Pharmacology of 13-cis-Retinoic Acid in Humans

Ian G. Kerr, Marc E. Lippman, Jean Jenkins, and Charles E. Myers

ABSTRACT

Vitamin A and its analogs (retinoids) have shown great promise for the chemoprevention of cancer as well as being a possible new class of chemotherapeutic agents. A Phase I and II trial of 13-cis-retinoic acid in advanced cancers was initiated, and the clinical pharmacology of the drug was studied. All patients received p.o. 13-cis-retinoic acid starting at 0.5 mg/kg/day, escalating over 4 weeks to a maximum dose of 8 mg/kg/day in divided doses. Although there was a linear correlation of plasma concentration with dose escalation, large interindividual variations in peak plasma concentrations were noted. At the maximum drug dose, the mean peak plasma concentration was $4 \times 10^{-6}$ M. There was little drug accumulation on this schedule, as trough concentrations between p.o. doses often dropped below $1 \times 10^{-6}$ M. The drug was metabolized extensively to a metabolite, the concentrations of which exceeded parent 13-cis-retinoic acid concentrations with chronic dosing. Retinol concentrations were below the normal range.

INTRODUCTION

Retinoids (vitamin A and its analogs) maintain epithelial cell differentiation, have antipromotional properties, and modify the growth and differentiation of transformed cells in culture (11-13, 16). On the basis of animal data, showing the efficacy of 13-cis-RA3 in preventing carcinogen-induced bladder carcinoma (17), it has been recommended that this retinoid be used as a chemopreventive agent in humans (9). The action of retinoids against established tumors is less clear, although there is some evidence that they may show activity even in this situation (1, 18). On the basis of these observations, a Phase I and II trial of 13-cis-RA was initiated in patients with advanced cancer (3).

A group of these patients underwent pharmacological studies. Although the pharmacology of retinoic acid has been well studied in animals (4, 5, 14), little is known of its time course and disposition in humans. In animals, retinoic acid is cleared rapidly from the body (14, 20). It also produces several metabolites, some of which have biological activity (4, 5, 13). Both of these considerations may be important determinants for the in vivo effects of retinoids. As a result of these concepts, the pharmacology of p.o. 13-cis-RA was studied in humans.

MATERIALS AND METHODS

Retinoids. 13-cis-RA (Ro 4-3780) and 4-oxo-trans-retinoic acid (Ro 12-4824/701) were kindly provided by Hoffman-LaRoche Inc. (Nutley, N. J.). Other retinoids, which included trans-retinoic acid, 4-hydroxy-trans-retinoic acid, and 5, 6-epoxy-retinoic acid, were provided by Dr. C. Frolik (National Cancer Institute, Bethesda, Md.). 13-cis-[11-3H]-Retinoic acid (1.75 Ci/mmol) was kindly provided by Midwest Research Institute (Kansas City, Mo.) through Dr. C. E. Smith (National Cancer Institute). All-trans-retinol was obtained from Sigma Chemical Company (St. Louis, Mo.).

All powdered retinoids were stored in the dark in a liquid nitrogen freezer until needed. Retinoid solutions were made up in methanol (reagent grade, filtered) and stored in containers covered with aluminum foil in the dark at $-35^\circ$. Any procedure utilizing retinoids was carried out in dim light in a fume hood. The fluorescent lighting in the same room was covered by 0.125-inch-thick UF3 plexiglass (Rohm and Haas, Philadelphia, Pa.).

The purity of 13-cis-[11-3H]retinoic acid was established by HPLC. The retention time of 13-cis-RA contained 95% of the radioactivity.

Chemicals and Instrumentation. All solvents were spectral grade, and the ammonium sulfate was reagent grade. Aquassure (New England Nuclear, Boston, Mass.) was used for liquid scintillation fluid.

A Waters Associates (Milford, Mass.) HPLC system was used for retinoid analyses. Readings were taken at 340 nm by a Model 440 absorbance detector. A model 660 solvent programmer was used to produce the eluting solvent gradient. For retinoid separation, a Waters radial compression module (RCM-100) was used with a C$_18$-Radial-Pak liquid chromatography cartridge (8-$\times$ 100-mm column; 10-$\mu$m spherical particle size).

