Advances in Brief

Time Course of Induction of Metabolism of all-trans-Retinoic Acid and the Up-Regulation of Cellular Retinoic Acid-binding Protein

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Abstract

A study of chronic i.v. dosing of all-trans-retinoic acid (all-trans-RA) was performed to determine whether induction of the capacity-limited elimination process for all-trans-RA occurred with long-term drug administration. Because up-regulation of the cellular retinoic acid-binding proteins (CRABP) may act to bind all-trans-RA intracellularly, the amount of CRABP in skin biopsy samples obtained during and following the course of all-trans-RA administration was also determined. Four adult rhesus monkeys received 50 mg/m² of all-trans-RA by bolus i.v. injection daily for 8 consecutive days and again on day 15 following a 7-day period without drug. Plasma clearance of all-trans-RA was characterized by a plateau phase, the duration of which decreased during the period of chronic drug administration, followed by a terminal exponential decay phase, which is consistent with a capacity-limited (saturable) elimination process. The Vmax of this process increased from 0.06 µmol/min on the first day to 0.17 µmol/min by the eighth day of all-trans-RA administration, consistent with induction of an enzymatic process. The amount of CRABP measured in skin biopsy specimens was rapidly induced, increasing to approximately 3-fold baseline levels by day 3 of all-trans-RA administration. It remained at this level throughout the period of chronic drug administration but diminished following the 7-day period without drug. These findings suggest that an intermittent schedule of administration for all-trans-RA has potential advantages over a continuous administration schedule. A period of time without drug administration would allow for return of plasma drug clearance toward baseline levels and down-regulation of CRABP, which could result in higher plasma drug concentrations and possibly less cytoplasmic binding of drug.

Introduction

Retinoids are naturally occurring and synthetic analogues of vitamin A (retinol) that are capable of inhibiting growth and inducing differentiation in experimental tumor models (1–3). In the clinical setting, topical and p.o. retinoids have been effective in the treatment of premalignant skin conditions (1, 2, 4), and, more recently, p.o. administration of all-trans-RA has been demonstrated to induce complete remissions in specific cell types (12, 13), the amount of CRABP in skin biopsy samples obtained during and following the course of all-trans-RA was also determined.

Time Course of Metabolism of all-trans-Retinoic Acid

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2To whom requests for reprints should addressed, at Pediatric Branch, National Cancer Institute, Bethesda, MD 20892.
3The abbreviations used are: RA, retinoic acid; CRABP, cellular retinoic acid-binding proteins; AUC, area under the concentration-time curve; HPLC, high-performance liquid chromatography.

dent and are described by a capacity-limited (saturable) process (11). To determine whether induction of this capacity-limited process could be responsible for the diminution in plasma drug concentrations observed with long-term drug administration, a study of chronic i.v. dosing of all-trans-RA was performed in the rhesus monkey.

The CRABPs (I and II) are two related high affinity all-trans-RA binding proteins found in the cytoplasm of many cell types, with CRABP I having a more widespread distribution than CRABP II. Because an increase in CRABP I may act to facilitate the intracellular metabolism of all-trans-RA in specific cell types (12, 13), the amount of total CRABP in skin biopsy samples obtained during and following the course of all-trans-RA administration was also determined.

Materials and Methods

Animals. Four adult rhesus monkeys (Macaca mulatta) ranging in weight from 4.7 to 11.7 kg were obtained from Buckshire Corporation (Perkasie, PA). The animals were fed Purina monkey chow and group housed according to the Guide for the Care and Use of Laboratory Animals (14). For pharmacokinetic studies, animals had catheters surgically placed into jugular or femoral veins and attached to an access port implanted s.c. (Vascular Access Port, Model LCI; Access Technology, Skokie, IL).

Drug Formulation and Administration. Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) except for the 4-oxo-13-cis-RA and 4-oxo-all-trans-RA metabolites, which were kindly provided by Hoffman La Roche (Nutley, NJ). The N-methylglucamine salt of all-trans-RA was prepared immediately prior to administration by a previously described method (11).

