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INHIBITION OF ALL-*TRANS*-RETINOIC ACID METABOLISM BY FLUCONAZOLE *IN VITRO* AND IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA*

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Abstract—All-*trans*-retinoic acid induces acute promyelocytic leukemia cell differentiation *in vitro*, and it produces greater than 90% complete remissions in patients with acute promyelocytic leukemia. Despite the high response rate, the majority of patients relapse with continued *trans*-retinoic acid therapy, and disease progression has been observed to be accompanied by an increase in the metabolism of *trans*-retinoic acid in the patients. In this study, the pharmacokinetic disposition of *trans*-retinoic acid was determined by HPLC in patients with acute promyelocytic leukemia before and after concurrent therapy with the triazole antimycotic agent fluconazole. Treatment with *trans*-retinoic acid for 1 week reduced the area under the plasma *trans*-retinoic acid concentration vs time curve in one patient by 67%, from 277 to 91 ng/mL/hr. *Trans*-retinoic acid pharmacokinetics were repeated after the second dose of fluconazole, administered 1 hour prior to the retinoid, and the AUC was found to be 401 ng/mL/hr, a greater than 4-fold increase from the pre-fluconazole level. A similar, though more modest, effect of fluconazole was seen in a second acute promyelocytic leukemia patient. The effect of fluconazole on *trans*-retinoic acid metabolism was examined *in vitro* using isolated human hepatic microsomes. Fluconazole inhibited the NADPH-dependent cytochrome P450-mediated catabolism of *trans*-retinoic acid in a concentration-dependent manner. Although fluconazole was approximately one-half as potent an inhibitor when compared with ketoconazole, a related antifungal drug, 60–90% inhibition was observed at the concentrations of fluconazole measured in the acute promyelocytic leukemia patients. Neither fluconazole nor ketoconazole inhibited lipid hydroperoxide-mediated metabolism of *trans*-retinoic acid. Since fluconazole is a well-tolerated agent frequently administered to leukemia patients, its use in combination with *trans*-retinoic acid merits further consideration.

Key words: all-*trans*-retinoic acid; fluconazole; acute promyelocytic leukemia; retinoic acid metabolism; retinoic acid pharmacokinetics; human hepatic microsomes

Studies of *trans*-retinoic acid in patients with leukemia and solid tumors suggested that the retinoid induces its own accelerated clearance with chronic therapy [1–4]. These observations were consistent with experiments in animals and with tissue culture cells in which the oxidative catabolism of *trans*-retinoic acid was induced rapidly with retinoid treatment [5–8]. One metabolic pathway for retinoid metabolism is retinoic acid 4-hydoxylation and subsequent conversion to 4-oxoretinoic acid, and these reactions are catalyzed by the microsomal cytochrome P450 oxidase system (reviewed in Ref. 9). The liver is the primary site of retinoid 4-oxidation, and 4-oxo-metabolites can be isolated from incubations of human hepatic microsomes and, along with the corresponding glucuronides, have also been detected in human serum and urine [1, 6, 9, 10]. Although it is not known if the progressive decline in serum *trans*-retinoic acid levels with continued therapy is responsible for the disease relapse in acute promyelocytic leukemia patients, strategies to reverse the acquired resistance are being evaluated. In this study, we describe the inhibitory effect of fluconazole, a triazole antifungal drug, on *trans*-retinoic acid metabolism. These studies demonstrated that fluconazole reverses the accelerated *trans*-retinoic acid clearance in a patient with acute promyelocytic leukemia.

MATERIALS AND METHODS

Materials

Authentic 4-oxo-*trans*-retinoic acid and Ro 23-4736 (internal standard) [11] were provided by Dr. Peter Sorter (Hoffmann-La Roche); fluconazole and UK54373 (internal standard) were provided by Pfizer Pharmaceuticals.

Patient treatment and collection of blood samples

Patient 1 was a 25-year-old female with acute promyelocytic leukemia (FAB classification M3), who was treated with daily oral *trans*-retinoic acid (22.5 mg/m^2 twice a day). Blood samples were collected in foil-lined tubes on day 1 (with the first dose of *trans*-retinoic acid) and on day 8 at the following times: immediately preceding *trans*-retinoic acid and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 6 hr after the *trans*-retinoic acid administration. On day 12, the patient was started on oral fluconazole (400 mg loading dose followed by a 200 mg daily dose) due to the presence of vaginal moniliasis; fluconazole was administered 1 hr prior to the daily dose of *trans*-retinoic acid. A set of blood samples was obtained on day 13

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(following the second dose of fluconazole) at the times indicated above. Plasma was separated from the blood by centrifugation, and it was stored in foil-lined tubes at −85° until analyzed. Patient 2 was a 50-year-old female acute promyelocytic leukemia patient (also FAB classification M3), who was treated with *trans*-retinoic acid and fluconazole as described for patient 1. Blood samples for pharmacokinetics were obtained on days 1, 8, and 13, as for patient 1, and an additional set of samples was obtained on day 14, with the third dose of fluconazole.