Drug Trial Design. 13-cis-RA (Ro 4-3780) was supplied by Hoffman-LaRoche as 20- or 40-mg gelatin capsules for p.o. administration. All patients in the study were started at a dose of 0.5 mg/kg p.o. The dose was escalated to 8 mg/kg/day p.o. in divided doses (4 mg twice a day) over the initial 4 weeks (Table 1). This dose level was subsequently maintained unless toxicity forced a dose reduction.

Ten patients underwent pharmacological studies. Nine patients were studied after the first dose (0.5 mg/kg p.o.), 1 patient at a dose of 1 mg/kg twice a day, 6 patients at 2 mg/kg twice a day, and 4 patients (1 of them twice) at 4 mg/kg twice a day. All patients that were studied subsequently to the first dose (0.5 mg/kg) had been taking the drug in a continuous fashion as outlined in Table 1.

All blood samples were collected in covered Vacutainer tubes containing 0.1 ml of 0.14 M sodium ascorbate and 0.07 M trisodium EDTA. This was a modification of the method of Frolik et al. (6). The tubes were centrifuged, and plasma was stored in the dark at $-35^\circ$ until analysis. One patient also had pleural fluid and another patient had ascitic fluid, which were analyzed in the same fashion.

Retinoid Analysis. All samples were thawed at room temperature, in the dark, just prior to analysis. A standard curve was prepared for every analysis. Standard solutions of 13-cis-RA, 4-oxo-trans-retinoic acid, and retinol were added to blank plasma, and the analysis was carried out in parallel with patient samples.

The analytical procedure was a modification of the method by Frolik et al. (6). To 1 ml of plasma were added 50 $\mu$l (containing approximately 4000 dpm) of 13-cis-$[\text{3H}]$RA as an internal standard. To this, 2 ml of

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2 Recipient of a Gordon E. Richards Fellowship from the Canadian Cancer Society. To whom requests for reprints should be addressed, at Building 10, Room 6N119, National Cancer Institute, Bethesda, Md. 20205.
3 The abbreviations used are: 13-cis-RA, 13-cis-retinoic acid; HPLC, high-pressure liquid chromatography; AUC, area under the concentration time curve.

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methanol were added, and the test tube was vortexed. The sample was centrifuged at 3500 rpm for 20 min at 5°, and the supernatant was removed and blown dry by a nitrogen flow under ambient temperature in the dark. To this, 100 μl of methanol were added, the tube was vortexed and centrifuged, and approximately 50 μl of the methanol solution were injected onto the HPLC. The sample was eluted with a solvent gradient (Waters Gradient No. 8) starting with 40% acetonitrile:60% ammonium acetate in water (1 %, w/v). This was increased to 80% acetonitrile:20% ammonium acetate (1 %) over a 10-min period. Each sample was eluted at 1.8 ml/min over 20 min. The 13-cis-RA peak was collected and counted to determine the percentage of recovery. The peak heights of the retinoids were measured for quantitation. The procedure was able to measure to at least 15 ng plasma per ml (5 x 10^-8 M) in a linear fashion with the use of the internal standard. The coefficient of variation was less than 10% for all 3 retinoids. The recovery of 13-cis-RA from plasma varied from 40 to 80%.

**RESULTS**

**Retinoid Analysis.** The analytical procedure easily measured the potential metabolites of 13-cis-RA as well as producing a clear separation of all-trans-retinoic acid from 13-cis-RA. No significant concentrations of trans-retinoic acid were detected in this study. The proposed metabolism of 13-cis-RA in humans (19) is shown in Chart 1. Only one metabolite peak could be detected, and this peak had the same retention time as did standard 4-oxo-trans-retinoic acid (Chart 2). This peak was not present in blank plasma or in samples obtained after the first p.o. dose of 13-cis-RA (0.5 mg/kg). It appeared in large quantities by 2 weeks of continuous drug administration (Chart 5). This peak, called "metabolite," remained a single peak after recycling through the HPLC 10 times using 50% acetonitrile:50% ammonium acetate (1%) as a solvent system. The relative peak height ratio (peak at 340 nm:peak at 365 nm simultaneously) was similar to that for standard 4-oxo-trans-retinoic acid (peak 340 nm:peak 365 nm = 0.8) and different from 4-hydroxy-trans-retinoic acid (ratio, 1.4). No 4-oxo-13-cis-retinoic acid was available for comparison. Others have reported that 4-oxo-trans-retinoic acid and 4-oxo-13-cis-retinoic acid have similar retention times and may be difficult to separate from each other (5, 19). Plasma retinol concentrations were also determined and are represented in Table 2.