Each animal received 50 mg/m² of all-trans-RA daily for 8 consecutive days. Following 7 days without drug, a single additional 50-mg/m² dose was administered on day 15. Drug was administered as a 5-min bolus dose via a percutaneously placed i.v. catheter.

Pharmacokinetics. Pharmacokinetic sampling was performed on days 1, 3, 5, and 8 and again on day 15 following a 7-day period without drug. All samples were obtained in a room with the lights dimmed. Blood samples were drawn through the venous port prior to and at 30, 60, 90, 120, 180, 240, and 360 min following drug administration. Plasma was separated from blood by centrifugation (3200 × g for 10 min at 10°C). All plasma samples were protected from light and stored at −20°C until assayed.

As shown previously, the plasma disappearance profile of all-trans-RA is best described by the Michaelis-Menten model (11). The km and Vmax of the Michaelis-Menten elimination process were estimated by fitting the mean concentration-time data to the differential equation

\[
\frac{dC}{dt} = \frac{V_{\text{max}} \times C}{K_m + C}
\]

using MLAB (15). The fit was weighted to the inverse square of the concentration. AUCs were calculated by integrating the Michaelis-Menten equation from time 0 to infinity and substituting the estimated Vmax and km into the equation

\[
AUC = \int_0^{\infty} C dt = \frac{C_0}{V_{\text{max}}} \left( K_m + \frac{C_0}{2} \right)
\]

(16), where C0 is the concentration at time 0 min, estimated based on the
30-min concentration and excluding the brief distributive phase. Clearance was determined by dividing the dose by the AUC.

Retinoic Acid Assay. The plasma concentration of all-trans-RA was measured by a modification of a previously described HPLC method (17). All procedures were performed in a room with the lights dimmed. Briefly, 50 µl of a 5% perchloric acid solution were added to each 500-µl plasma sample and vortexed for 30 s. To this, 500 µl of ethyl acetate were added and vortexed for 60 s. Following centrifugation (10,000 x g for 60 s at 20°C), the organic layer was removed and analyzed by HPLC. The HPLC system included two Waters model 510 pumps (Milford, MA) and a Beckman 5-µm steel ultra-sphere column (San Ramon, CA) coupled with a Waters Resolve 5-µm C18 radial compression module. The mobile phase consisted of 95% acetonitrile and 5% ammonium acetate buffer (1% v/v) at a flow rate of 2.5 ml/min. Eluant was monitored with a Waters 490 multil wavelength detector at wavelengths of 340 and 360 nm. Retention times under these conditions for 4-oxo-13-cis-RA, 4-oxo-all-trans-RA, 13-cis-RA and all-trans-RA were approximately 5.0, 5.6, 7.7, and 10.3 min, respectively. Recovery of all-trans-RA was approximately 90% and its lower limit of quantification was 0.1 µM.

CRABP Determination. Skin samples were obtained by 6-mm punch biopsies on days 1, 3, 5, and 8 of chronic all-trans-RA administration and again on day 15 following 7 days without drug administration. Specimens were placed in 100 µl of normal saline and stored at −20°C until time of analysis. Total CRABP (CRABP I and II) levels were quantitated by a previously described fast protein liquid chromatography method (12). Briefly, cytoplasmic protein was extracted and 200 to 600 µg of protein were incubated overnight at 4°C in a 500-µl solution of 50 mM [3H]retinoic acid (New England Nuclear, Boston, MA) and 2 mM dithiothreitol. Unbound [3H]retinoic acid was removed with charcoal/dextran, and extracts were fractionated over a Superose 12 HR 10/30 size exclusion column (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C maintaining a flow rate of 0.5 ml/min with 5 mM sodium phosphate, pH 7.4–10 mM thioglycerol-10% glycerol buffer-plus 0.4 M KC1. Fractions were collected and quantitated by liquid scintillation.

Results

The mean plasma concentration-time curves for the four animals studied on days 1, 3, 5, and 8 of chronic all-trans-RA administration are shown in Fig. 1. Plasma disappearance of all-trans-RA was characterized by an initial plateau phase in the curve, followed by a terminal exponential decay phase. This profile is consistent with a capacity-limited (saturable) elimination process. The duration of the plateau phase decreased over the period of chronic drug administration. The V_max of the capacity-limited process increased from 0.06 µmol/min on the first day of all-trans-RA administration to 0.17 µmol/min by day 8 of drug administration (Fig. 2), consistent with induction of an enzymatic process. The V_max decreased to 0.1 µmol/min on day 15, after a 7-day period without drug.

