Clinical Pharmacology of Oral All-trans Retinoic Acid in Patients with Acute Promyelocytic Leukemia

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ABSTRACT

All-trans retinoic acid (RA) induces leukemic cell differentiation and complete remission in a high proportion of patients with acute promyelocytic leukemia (APL). However, remissions induced by all-trans RA tend to be brief, and relapses are associated with resistance to further treatment in vivo, although the leukemic cells appear to retain sensitivity to the cytodifferentiating effects of all-trans RA in vitro.

The clinical pharmacology of all-trans RA was examined in 13 patients with APL. The drug was administered at a constant dose of 45 mg/m2/day, given as a single dose on the first day of therapy and in two divided doses thereafter. Plasma and urinary concentrations of the parent drug and metabolites were quantitated by reverse-phase high-performance liquid chromatography and, where required, by a combination of normal-phase liquid chromatography/negative chemical ionization mass spectrometry. In patients with APL, basal levels of endogenous retinol and natural retinoids were within the normal range. Peak plasma levels of all-trans RA (347 ± 266 ng/ml, mean ± SD) were reached 1–2 h after drug ingestion and decayed in a monoeponential fashion with a half-life of 0.8 ± 0.1 h. The only drug metabolite detected in plasma or urine was 4-oxo-all-trans RA (present in urine as the glucuronide conjugate). This metabolite accounted for <10% of the circulating drug in plasma, and its cumulative urinary excretion accounted for <1% of the administered dose. The drug was not found in cerebrospinal fluid.

Continued oral administration of all-trans RA was associated with a significant decrease in both the plasma peak levels and the area under the concentration-time curve (P = 0.01 and 0.004, respectively) when measured after 2–6 weeks of treatment. We previously reported that a decrease in plasma area under the concentration-time curve was highly correlated with clinical relapse. Observations in a subset of patients in this study suggested that, in fact, the major decrease occurred early, within the first 7 days of treatment. These changes were associated with a 10-fold increase in urinary excretion of 4-oxo-all-trans RA glucuronide, suggesting that the accelerated clearance from plasma was associated with increased drug catabolism.

The rapid disappearance may explain early relapse from remissions induced by all-trans RA; clinical "resistance" to all-trans RA may either wholly or in part result from an inability to sustain effective plasma concentrations of all-trans RA during continuous treatment. Induction of accelerated catabolism by a cytochrome P-450-like enzyme system could account for this phenomenon; however, alternative explanations, particularly a drug-induced increase in cellular retinoic acid-binding proteins in normal tissue, cannot be excluded. Results from this study suggest that treatment with all-trans RA in APL might be improved by discontinuous dosing schedules or by concurrent treatment with P-450 enzyme inhibitors.

INTRODUCTION

RA 3 induces complete remissions in a high proportion of patients with APL (1–3). The initial clinical response to treatment in this disease is clearly associated with the induction of cytodifferentiation of the malignant cells (3). APL is characterized by a specific cytogenetic abnormality that involves reciprocal translocations between the long arms of chromosomes 17 and 15 ([t(15;17) (q22;q21)] (4). The breakpoint for this translocation fuses the RAR-α gene located on chromosome 17 with a previously unidentified gene that may act as a transcription factor on chromosome 15 (initially termed myl, now called PML) (5–9). These clinical and molecular studies have suggested that the gene product of this fusion may serve as a molecular target of all-trans RA therapy.

Despite the high rate of initial remission induction, the duration of complete remission has been relatively brief, averaging 4–5 months in our series 3 (3). Recently, we showed that patients who relapsed from a remission induced by all-trans RA were clinically "resistant" to further treatment, although their leukemic cells appeared to retain sensitivity to the cytodifferentiating actions of the drug in vivo (10). Relapsing patients had not acquired further molecular abnormalities in PML-RAR-α; however, these patients had unexpectedly low plasma drug concentrations that were not augmented despite a 2-fold dose escalation (10). Although all-trans RA is now being widely used for treatment of patients with APL, the pharmacokinetics of this agent in human subjects have not previously been described. In this study, we report the clinical pharmacology of all-trans RA in patients who underwent induction therapy for APL. We also evaluated plasma retinol and retinoid levels to test whether endogenous levels of these natural compounds were affected by the presence of the mutated PML–RAR-α receptor in this disease or treatment with all-trans RA.