**Plasma Concentrations of 13-cis-RA after p.o. Administration.** The peak plasma concentrations of 13-cis-RA were quite variable (Chart 3). After the first treatment dose (0.5 mg/kg p.o.), peak plasma concentrations ranged from 0 (a patient with ascites) to 740 ng/ml (2.5 x 10^-6 M). After 4 mg/kg p.o., the maximum dose used, peak plasma concentrations ranged from 828 ng/ml (2.8 x 10^-6 M) to 1950 ng/ml (6.5 x 10^-6 M) with a mean of 1160 ng/ml (3.9 x 10^-6 M). At this dose, the trough concentration (the minimum levels detected prior to absorption of the next p.o. dose) ranged between 75 ng/ml (0.25 x 10^-6 M) and 400 ng/ml (1.3 x 10^-6 M) with a mean of 242 ng/ml (0.8 x 10^-6 M).

Despite the variable interindividual peak plasma concentrations, there was an overall linear correlation in peak plasma concentration with increasing dose when patients were studied.

![Chart 1: Proposed metabolism of 13-cis-RA in humans (modified from 33).](chart1)

![Chart 2: HPLC separation of retinoids. There was clear separation of retinoid standards using the HPLC gradient system described in the text. Retinoids were dissolved in methanol as a mixture and read at sensitivity of 0.01 at 340 nm. Peaks, 30 to 40 ng of retinoids. Trans RA, trans-retinoic acid; cis RA, 13-cis-retinoic acid; 5,6-Epox RA, 5,6-epoxyretinoic acid; 4-oxo RA, 4-oxoretinoic acid; 4-Hydrox RA, 4-hydroxyretinoic acid; INJ, injection; RT, retention time.](chart2)
Table 2
Plasma retinol concentrations

<table>
<thead>
<tr>
<th>Day</th>
<th>ng retinol/ml plasma</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>284 ± 39*</td>
<td>9</td>
</tr>
<tr>
<td>14</td>
<td>233 ± 50</td>
<td>6</td>
</tr>
<tr>
<td>28</td>
<td>267 ± 76</td>
<td>5</td>
</tr>
</tbody>
</table>

* n, number of determinations for each time point (each determination represented the mean of 3 to 6 samples from the same patient on a particular day of analysis). The lower limit of normal in humans is approximately 300 ng retinol per ml plasma (15).

Mean ± S.E.

Chart 3. Concentrations of 13-cis-RA in plasma versus dose. Concentrations were measured as ng 13-cis-RA per ml plasma with the molar scale shown for comparison. Peak concentrations were determined as the maximum concentration after p.o. dose. The time to reach the peak was variable (1 to 6 hr). Trough concentrations were the minimum drug concentrations detected before drug absorption occurred. Numbers in parentheses, number of patients at each point. Bars, S.E.

serially (Chart 3, solid circles), at least to a maximum dose of 4 mg/kg twice daily. The times to attain these peak plasma concentrations were quite variable after the first 0.5-mg/kg dose (2, 3, 4, 4, 4, 6, and 6 hr) as well as for the 2-mg/kg dose (1, 1, 2, 2, 3, and 4 hr) and 4-mg/kg (1.5, 2, 2.5, 3, and 6 hr) dose.

