domains are less conserved across receptors but remain highly conserved for the same receptors across different species.

Two families of retinoid nuclear receptors have been described, the retinoic acid receptors (RARs) [18, 19] and the retinoid X receptors (RXRs) [20, 21]. The RARs (α, β, and γ) bind the naturally occurring retinoid ATRA with high affinity, whereas the RXRs (α, β, and γ) do not bind ATRA [22]. 9-cis-retinoic acid (9cRA) is a naturally occurring, biologically active isomer of ATRA [23] that is capable of binding and transactivating both the RXRs as well as the RARs. This multiplicity of receptors and gene pathways may in part explain the diverse effects of retinoids on a wide range of cellular processes.
Under normal physiologic conditions, the concentration of ATRA and other naturally occurring retinoids is under tight metabolic control. The physiologic plasma concentration of ATRA is approximately 5 nM [24]. Although circulating ATRA enters cells via passive diffusion, its contribution to intracellular ATRA levels is likely to be inconsequential under normal conditions because cells derive retinoic acid from intracellular oxidation of retinaldehyde, a metabolite of retinol [25]. Intracellular ATRA is bound to specific binding proteins, the cellular retinoic acid binding proteins (CRABPs) [26] (Fig. 3). CRABP I and CRABP II are highly conserved throughout evolution and appear to regulate the amount of retinoic acid capable of binding to their nuclear receptors [27]. Binding of ATRA to CRABP appears to facilitate intracellular oxidative catabolism of ATRA to the inactive metabolite, 4-hydroxy-retinoic acid [28].

**MECHANISMS OF ACTION**

There is considerable evidence to indicate that remission induction by ATRA in patients with APL is associated with the differentiation of immature promyeloblasts into mature granulocytes, followed by the recovery of normal hematopoiesis [29]: A) APL patients treated with ATRA do not experience a period of marrow hypoplasia during induction; B) immunophenotypically intermediate cells which express both immature (CD33) and mature (CD15) cell surface antigens are observed during induction therapy [8]; and C) in situ hybridization studies demonstrate the presence of the (15;17) translocation in maturing cells [9].

The hallmark cytogenetic alteration found in the leukemic cells of patients with APL is the reciprocal translocation between the long arms of chromosomes 15 and 17 [30, 31]. In 1987, the gene encoding the retinoic acid receptor-alpha (RARα) was mapped to chromosome 17q21 [32]. Soon thereafter, it was demonstrated that in APL the breakpoint on chromosome 17 involved the gene encoding for the RARα receptor [33]. The breakpoints on chromosome 15 cluster in a region containing a previously undefined gene initially termed myl [34] but subsequently renamed PML [35-37]. The fusion of the RARα and PML genes on chromosomes 15 and 17 results in the generation of two reciprocal transcripts - PML/RARα (Fig. 2), which is expressed in all patients studied to date, and the RARα/PML transcript, expressed in approximately two-thirds of patients [29].

The PML/RARα fusion protein acts as an ATRA-dependent transcription factor and appears to interfere with retinoic acid transcriptional regulation [38]. In myeloid leukemic cell lines, overexpression of PML/RARα blocks normal maturation and differentiation [39]. It thus appears that disruption of RARα in APL is linked to leukemogenesis, and that this disruption can be overcome by superphysiologic concentrations of ATRA. The exact mechanism by which ATRA reverses this maturation block, however, has not been fully elucidated.

**PHARMACOKINETICS**

Patients with APL who respond to ATRA as a single agent invariably relapse, usually within three to four months of diagnosis. Pharmacokinetic studies of ATRA have shown that plasma concentrations of the drug at the time of relapse are substantially lower than concentrations on the first day of treatment, leading to speculation that pharmacologic factors were the cause of treatment failure [13].

Plasma ATRA concentrations after oral administration are notable for their high degree of interpatient variability [40]. Following a 45 mg/m² oral dose of ATRA, peak plasma concentrations on the first day of treatment range from 0.1 to 8 µM, with a median peak concentration of approximately 1 µM [41-43]. Elimination of ATRA is rapid, with a terminal half-life of approximately 45 minutes [13, 41, 43].

With daily dosing, ATRA plasma concentrations diminish rapidly. Although lower plasma concentrations were initially noted at the time of relapse in patients with APL [13], subsequent studies have demonstrated that the decline in plasma drug concentrations occurs within one to two weeks of initiation of treatment [42, 44], and possibly as early as three days [45]. A significant decrease in plasma drug exposure, as measured by the area under the concentration-time curve (AUC), has been observed by numerous investigators (Table 1).