MATERIALS AND METHODS

Pharmacokinetic Studies. Patients in this study were undergoing remission induction treatment for acute promyelocytic leukemia with all-trans RA as recently described (3). All-trans RA was formulated in soft gelatin capsules and was supplied by Hoffmann LaRoche, Nutley, NJ. On day 1 of therapy, patients received 45 mg/m2 of all-trans RA as a single oral dose. Heparinized blood samples were obtained prior to drug administration and at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h. Urine was collected in three aliquots from 0-6-, 6-12-, and 12-24-h intervals after drug ingestion. Thereafter, the same total dosage of all-trans RA was administered in two equally divided doses approximately 6 h apart. In selected patients, plasma samples were obtained immediately before and 2 h after the morning dose for the next 6 days. Complete 24-h urine samples were also collected during this 6-day period. Cerebrospi-
nal fluid was collected from patients who underwent lumbar puncture for clinical reasons. Patients gave written informed consent, and the study was reviewed and approved in advance by the Institutional Review Board of this center.

Reagents. All-trans-9-nositol-3,7-dimethyl-2,4,6,8 nona-tetraenoic acid (the internal standard), 4-oxo-13-cis-RA, and 4-oxo-all-trans RA were kindly supplied by Dr. Floie Vane of Hoffmann-LaRoche (Nutley, NJ). β-Glucuronidase type VII-A (from Escherichia coli), all-trans RA, and 13-cis RA were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from either Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Fair Lawn, NJ) and were of HPLC or the highest grade available. Since most retinoids are light sensitive, standard compounds and biological samples were transported in amber-colored containers and processed under low-light conditions where possible.

HPLC Analysis. Plasma samples were extracted and assayed for retinoid acid and metabolites by HPLC as described by Bugge et al. (11). Briefly, 175 μl of a 1:1 mixture of acetonitrile and isobutyl alcohol was added to 250 μl of plasma containing 125 ng of the internal standard and vortexed. After the addition of 150 μl of saturated K2HPO4 solution (1 g/liter) and mixing, the samples were microcentrifuged for 2 min at 24°C. The organic upper layer (50 μl) was analyzed by HPLC. All-trans RA and its metabolites were separated by HPLC on a C18 5 microcartridge (250 x 4.6 mm) from Alttech (Deerfield, IL) with an ODS-10 micro-guard, (3 cm x 4.6 mm) from Bio-Rad (Richmond, CA) using a linear gradient with initial conditions of 50% phase A and 50% phase B to 20% A and 80% B over 10 min. These final conditions were maintained for 8 min prior to a 6 min reequilibration to initial conditions. Mobile phase A consisted of a mixture of 1:1 acetonitrile:water, while B was 19:1 acetonitrile:water. Mobile phases A and B contained ammonium acetate (20 mM) with the pH adjusted to 6.65 and 7.5, respectively. Retinoids were detected by UV absorption at 365 nm (attenuation = 0.01). Selected plasma samples that decreased below the limits of sensitivity of this HPLC technique (10 ng/ml) were assayed by a recently described method using liquid chromatography/mass spectrometry (12). Standard curves for all-trans RA, 13-cis RA, and their 4-oxo metabolites were established using pooled normal plasma and were linear over a range from 0.5 to 1000 ng/ml. The plasma half-life was estimated using computerized software (PCNONLIN84; Statistical Consultants, Inc., Lexington, KY), and the AUC was determined by trapezoidal approximation.

Urinary retinoids were assayed following alkaline or enzymatic hydrolysis with β-glucuronidase. Alkaline hydrolysis was achieved by adding 1 ml of 0.5 M NaOH to 1 ml of urine for 10 min, whereas enzymatic hydrolysis required 18-h incubation at 37°C with 1000 units/ml of β-glucuronidase at pH 6.0. The samples were extracted twice with 5 ml of ether after the internal standard was added and the pH was adjusted to 6.0. Ether extracts were evaporated to dryness under a nitrogen stream at 37°C. The residue was redissolved in 175 μl of methanol and assayed by HPLC.