Measurement of drug levels

Quantitation of *trans*-retinoic acid and fluconazole in plasma was done by HPLC using modifications of previously described procedures [11, 12]. *Trans*-retinoic acid was extracted under subdued lighting from patient samples by combining 0.5 mL of plasma with internal standard (0.5 μg in 25 μL acetonitrile) and 0.35 mL of a solution containing 1-butanol:acetonitrile (50:50). After mixing thoroughly, 0.3 mL of a saturated solution of K_2HPO_4 was added and the tubes were mixed again. After centrifugation at 3000 *g* for 10 min, the organic layer was transferred to an amber auto-sampler vial. An aliquot was injected on an Adsorbosphere C_{18} column (Alltech) using a Hewlett–Packard HP1090 HPLC. Elution was with a gradient of 50 to 95% acetonitrile (containing 10 mM ammonium acetate) over a period of 10 min; the flow rate was 1.5 mL/min. The eluant was monitored at 365 nm, and peak heights were quantitated with a recording integrator. A standard curve, with concentrations up to 1000 ng/mL, was prepared from analysis of normal human plasma spiked with *trans*-retinoic acid. The lower limit of sensitivity of the assay was 10 ng/mL, and the intra- and inter-day coefficients of variation were 2.4 and 12%, respectively, for a 50 ng/mL sample.

For the HPLC measurement of fluconazole, aliquots of plasma (0.5 mL) were combined with internal standard (5 μg in 0.5 mL of 10% methanol) and 0.25 mL of 1 M ammonium hydroxide. After mixing, the fluconazole was extracted with 5 mL of ethyl acetate. After mixing and centrifugation, the organic layer was acidified with 1 mL of 1 M HCl. Following centrifugation, the aqueous layer was re-extracted with 1.5 mL of 6 M ammonium hydroxide and 5 mL of ethyl acetate. The organic layer was removed and evaporated to dryness under N_2 at 40°. The residue was dissolved in mobile phase (50:50 methanol: 10 mM phosphate buffer, pH 7), and injected on an Adsorbosphere C_{18} column (Alltech). The eluant was monitored at 260 nm and quantitated with a recording integrator. A standard curve with concentrations up to 100 ng/mL was prepared from analysis of normal human plasma spiked with fluconazole. The lower limit of sensitivity of the assay was 1 ng/mL, and the coefficient of variation was 9.5% for a 10 ng/mL sample.

Measurement of trans-*retinoic acid metabolism* in vitro

Human hepatic microsomes (100,000 *g* pellet) were prepared from donor liver. Reaction mixtures contained, in 250 μl, 0.1 M KH_2PO_4 (pH 7.4), 200 μg microsomal protein, 1 μM [11, 12-^{3}H(N)]*trans*-retinoic acid (4 Ci/mmol; New England Nuclear), and 2 mM NADPH (Sigma). Lipid hydroperoxide-mediated metabolism of *trans*-retinoic acid was assayed by replacing the NADPH with 30 μM 13(*S*)-HpODE* (Sigma). Reactions were incubated for 10 min at 37° and were terminated with the addition of butanol:acetonitrile (50:50). The extraction and HPLC analysis of [^{3}H]*trans*-retinoic acid and its metabolites were as described above; 0.5-min fractions of the eluant were collected and combined with liquid scintillation fluid, and the radioactivity was determined using an LKB Rackbeta Liquid Scintillation Counter. Authentic *trans*-retinoic acid and its 4-oxo metabolite were analyzed by HPLC under identical conditions and monitored at 365 nm. Reactions were linear with respect to time and protein concentration.