![Figure 3. ATRA enters cells via passive diffusion (1). Intracellularly, ATRA is bound by CRABP (2) which appears to facilitate its oxidation (3), via P450 cytochromes (4), to 4-oxo-metabolites. ATRA can also undergo isomerization to 9cRA and 13cRA (5). In order for it to exert its effect, ATRA must bind nuclear receptors (6). Following dimerization with other receptor/ligand complexes, transcriptional activation can take place via binding to RAREs in DNA (7).](http://theoncologist.alphamedpress.org/.../Figure%203.png)
A more detailed analysis of the pharmacokinetic behavior of retinoids in humans has been limited by the low and variable plasma concentrations achieved with oral administration and the lack of an i.v. formulation of ATRA for human use. We, therefore, developed an i.v. formulation that could be administered in a nonhuman primate model in order to better assess the pharmacokinetic behavior of ATRA. This model has been highly predictive of the pharmacokinetics of retinoids [46-48] as well as other anti-cancer drugs in humans [49-51].

The plasma concentration-time profile of ATRA after an i.v. bolus dose in the primate model displayed three distinct phases: an initial rapid distributive phase; a plateau phase, the duration of which was proportional to the dose; and a rapid exponential elimination phase (Fig. 4) [46]. This curve shape is most consistent with a capacity-limited (saturable) elimination process. At high drug concentrations, the elimination process is saturated and the rate of elimination is independent of drug concentration (zero-order elimination). As the drug concentration falls toward the Michaelis constant $K_m$, the drug itself becomes rate-limiting and drug elimination becomes proportional to drug concentration (first-order elimination). Fitting the Michaelis-Menten model for a capacity-limited process to the plasma concentration data yields a $K_m$ of between 1 and 5 µM, approximating the peak plasma concentrations observed in patients receiving oral doses of ATRA. With i.v. daily dosing in the nonhuman primate model, ATRA clearance is rapidly upregulated, with a significant decrease in plasma AUC noted as early as the third day of drug administration [47]. Induction of this capacity-limited elimination process for ATRA could account for the decrease in plasma drug concentrations observed over time in patients with APL. Of note is the fact that this upregulation in ATRA elimination occurred within several days of initiation of drug administration, and not weeks to months later, the time frame when relapses are usually observed in APL patients.

Several lines of evidence suggest that the capacity-limited elimination step in ATRA metabolism is mediated by

![Figure 4. The top panel is the concentration time profile of ATRA following a 100 mg/m² i.v. bolus dose. The shape of the curve is most consistent with a capacity-limited (saturable) elimination process. The bottom panel displays the predicted curves for a drug cleared via a more common first-order elimination pathway, shown as bi-exponential elimination, versus a drug cleared via a capacity-limited pathway, which is best described by the Michaelis-Menten equation.](imageurl)
cytochrome-P450 enzymes. Preclinical studies have identified 4-hydroxy- and 4-oxo-retinoic acid as major metabolites of ATRA in animal models [52-54]. In humans, the only metabolite observed in plasma after oral administration of ATRA has been 4-oxo-retinoic acid, albeit at concentrations significantly below (<10%) that of ATRA [42]. After 28 days of daily dosing, a point at which plasma concentrations of ATRA have decreased substantially, administration of P450 inhibitors (e.g., ketoconazole, liarozole) can transiently increase plasma ATRA concentrations [55, 56]. Although the rate-limiting step in ATRA elimination appears to be oxidation, it is unlikely that this is the final step in ATRA catabolism. Preclinical studies performed in cynomolgus monkeys demonstrated that glucuronidation of both ATRA and 4-oxo-retinoic acid was a major metabolic pathway in retinoid metabolism [54, 57-59]. In patients, the predominant form of retinoid recovered in the urine following ATRA administration is glucuronide [42]. Thus ATRA appears to be eliminated by the well-described detoxification pathway of oxidation followed by glucuronidation [60] (Fig. 5).

**RESISTANCE TO ATRA**

Although ATRA is a highly efficacious induction agent in APL, resistance almost invariably develops when ATRA is administered as a maintenance agent on a daily basis. The precise mechanism(s) of resistance in APL has not been determined, but there are several potential mechanisms, based on the assumptions that a threshold concentration of free retinoic acid must be achieved at the site of nuclear receptors and that receptor-bound ligands are required to activate target genes. Thus drug resistance could develop as a result of: A) alteration to the retinoic acid receptor, RARα; B) insufficient concentrations of ATRA; or C) alterations in the mechanisms regulating intracellular retinoic-acid binding and metabolism.