The clearance of all-trans-RA, determined on days 1, 3, 5, and 8 of drug administration and again following a 7-day period without drug, is shown in Fig. 3. Clearance increased during the 8-day period of drug administration, and following a 7-day interval without drug returned to near baseline levels. The 4-oxo-all-trans-RA metabolite was not quantifiable (<0.2 µM) in any plasma sample.

The amount of CRABP measured in skin biopsy specimens is shown in Fig. 4. CRABP levels rapidly increased to approximately 3-fold baseline levels by day 3 of all-trans-RA administration and remained at this level for the remainder of the period of chronic drug administration. After 7 days without therapy, CRABP levels had decreased but had not returned to baseline levels.

Discussion

All-trans-RA has proven to be an effective agent for inducing complete remissions in patients with acute promyelocytic leukemia, but as a single agent it does not appear able to maintain patients in remission (6). Recent pharmacokinetic studies have demonstrated that systemic exposure to all-trans-RA as measured by the AUC decreases over time when drug is administered on a chronic daily schedule, such that by day 28 of therapy the AUC may be as much as 5-fold lower than that on day 1 of therapy (8, 9). It has been speculated that recurrence of disease may be secondary to this diminution in plasma drug concentrations. The basis for the observed decrease in plasma all-trans-RA concentration, however, has not been determined. One potential explanation is that gastrointestinal absorption is down-regulated with long-term administration of drug. Because there is currently no i.v. formulation of all-trans-RA for human trials, the absolute bioavailability of p.o. all-trans-RA is unknown, and thus this hypothesis has not been tested. The study presented here demonstrates that an alternative explanation, induction of a capacity-limited elimination process for all-trans-RA, could also account for a decrease in plasma drug concentrations observed over time.

In the current study, a decrease in plasma concentrations of all-trans-RA was noted as early as the third day of i.v. drug administration. The basis for this decrease was an increase in plasma all-trans-RA clearance, such that after 1 week of drug administration plasma clearance increased almost 2-fold. Induction of the capacity-limited elimination process, as evidenced by the approximately 3-fold
increase in the $V_{\text{max}}$ (Fig. 2), resulted in a progressive shortening in the duration of the plateau (zero-order) phase of the all-trans-RA disappearance curve, and a reduction in the plasma drug concentrations.

In a recent study performed in humans, the plasma concentration of all-trans-RA 2 h following a p.o. dose of drug decreased throughout the first week of drug administration (18). A decrease in the AUC may also have occurred, but the correlation between the 2-h plasma drug concentration and AUC was not defined. Inasmuch as drug was administered p.o. in that study, down-regulation of absorption could also have accounted for the observed decrease. The time course of changes in plasma all-trans-RA concentrations observed in the rhesus monkey following chronic administration of drug, however, appears to parallel those observed in humans, suggesting that the decrease in plasma all-trans-RA concentration that occurs in patients is also the result of induction of a metabolic pathway.

The nature of the capacity-limited elimination process for all-trans-RA is not known, but there are at least two previously described metabolic pathways that might account for the observed elimination profile. Administration of P-450 inhibitors can increase the plasma concentrations of all-trans-RA in animal models (19) and in patients (20), suggesting that oxidation of all-trans-RA is one pathway for its catabolism. However, the plasma concentrations of 4-oxo-all-trans-RA in this study were extremely low (<0.2 $\mu$M), with no increase observed with chronic drug administration. Other studies have also found that plasma concentrations of 4-oxo-all-trans-RA are low or undetectable following a single dose of all-trans-RA administration (8, 18, 21–23). It thus appears unlikely that oxidation alone is the major route of drug elimination for all-trans-RA.