A complete bioavailability study could not be carried out as no i.v. preparation of 13-cis-RA was available to compare to the results obtained from p.o. administration. Drug bioavailability results from the opposing processes of drug absorption and drug clearance by the gastrointestinal mucosa and liver during its initial exposure to these organs immediately after absorption (designated the first-pass effect). Although drug absorption could not be directly measured, an estimate of a maximum first-pass effect can be made from the data depicted by Chart 4. An intrinsic initial clearance can be calculated from the drug dose given p.o. (0.5 mg/kg) divided by the AUC shown by Chart 4. This was calculated with MLAB, a computer program used at the NIH. Although the clearance was estimated to be 710 ml/min, this was a maximum clearance value as the AUC beyond 7 hr (Chart 4) is likely to be significant, and the result obtained here was calculated from the relationship of p.o. dose/AUC to 7 hr only. From this result, an estimate of the first-pass extraction ratio (E) can be made by the relationship:

\[ E = \frac{CI}{CI + Q} \]

in which Q represents hepatic blood flow and CI represents intrinsic clearance. If Q is given a value of 1.5 l/min, then the maximum extraction ratio, E, would be 0.32. Relatively low values of E are unlikely to contribute to major variations in bioavailability secondary to changes in hepatic function. As a result, a first-pass effect is probably not of major importance in hindering systemic bioavailability of 13-cis-RA after p.o. administration.

Time Course of 13-cis-RA and Metabolite in Plasma. Plasma concentrations of 13-cis-RA after the first dose (0.5 mg/kg) are shown in Chart 4. One patient with ascites was the only patient who did not have any detectable drug either in plasma or ascitic fluid over the 6-hr study period. This may have resulted from the lack of drug absorption or a larger VD, resulting from a third space effect, which would lower drug concentration. In addition, 4-oxo-retinoic acid was not detectable after the first p.o. dose in any of the patients studied.

An estimate of a β-elimination t1/2 of 4 hr was determined from 3 patients (2.6, 4, and 5.5 hr) after this initial dose. Several patients at this and subsequent dose levels had an insufficient number of data points beyond the plasma peak to accurately determine a β-elimination t1/2. This assumes a first-compartment open model. The composite curve shown in Chart 4 is fairly flat as a result of the variability in the times to peak concentration and does not reflect a long β-elimination t1/2.

After the first p.o. dose (0.5 mg/kg), only 2 patients had blood collected beyond 7 hr. One patient had no detectable drug at 25 hr, while the second patient had 26% of her peak
concentration were often late and variable (4 to 8 hr). Also, if variations in peak plasma concentrations of 13-cis-RA and fluid, after receiving 1 mg/kg p.o. This was calculated from the time to attain these peak concentrations. Although differences in drug bioavailability could account for this, there was an estimation of peak concentration in the patients when studied serially. Data represented at each point. Points, mean. Bars, S.E. Metabolite concentrations were calculated from the 13-cis-RA standard curve. The metabolite had the same retention time as did 4-oxo-trans-retinoic acid.

The plasma concentrations attained after 2 and 4 mg/kg are shown in Chart 5. These patients had been taking 13-cis-RA for 2 and 4 weeks, respectively (Table 1). Again, variability in the time to peak plasma concentrations after a p.o. dose caused some flattening of the composite curves. Estimated β-elimination t1/2 for the 2-mg/kg dose (2.4 and 3.8 hr) and the 4-mg/kg dose (2.5, 3.0, and 7.4 hr) were variable. Aside from the peak and trough concentrations that have been noted, the major observation is that there are significantly higher plasma concentrations of "metabolite" than of parent 13-cis-RA. The "metabolite" concentrations were calculated from the 13-cis-RA standard curve.

One patient, not shown, with a pleural effusion achieved only 60% of the comparative plasma concentration in the pleural fluid, after receiving 1 mg/kg p.o. This was calculated from comparative AUC values over a 6-hr postdose period.

DISCUSSION

After p.o. administration, there were large interindividual variations in peak plasma concentrations of 13-cis-RA and time to attain these peak concentrations. Although differences in drug bioavailability could account for this, there was an overall linear relationship between p.o. dose and peak plasma concentration in the patients when studied serially. Data reported by Frolick et al. (6) support this, as their times to peak concentration were often late and variable (4 to 8 hr). Also, if

their doses are calculated for a 70-kg person, the peak concentrations of 13-cis-RA achieved are in agreement with values in Chart 3. Theoretically, larger p.o. doses may achieve more optimal plasma concentrations, although some as yet undiscovered saturable process might supervene at higher administered doses. Also, toxicity may then become dose limiting since, even at 4 mg/kg twice a day, the maximum dose studied, some degree of toxicity occurred in all patients (3).