**Additional Mutations in RARα**

The presence of additional mutations in RARα is an obvious potential source of ATRA drug resistance. Such mutations have been described in vitro in a resistant HL60 cell line [61, 62]. However, this cell line, similar to other resistant cell lines [63], was developed only in the presence of sustained high concentrations of ATRA. These high concentrations of ATRA are much higher than concentrations achieved in patients with standard doses of ATRA, and to date no additional RARα mutations have been described in the leukemic blasts from patients with APL at the time of recurrence [10, 64]. Clinically, resistance to ATRA can be reversible. Patients whose disease has previously recurred on ATRA but who have not received the drug for a prolonged period of time may have a response to ATRA after retreatment [11]. This would not be consistent with a stably carried mutation. It thus appears unlikely that additional mutations in RARα are responsible for retinoid resistance in most patients with APL.

**Pharmacokinetics of ATRA**

The inability to maintain adequate plasma concentrations of ATRA because of rapid upregulation of its catabolism is an attractive hypothesis to explain the inevitable recurrences in patients with initially responsive disease, but more recent data suggest that this mechanism alone is unlikely to be responsible for drug resistance. As previously discussed, the low plasma concentrations of ATRA initially observed at the time of relapse are actually present since very early in treatment course, usually within the first week of therapy. Thus the plasma ATRA concentrations observed at relapse are similar to the concentrations achieved during most of the induction phase of therapy.

An early report suggested that APL blasts obtained from patients at time of relapse retained sensitivity to ATRA in vitro [13]. However, in this study leukemic cells were exposed to an ATRA concentration of 1 µM, which is a relatively high concentration. Most APL blasts from patients at the time of diagnosis are exquisitely sensitive to ATRA and undergo differentiation with concentrations of 0.1 µM [65]. A more detailed analysis has shown that there is indeed a shift in the dose-response curve when comparing leukemic blasts obtained at relapse with those obtained at diagnosis (Fig. 6) [66]. These data suggest that retinoid...
resistance is more likely to be primarily due to mechanisms which remain intrinsic to the leukemic cell, and not due to an extrinsic change in drug clearance.

Intracellular ATRA Concentration

CRABPs play a critical role in regulating the amount of free retinoic acid capable of reaching and activating nuclear receptors. When the F9 teratocarcinoma cell line (a murine cell line which differentiates into parietal endoderm in response to ATRA) was stably transfected with the gene encoding for CRABP-I, the differentiating effect of ATRA was greatly abrogated by the resultant increase in intracellular levels of CRABP-I protein [67]. This suggests that CRABP may serve to regulate the intracellular concentration of ATRA. This hypothesis is supported by experimental data demonstrating that CRABP binding enhances the intracellular oxidative catabolism of ATRA [24], decreasing the amount of ATRA available for binding to nuclear receptors. CRABP tissue levels are rapidly upregulated after systemic administration of standard doses of ATRA in a preclinical animal model [47]. In leukemic blasts obtained at diagnosis from patients with APL, CRABP is not detectable [68], but within 30 days of treatment, cytosolic extracts from bone marrow of patients receiving ATRA contain detectable amounts of CRABP [68]. In addition, leukemic blasts from four patients at the time of relapse were found to contain significant amounts of CRABP-II [66].

The demonstration of a shift in the ATRA dose-response curve in vitro at time of relapse, combined with the upregulation in the expression of CRABP-II in leukemic cells, support the hypothesis that treatment failure of ATRA may occur as a result of changes in the intracellular regulation of the drug.

Overcoming ATRA Resistance

Based on the data suggesting that there may be a pharmacological basis for retinoid resistance, three strategies to overcome this form of resistance have undergone preliminary investigation. The first strategy is aimed at interfering with the oxidative catabolism of ATRA by administering P450 inhibitors. This strategy might increase plasma concentrations of ATRA but may not increase intracellular concentrations of drug. A second approach is to administer ATRA on an intermittent dosing schedule, which would allow for downregulation of ATRA metabolism and CRABPs. This strategy could potentially increase plasma concentrations of ATRA and might also increase intracellular concentrations of drug if CRABPs were decreased during the drug holiday. The final strategy is to identify retinoids that are not subjected to increased rates of elimination over time and are not sequestered intracellularly by CRABP.

P450 Inhibitors

The effects of P450 inhibitors on ATRA metabolism have been examined in preclinical studies [69, 70] and in clinical trials [55, 56]. A single dose of a P450 inhibitor (ketoconazole, liarozole) after 29 days of continuous oral ATRA administration partially restored plasma ATRA concentrations to levels observed on the first day of drug administration [55, 56]. However, when this approach was tested on a continuous basis by administering ketoconazole daily with the ATRA, after 14 days there were no differences in plasma ATRA concentrations in patients randomized to receive ATRA plus ketoconazole compared with patients who received ATRA alone [71]. In addition, more toxicities were observed in patients receiving ketoconazole.