RESULTS

The pharmacokinetic disposition of *trans*-retinoic acid was examined in two patients receiving the differentiation-inducer for treatment of acute promyelocytic leukemia (Fig. 1). The measured peak *trans*-retinoic acid level in the first patient was 157 ng/mL and occurred 3 hr after the first dose of the drug, representing a somewhat slower absorption compared with the average of other patients; the value for AUC with the first dose (277 ng/mL/hr) was comparable to those previously reported for other acute promyelocytic leukemia patients after correction for the dose of *trans*-retinoic acid administered (Fig. 1 and Table 1) [1, 2, 4]. The effect of continuous oral dosing of *trans*-retinoic acid on the disposition of the retinoid was then evaluated (Fig. 1 and Table 1). Both the peak concentration of *trans*-retinoic acid and the value for AUC were decreased by approximately 70% on day 8. The patient was treated subsequently with fluconazole, and *trans*-retinoic acid disposition was re-examined on day 13, concurrent with the second dose of fluconazole. Treatment with fluconazole 1 hr prior to the oral dose of *trans*-retinoic acid led to a greater than 6-fold increase in the peak *trans*-retinoic acid concentration, and a 4-fold increase in the *trans*-retinoic acid AUC, to 401 ng/mL/hr. The value for the *trans*-retinoic acid AUC with fluconazole was also 45% higher than that measured on day 1.

Similar observations were noted for the second patient analyzed (Fig. 1 and Table 1). Plasma levels of *trans*-retinoic acid were lower in this individual than in patient 1; the decline on day 8 of continued retinoic acid therapy was approximately 60%, and fluconazole given on day 13 nearly completely reversed the decline in plasma retinoic acid levels (94% of day 1). Compared with the peak plasma and AUC values measured on day 8, those on day 13 with fluconazole were 95 and 127% higher, respectively. A second set of samples during the co-administration of *trans*-retinoic acid and fluconazole was obtained on day 14, and the pharmacokinetic parameters observed for *trans*-retinoic acid were also elevated compared with day 8, although they were not as high as seen on day 13.

To determine if the fluconazole effect was related to the modulation of *trans*-retinoic acid metabolism, the oxidative metabolism of [^{3}H]*trans*-retinoic acid was measured *in vitro* using isolated human hepatic microsomes. [^{3}H]*trans*-retinoic acid and its metabolites were separated by HPLC, and a microsome- and

* Abbreviation: 13(*S*)HpODE, 13(*S*)-hydroperoxy-(9Z,11E)-octadecadienoic acid.

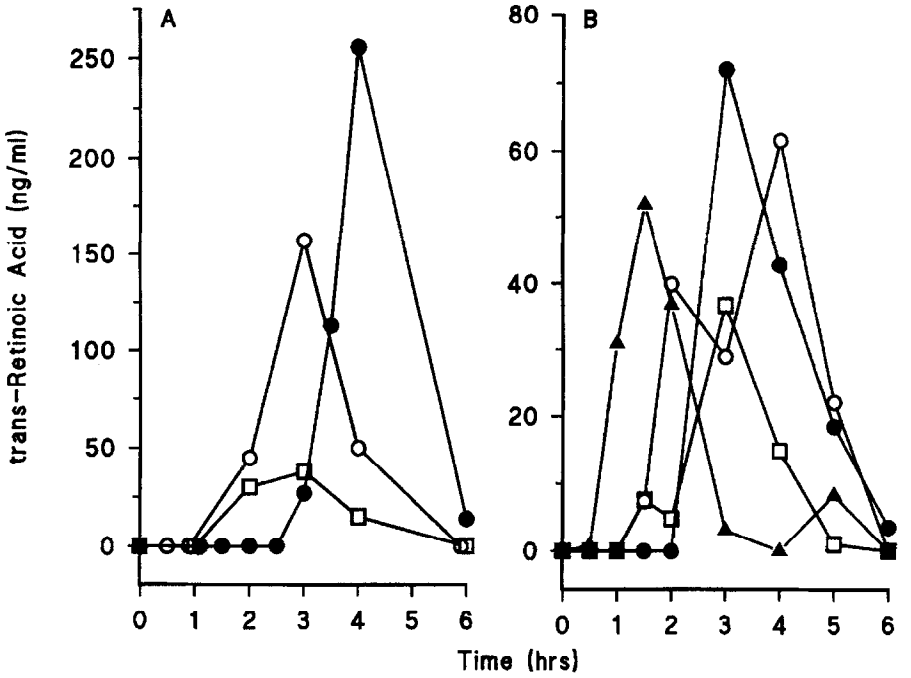

Fig. 1. Effect of fluconazole on the plasma disposition of all-*trans*-retinoic acid in two patients with acute promyelocytic leukemia. Two patients with acute promyelocytic leukemia were treated with daily oral *trans*-retinoic acid (22.5 mg/m^2 twice a day). Blood samples were collected in foil-lined tubes at the indicated times after *trans*-retinoic acid administration, and *trans*-retinoic acid levels were measured by HPLC as described in Materials and Methods. On day 12, the patients were started on oral fluconazole (400 mg loading dose followed by a 200 mg daily dose), which was administered 1 hr prior to the daily dose of *trans*-retinoic acid. Samples were analyzed on day 1 (○) (the first dose of *trans*-retinoic acid); on day 8 of daily oral *trans*-retinoic acid dosing (□); and on days 13 and 14 of *trans*-retinoic acid administration, corresponding to the second (●) and third (▲) daily doses of fluconazole. Panel A: patient 1; Panel B: patient 2.