The trough (predrug absorption) levels of 13-cis-RA after continuous administration were quite low, inasmuch as they were often less than 1 × 10⁻⁶ M. This occurred even at the maximum dose of 4 mg/kg twice a day. If the estimated β-elimination half-life is 4 hr, the dosing interval was suboptimal. For significant drug accumulation to occur, 13-cis-RA should be given approximately every elimination half-life or every 4 hr. Unfortunately, a γ-elimination t1/2 could not be determined. If the AUC of the γ-phase is substantial when compared to the β-phase, it could play a major role in drug clearance. Although a γ-elimination t1/2 of at least 24 hr has been reported (7) and Frolick et al. (6) have shown residual drug at 24 hr (one-third or less of peak for 4 of 5 patients), the significance of this in drug accumulation is unclear because there was little measurable accumulation (reflected by trough levels) of 13-cis-RA in our study. If the γ-elimination phase was predominant, there should be better evidence for drug accumulation because the 4-mg/kg twice a day dose (maximum dose) was studied on the fourth day (Table 1, Day 28) at that dose level or at least 3 t1/2 (if γ-elimination t1/2 is 24 hr). This should allow attainment of 88% of the plateau level achievable with chronic dosing if the plateau principle is utilized. Therefore, a more frequent dosing interval would seem to be required to ensure more adequate drug accumulation.

In addition to measuring parent 13-cis-RA in plasma, we attempted to measure any substantial metabolites that were formed. It seemed reasonable to do this since in animals retinoic acid undergoes extensive metabolism, with several of the metabolites being efficacious as well as toxic (4, 6, 13). In fact, plasma concentrations of a metabolite were considerable and exceeded parent drug concentration after chronic p.o. administration of 13-cis-RA (Chart 5). Vane and Bugge (19) have reported recently that 4-oxo-13-cis-retinoic acid is the predominant metabolite formed in humans with 4-oxo-trans-retinoic acid being the second most abundant metabolite (Chart 1). Although the metabolite peak noted in our study appeared to be single and to have the same retention time as that noted in our Figure 2, this is not definitive proof of its identity. In addition, 4-oxo-13-cis-retinoic acid was not available for comparison. Further work is needed to clarify the structure of the metabolite, and subsequently its biological activity, as it may play a significant role in the biological effect of 13-cis-RA.

Plasma retinol concentrations were also measured (Table 2) and were found to be generally below the normal range expected in humans (15). Although retinoic acid cannot be biotransformed to retinol, published animal studies have reported that chronic retinoid acid treatment may, in fact, lower plasma retinol concentrations (10). No change was seen over our study period, although the observation period was too short to be definitive. This should be examined in conjunction with a more chronic retinoid administration. However, the significance of low plasma retinol concentrations in cancer patients is unclear, although it may be associated with increased cancer rates in
humans (9) and in decreasing the response to chemotherapy, at least in breast cancer patients (2).

As a result of the above observations, 1-3-cis-RA levels attained by this protocol were probably not adequate to control malignant cell proliferation. Although all patients at the maximum dose studied (4 mg/kg twice a day) had some degree of toxicity, no direct relationship between retinoid levels and toxicity could be determined from the small numbers of patients examined. No patients had an objective tumor response (3).

Toxicity could be determined from the small numbers of patients attained by this protocol were probably not adequate to control the growth of transformed cell lines in culture may well exceed $10^{-6}$ to $10^{-5}$ M (11, 12). Reports from the literature, assessing optimal retinoid concentrations for optimal growth suppression. Also, the low trough concentrations reported in our study (often below $1x10^{-6}$ M), as a result of little drug accumulation, may have allowed inadequate growth suppression of sensitive tumor cells and/or regrowth between dose cycles. Although a more optimal dosing schedule may produce higher concentrations of 13-cis-RA, this may not be wise with this retinoid, because toxicity may become a significant problem.

REFERENCES

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