Ultrafiltration Studies. Plasma from a healthy volunteer was incubated for 30 min with 0–500 ng/ml of all-trans RA dissolved in ethanol at 24°C, in the dark. The final concentration of ethanol in the incubation mixture was 0.5%. This concentration of ethanol did not change the physical and chemical characteristic of lipoproteins as evidenced by electrophoretic mobility and triglyceride content of the individual lipoprotein fractions. Ultrafiltration was achieved by centrifugation at 2000 × g for 20 min at 24°C using a micropartition system (MPS-1) and anisotropic hydrophilic YMT ultrafiltrate membranes (Amicon, Denver, MA). The membranes were rinsed with 3 ml of phosphate-buffered saline before use. Ultrafiltrates were extracted and assayed for all-trans RA as described above.

Characterization of RA-Protein Association. The distribution of all-trans RA among various human serum lipoprotein classes was examined on spiked and clinical plasma samples using a KBr density gradient ultracentrifugation method (13).

RESULTS

HPLC Retinoid Separation. Retention times for authentic retinol, all-trans RA, 13-cis RA, the internal standard, and 4-oxo-all-trans RA were 13.0, 12.4, 11.0, 9.0, and 4.8 min, respectively (Fig. 1). The lower limit of detection for both all-trans RA and 4-oxo-all-trans RA was 10 ng/ml.

Pharmacokinetics of All-trans RA. Following a single oral dose of all-trans RA at a dose of 45 mg/m², plasma levels measurable by HPLC (>10 ng/ml) were observed 30 min after drug ingestion. The Cpmax was generally reached after 1–2 h; thereafter, the plasma content of all-trans RA decreased rapidly and was <10 ng/ml by 8 h (Fig. 2). Oral administration was characterized by marked interpatient variation in bioavailability; detailed pharmacokinetic parameters for the day 1 study are shown in Table 1. The peak plasma concentration of all-trans RA was 346 ± 266 ng/ml (mean ± SD). The parent drug was rapidly eliminated from plasma in a monoexponential fashion with a harmonic half-life of 0.8 ± 0.1 h. The AUC0.8h on day 1 was 682 ± 500 ng/h/ml. Trough plasma concentrations prior to each subsequent morning dose were routinely within the physiological range.

In 7 patients, full pharmacokinetic studies after a single dose of 45 mg/m² (similar to day 1) were repeated after 2–6 weeks of continuous drug treatment. In this subset of patients, the RA Cpmax decreased from 294 ± 89 on day 1 to 138 ± 139 ng/ml.

Fig. 1. Chromatogram showing separation of 4-oxo-all-trans RA (peak A), internal standard (peak B), 13-cis-RA (peak C), all-trans RA (peak D), and retinol (peak E).

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could not be determined whether this metabolite circulates in plasma in a free or conjugated form. The median peak plasma level of 4-oxo-all-trans RA was 25 ng/ml and occurred coincident with the peak concentration of parent drug. Chronic administration of all-trans RA did not increase plasma concentrations of 4-oxo-all-trans RA. Trace amounts of 13-cis RA measurable by HPLC were observed in plasma from only 2 of the 13 patients studied.

Analysis of nonhydrolyzed urine samples revealed neither all-trans RA nor 4-oxo-all-trans RA. However, following nonspecific alkaline and specific β-glucuronidase hydrolysis, the 4-oxo-RA metabolite was detected, indicating the presence of this derivative in a glucuronide-conjugated form. Following the initial dose on day 1, <0.1% of the ingested all-trans RA was excreted in urine as 4-oxo-RA glucuronide. However, in 5 of the 7 patients who underwent repeat pharmacokinetic studies with urinary collections after 2–6 weeks of continuous dosing, the 24-h urinary excretion of 4-oxo-RA glucuronide increased from 32 ± 12 μg after the first dose to 318 ± 116 μg during the subsequent study (P < 0.007). Biliary and fecal excretion was not examined in this study.

**Plasma Protein Binding of All-Trans RA.** Since physiological levels of all-trans RA circulate bound to albumin, we evaluated whether pharmacological concentrations were similarly carried. All-trans RA was not found in the ultrafiltrate, suggesting extensive plasma protein binding. KBr density gradient ultracentrifugation showed that >95% of all-trans RA was found in the nonlipoprotein fraction with only small amounts present in the high-density lipoprotein. Serum from a patient receiving all-trans RA showed similar results with trace amounts of all-trans RA in the very low-density lipoprotein fraction (data not shown).