The glucuronide conjugate of all-trans-RA has been identified as a primary metabolite detected following both i.v. and p.o. all-trans-RA dosing in animals (22–25). In studies of long-term administration of p.o. all-trans-RA to cynomolgus monkeys (23), the amount of all-trans-RA-glucuronide formed increased over time. The 4-oxo-all-trans-RA glucuronide has been tentatively identified as the only urinary metabolite detected in humans following a p.o. dose of

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**Fig. 2.** $V_{\text{max}}$, calculated by fitting the average plasma concentration-time data to the Michaelis-Menten equation for a capacity-limited process ($r^2 = 0.98, 0.94, 0.89, 0.85, 0.99$ for days 1, 3, 5, 8, and 15, respectively), is plotted as a function of days of all-trans-RA administration. The increase in $V_{\text{max}}$ that occurs from day 1 through day 8 is consistent with induction of a capacity-limited elimination process.

**Fig. 3.** Plasma clearance of all-trans-RA plotted as a function of days of drug administration.
all-trans-RA, and increases with long-term all-trans-RA administration (18). These studies suggest that glucuronidation may be a major pathway of all-trans-RA catabolism.

Unfortunately, the analytical methods used in the present study were not capable of detecting glucuronidated metabolites of all-trans-RA in plasma samples. Because both oxidation or glucuronidation can be up-regulated, the capacity-limited process induced with long-term drug administration described here may be either oxidation or glucuronidation. This would not be unexpected because many drugs are detoxified by phase I (oxidation) followed by phase II (conjugation) enzyme systems (26). If oxidation of all-trans-RA were the rate-limiting step in its metabolism, significant plasma concentrations of 4-oxo-all-trans-RA would not be observed even following enzyme induction because of the subsequent, and presumably more efficient, glucuronidation step.

New evidence is evolving that the high-affinity retinoic acid-binding proteins CRABP I and II may not only modulate the differentiating effects of retinoids but also play a role in their biotransformation. Up-regulation of CRABP I in in vitro studies led to a decrease in the ability of all-trans-RA to induce differentiation (12) and facilitated the intracellular metabolism of all-trans-RA to more polar metabolites (13). In another study using an in vitro microsomal assay (27), holo-CRABP I was shown to serve as a substrate in all-trans-RA metabolism. In the current study, the levels of CRABP skin increased rapidly, peaked by the third day, and remained at this level throughout the period of all-trans-RA administration.

Although the liver appears to be the primary site of all-trans-RA catabolism (28, 29), it is not clear what role CRABP plays in the hepatic metabolism of the drug. From our results, the increase in plasma drug clearance observed with continuous all-trans-RA administration appears to be related to catabolic enzyme induction and not increased CRABP expression, as suggested by the differences in the time course of both the up- and down-regulation of all-trans-RA clearance compared with CRABP skin levels. There thus appear to be two parallel but potentially related processes that may diminish the effectiveness of all-trans-RA when administered on a continuous basis: a decrease in plasma drug concentrations secondary to increased plasma clearance; and an increase in the binding and subsequent metabolism of drug within the cytosolic compartment of cells by up-regulated CRABP.

The findings in the present study, coupled with recent data obtained in human trials suggesting that systemic exposure to drug during long-term p.o. administration all-trans-RA also decreases rapidly (18), raise questions regarding the plasma all-trans-RA concentrations required to achieve a clinical response in patients with acute promyelocytic leukemia. For example, it is unknown whether the clinical responses observed in patients with acute promyelocytic leukemia are achieved with brief exposure to high concentrations of all-trans-RA or with the more lengthy exposure to the relatively low drug concentrations that occur within days of starting administration of drug on a daily schedule. One critical piece of data that would be beneficial in clinically applying the pharmacokinetic information presented here is determination of both the concentrations of all-trans-RA required in vitro for antitumor efficacy and the minimum required duration of exposure to these concentrations. Findings from the present study suggest that an intermittent schedule would have two potential advantages over a continuous administration schedule. A period of time without drug administration would allow for return of plasma drug clearance towards baseline levels, and hence an increase in plasma all-trans-RA concentrations. Furthermore, down-regulation of CRABP that accompanies discontinuation of drug could result in less cytoplasmic binding of drug and a potential increase in all-trans-RA effectiveness. Based on the results of the present study, clinical trials of intermittent administration of all-trans-RA appear warranted and are being planned.

References


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