NADPH-dependent peak was identified and found to co-elute with authentic 4-oxo-*trans*-retinoic acid (Fig. 2). The identification of this peak as 4-oxo-*trans*-retinoic acid was not unequivocal, however, as other oxidative metabolites of *trans*-retinoic acid (e.g., 4-hydroxy-*trans*-retinoic acid, 4-oxo-*cis*-retinoic acid, as well as possibly other compounds) have similar elution times [11]. Formation of the metabolite was also found to be linear with respect to both time and microsomal concentration (data not shown). When fluconazole was included in the *in vitro* reaction, there was a concentration-dependent inhibition of [^{3}H]*trans*-retinoic acid metabolism observed, with a 50% reduction at a concentration of approximately 10 μM fluconazole and 90% inhibition at 36 μM (Fig. 3). The effect of fluconazole was then compared with that of ketoconazole, a related antimycotic drug previously shown to inhibit *trans*-retinoic acid metabolism [4, 5, 13, 14]. Ketoconazole was more potent than fluconazole, inhibiting *trans*-retinoic acid metabolism by 50 and 90% at concentrations of <5 and 17 μM, respectively (Fig. 3). Lipid hydroperoxide-mediated metabolism of *trans*-retinoic acid was 10-fold higher in the microsomes when compared with NADPH-dependent metabolism; however, there was less than 20% inhibition of 13(*S*)-HpODE-dependent metabolism of tRA by the antifungal agents, even at concentrations of 80 μM (Fig. 3).

To relate the inhibitory effect of fluconazole observed *in vitro* with potential actions *in vivo*, the plasma levels of fluconazole were measured in the acute promyelocytic leukemia patients. The concentrations of fluconazole reached peaks of 30 and 80 μM in patients 1 and 2, respectively, at approximately 3–4 hr after the oral dose of fluconazole (2–3 hr after the administration of the *trans*-retinoic acid) (Fig. 4). The decline in plasma levels was relatively slow, such that the concentration remained above approximately 15 and 40 μM in patients 1 and 2, respectively, for at least 7 hr. Both the concentrations of fluconazole and the rate of elimination observed in this patient were consistent with those reported previously [12]. Since *trans*-retinoic acid is generally completely eliminated by 6–8 hr, it is clear that the therapeutic concentrations of fluconazole that are generally achieved would be sufficient to inhibit *trans*-retinoic acid metabolism by 60% or greater during the period that *trans*-retinoic acid is present in the systemic circulation. Although they were higher in the presence of fluconazole, the serum levels of *trans*-retinoic acid did eventually decline despite the continued presence of fluconazole in the plasma; this suggests that fluconazole did not inhibit *trans*-retinoic acid metabolism completely.

DISCUSSION

The retinoids are playing an increasingly important role in the treatment of malignancies. Initial enthusiasm over the high rate of complete responses to *trans*-retinoic acid in acute promyelocytic leukemia patients has been tempered by the observation that the duration of remis-

Table 1. Effect of fluconazole on *trans*-retinoic acid disposition in patients with acute promyelocytic leukemia

Day	Patient 1	Patient 2
	Peak plasma retinoic acid concn (ng/mL)	
1	157 (100%)	62 (100%)
8	38 (24%)	37 (60%)
13*	256 (163%)	72 (116%)
14†	ND‡	52 (84%)
	Retinoic acid AUC (ng/mL/hr)	
1	277 (100%)	144 (100%)
8	91 (33%)	60 (41%)
13*	401 (145%)	136 (94%)
14†	ND	82 (57%)

Two patients with acute promyelocytic leukemia were treated with daily oral *trans*-retinoic acid (22.5 mg/m^2 twice a day). Blood samples were collected, and *trans*-retinoic acid levels were determined as described in the legend to Fig. 1 and in Materials and Methods. On day 12, the patients were started on oral fluconazole (400 mg loading dose followed by a 200 mg daily dose), which was administered 1 hr prior to the daily dose of *trans*-retinoic acid. Samples were analyzed on day 1 (the first dose of *trans*-retinoic acid); on day 8 of daily oral *trans*-retinoic acid dosing; and on days 13 and 14 of *trans*-retinoic acid administration, corresponding to the second and third daily doses of fluconazole. The area under the *trans*-retinoic acid concentration vs time curve (AUC) was determined by the trapezoid method.