**Endogenous Plasma Retinoids.** Baseline plasma levels of all-trans RA, 13-cis RA, their 4-oxo metabolites, and retinol (vitamin A) were similar to those found or reported for healthy volunteers (Table 3). Treatment with all-trans RA had no effect on plasma retinol levels (data not shown).

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**(P < 0.01; paired t test).** The plasma RA AUC decreased from 537 ± 191 to 249 ± 185 ng/h/ml (P = 0.004) (Table 2). Sequential mean peak plasma concentrations of all-trans RA sampled 2 h after ingestion of the morning dose on treatment days 2 through 7 are shown in Fig. 3. While again showing a high degree of intersubject variability, mean peak levels in plasma decreased from 140 ng/ml on day 2 to 60 ng/ml on days 5–7.

**Cerebrospinal fluid** was sampled on 3 patients who experienced intracranial hypertension; however, no levels of all-trans RA were detected by liquid chromatography/mass spectrometry, even in these symptomatic patients.

**Plasma and Urinary Metabolites.** 4-Oxo-all-trans RA was the only metabolite detected in plasma of patients receiving all-trans RA, and it accounted for <10% of circulating drug. Since the extraction method hydrolyzes glucuronide conjugates, it would not be expected to detect this metabolite. Following the initial dose on day 1, 0.1% of the ingested all-trans RA was excreted in urine as 4-oxo-RA glucuronide. However, in 5 of the 7 patients who underwent repeat pharmacokinetic studies with urinary collections after 2–6 weeks of continuous dosing, the 24-h urinary excretion of 4-oxo-RA glucuronide increased from 32 ± 12 μg after the first dose to 318 ± 116 μg during the subsequent study (P < 0.007). Biliary and fecal excretion was not examined in this study.

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**DISCUSSION**

All-trans RA induces complete remission in a very high proportion of patients with APL (1–3). Treatment with this agent causes unique changes in the morphological and immunophenotypic characteristics of the malignant cells that are clearly related to maturation of the leukemic clone (3). The exquisite sensitivity of this disease may in part owe to the proportion of patients with APL (1–3). Treatment with this

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>APL patients (n = 12)</th>
<th>Normal Volunteers (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans RA</td>
<td>1.94 ± 0.33</td>
<td>1.94 ± 0.54</td>
</tr>
<tr>
<td>13-cis RA</td>
<td>1.22 ± 0.39</td>
<td>1.51 ± 0.35</td>
</tr>
<tr>
<td>4-oxo-all-trans RA</td>
<td>0.82 ± 0.35</td>
<td>0.67 ± 0.16</td>
</tr>
<tr>
<td>4-oxo-13-cis RA</td>
<td>1.87 ± 1.25</td>
<td>2.86 ± 1.32</td>
</tr>
<tr>
<td>Retinol</td>
<td>728 ± 395</td>
<td>649 ± 220*</td>
</tr>
</tbody>
</table>

* The normal retinol range was derived from Ref. 35.

In vitro studies have examined the ability of all-trans RA to induce cytodifferentiation in cell lines and in cultures of fresh human leukemic cells. Largely, these studies have used continuous cell culture techniques at various RA concentrations, with most studies indicating activity in the range of 10^-7–10^-6 M (1, 14, 15) (1 ßM = 300 ng/ml). Thus, the pharmacokinetic behavior of all-trans RA exhibited in this study of human subjects with APL was somewhat surprising relative to the striking clinical activity of the drug. In our patients, plasma concentration of all-trans RA on the initial day of therapy exceeded 10^-7 M (30 ng/ml) for approximately 4 h. On subsequent days, this concentration was inconsistently achieved even at peak times after drug ingestion.