Because of the inability of P450 inhibitors to maintain ATRA concentrations on a continuous basis, the increased toxicity, and the unlikelihood of significantly impacting intracellular ATRA concentrations, the use of P450 inhibitors in combination with ATRA cannot be recommended.

Schedule of Administration

The 45 mg/m² daily dosing schedule of ATRA used in patients with APL was chosen empirically. Fortunately, APL proved to be exquisitely sensitive to retinoids, as it appears that both lower doses of ATRA [72, 73] and shorter schedules of administration (anecdotal reports [74]) might be equally efficacious as remission induction therapy. With the remarkably high remission induction rate with ATRA administered on a daily schedule, it would not be prudent to test alternative dosing schedules during induction therapy.
because improvements in outcome would be extremely difficult to demonstrate. The role of ATRA in the maintenance of remissions, however, has not been defined, and in this setting, as well as in other disease states where low plasma concentrations of ATRA are unlikely to exert a differentiating effect, evaluating alternative schedules of administration has merit.

Based on preclinical studies [47], we investigated intermittent schedules of ATRA administration, including a dosing schedule of 7 days on/7 days off [44]. In this study, the plasma AUC declined significantly during the first week of drug administration, from a mean (± SD) AUC of 145 ± 26 μM•min on days 1 to 18 ± 4 μM•min on day 7. Plasma ATRA concentrations at the start of weeks 3 and 11 of this every-other-week schedule, however, were equivalent to those achieved on day 1 of treatment. Mean AUCs were 177 ± 39 and 128 ± 30 μM•min on day 1 of weeks 3 and 11, respectively (Fig. 7). This rapid upregulation of ATRA clearance followed by downregulation during the phase of the intermittent schedule when the patient is off therapy has been observed in other studies [71, 75, 76]. Thus, intermittent schedules of ATRA administration appear to circumvent the low plasma drug exposure that results from the sustained upregulation of catabolism on chronic daily dosing schedules.

Another potential benefit of intermittent ATRA administration may be its impact on CRABP expression. In a preclinical study, the level of CRABP expressed in skin biopsy specimens decreased following a seven-day period without ATRA administration, although it did not return to baseline levels [47]. In patients with APL, the level of CRABP expression in cytosolic extracts from bone marrow returned to baseline levels three to six months after ATRA discontinuation [68]. It is not clear, however, that schedules that allow for only one- to two-week periods without drug would be sufficient for CRABP expression to return to levels observed pretreatment.

**Other Retinoids**

The search for retinoids that specifically activate desired signaling pathways but that are not subject to metabolic regulation that tightly controls intracellular and extracellular levels of ATRA is an area of ongoing research. 9cRA, a retinoid currently in clinical trials, may possess pharmacological characteristics more favorable than ATRA. 9cRA is a naturally occurring, geometric isomer of ATRA capable of binding and transactivating both the RXRs and the RARs [23, 77]. In many in vitro models, 9cRA is as potent as, or more potent than, ATRA in inducing a differentiated phenotype [78-82].

9cRA elimination in preclinical studies was first-order at doses up to 100 mg/m², suggesting a lower likelihood that daily dosing with 9cRA would result in decreased plasma concentrations over time [48]. Patients treated at doses less than 140 mg/m²/day have experienced only small decreases in plasma drug exposure over time in the initial clinical trial [83]. In contrast to ATRA, CRABPs do not bind 9cRA [84]. Therefore, if upregulated CRABP is an important mechanism of resistance to ATRA in APL, 9cRA may offer an advantage over ATRA. However, in a study of seven multiply relapsed APL patients treated with a range of doses, 9cRA was unable to induce remissions in the majority of patients [83].

Numerous other retinoids are currently in preclinical testing [85, 86], with a small number in early stages of clinical testing [87]. These retinoids possess a range of specificities for the RAR and RXR proteins. The role of these receptor-specific analogs as differentiation agents remains to be determined.

**CONCLUSIONS**

ATRA has become central to the management of patients with APL. An understanding of the clinical pharmacology of ATRA has been an area of intensive research, as it initially appeared to be linked to treatment failure. Our current knowledge suggests that ATRA resistance is not simply an inability to maintain therapeutic plasma concentrations of drug, but rather may be linked to the intracellular regulation of drug. The intricate nature of the homeostatic mechanisms that maintain tight control over retinoids, combined with the multiplicity of retinoid receptors and signaling pathways, leave open the possibility of a yet-to-be-defined mechanism of resistance that is independent of the clinical pharmacology of ATRA.
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