The clinical pharmacokinetic behavior of all-trans RA differs substantially from that of its isomer, 13-cis RA. We found that plasma levels of all-trans RA disappeared from plasma quite rapidly with a mean half-life of <1 h, compared to reported half-lives ranging from 12–14 h for 13-cis RA (16–18). While in vivo treatment with 13-cis RA has been associated with 20–30% isomerization to all-trans RA (16, 17), in this study we found minimal conversion of all-trans RA to 13-cis RA. The sole metabolite detected in plasma was 4-oxo-all-trans RA. This compound accounted for approximately 10% of the drug in circulation. In urine, this metabolite was conjugated as a glucuronide; however, urinary retinoid excretion accounted for <0.1% of the administered dose. The inter- and intrapatient variation in drug bioavailability observed in this study has been a characteristic feature of retinoid pharmacology and is similar to that observed in studies of 13-cis RA (16–18). The bioavailability of most retinoids is increased with ingestion of food (19, 20). Since a strict dietary regimen was not enforced in the present study, dietary anomalies may also have contributed to the observed variability. Unlike 13-cis RA, the pharmacokinetics of all-trans RA have been reported to be dose dependent in rodents (21). The use of a single fixed-dose regimen in this study precluded testing this assumption in our patients.

We also evaluated whether the presence of an aberrant RAR-α might have led to a state of relative or absolute deficiency of endogenous retinol or all-trans RA. If this hypothesis were correct, this disease might become clinically evident only after endogenous stores had been depleted below some critical threshold. However, pretreatment plasma retinol and all-trans RA levels in APL patients were within the normal range, and no correlation was observed between pretreatment plasma levels of all-trans RA and response to this therapy. Thus, the responsiveness of this disease to all-trans RA therapy cannot be attributed to a quantitative deficiency of endogenous retinoids. Unlike the effects of treatment with synthetic retinoids such as fenretinide (22, 23), treatment with all-trans RA did not alter plasma retinol concentrations.

A potentially critical observation from this study was that chronic oral administration of all-trans RA resulted in a progressive decrease in peak plasma concentrations and in AUC values. This finding is in contrast to studies involving the protracted oral administration of other retinoids such as 13-cis RA and etretinate that have shown no changes in AUC levels (17, 18). However, a decrease in AUC after oral administration of all-trans RA, but not 13-cis RA, has previously been described in both mice (24, 25) and monkeys (26), and thus our observations are not species specific. We recently reported that clinical relapse of patients with APL induced into remission with all-trans RA was associated with a significant reduction in plasma drug concentrations (10). Results from this study indicate that this reduction may occur early during the course of treatment, possibly within the first week as suggested by data in Fig. 3.

Chronic ingestion of all-trans RA was associated with approximately a 10-fold increase in urinary excretion of 4-oxo-all-trans RA, a finding that suggests increased catabolism with time rather than a reduction in oral bioavailability. (The current lack of a suitable parenteral or radiolabeled formulation precluded other bioavailability studies.) All-trans RA is known to be catabolized by cytochrome P-450-like enzyme systems (27, 28) that are presumably autoinducible. Recently, two inhibitors of P-450 (ketocazole and RW 27,251) were shown to significantly prolong the plasma half-life of all-trans RA in animals (29, 30). Conceivably, coadministration of P-450 inhibitors to patients with APL might also prevent the reduction in plasma drug levels and possibly extend remission duration (36).

All-trans RA has a high affinity for cytosolic CRABP (31, 32) which rapidly clear the drug from circulation. Topical application of all-trans RA and systemic administration of the synthetic retinoid acitretin have been shown to increase CRABP content in human skin (33, 34). A drug-induced increase of CRABP in normal tissues could result in accelerated disappearance of the drug from plasma and could also account for the low plasma concentrations observed in this study after continuous therapy.

In summary, the human pharmacokinetics of all-trans RA are quite distinct from other natural or synthetic retinoids. Relative to recent experience with its isomer 13-cis RA, the plasma half life of all-trans RA was quite brief and the plasma AUC decreased significantly with continuous drug administration. Whether this phenomenon is associated with induction of accelerated enzymatic catabolism or increased binding to cytosolic proteins in normal tissues is unclear, and both of these hypotheses are currently under study. Nonetheless, these observations suggest a potentially critical explanation for relapse and retinoid “resistance” in patients with APL treated with this agent.
Note Added in Proof

Lefebvre et al. have recently reported similar Day 1 pharmacokinetic data (37).

ACKNOWLEDGMENTS

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