* Second dose of fluconazole.

† Third dose of fluconazole.

‡ ND, not determined.

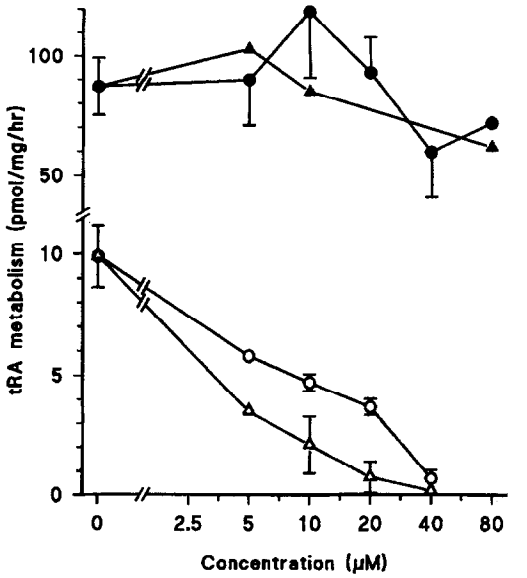

Fig. 3. Effects of fluconazole and ketoconazole on the metabolism of [^{3}H]*trans*-retinoic acid (tRA) to more polar metabolites by isolated human microsomes *in vitro*. NADPH-dependent (○, △) or 13(*S*)-HpODE-dependent (●, ▲) *trans*-retinoic acid metabolism was measured as described in the legend to Fig. 2 in the presence of the indicated concentrations of fluconazole (○, ●) or ketoconazole (△, ▲); enzyme activity is expressed as picomoles of [^{3}H]*trans*-retinoic acid metabolite formed per milligram of protein per hour. Data are means ± SEM from three experiments.

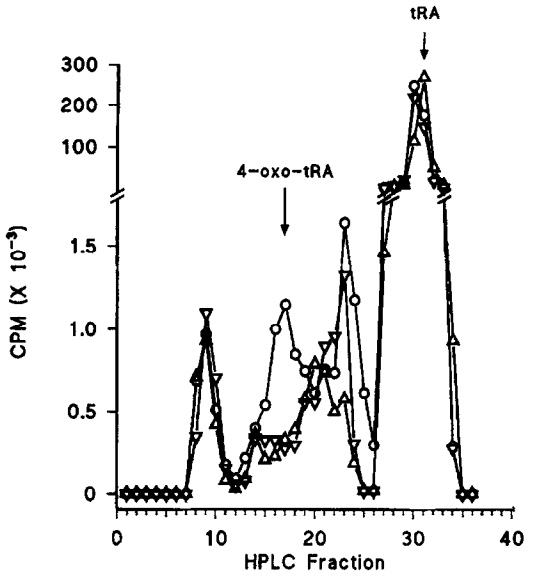

Fig. 2. Metabolism of [^{3}H]*trans*-retinoic acid (tRA) to more polar metabolites by isolated human microsomes *in vitro*. Human hepatic microsomes (100,000 *g* pellet) were incubated in mixtures containing 0.1 M KH_2PO_4 (pH 7.4), 200 μg of microsomal protein, 1 μM [11,12-^{3}H(N)]*trans*-retinoic acid (4 Ci/mmol), and 2 mM NADPH. Reactions were incubated for 10 min at 37° and were terminated with the addition of butanol: acetonitrile (50:50). The extraction and HPLC analysis of [^{3}H]*trans*-retinoic acid and its metabolites were as described in Materials and Methods; 0.5-min fractions of the eluant were collected and the radioactivity was determined. The locations of authentic *trans*-retinoic acid and 4-oxo-*trans*-retinoic acid are shown. Key: (○) complete reaction; (△) reaction in the absence of microsomes; and (▽) reaction in the absence of NADPH.

sions is short in most patients [15–17]. Clinical relapses are characterized by resistance to further *trans*-retinoic acid therapy, despite the fact that leukemic bone marrow blast cells from some relapsed patients remain sensitive to the differentiation-inducing activity of *trans*-retinoic acid [2, 18, 19]. Recent studies indicate that chronic *trans*-retinoic acid therapy leads to substantially diminished plasma levels of *trans*-retinoic acid, most likely due to induction of enzymes responsible for *trans*-retinoic acid catabolism [1–4]. This phenomenon occurs rapidly, within days of beginning daily oral *trans*-retinoic acid therapy, and can result in essentially undetectable levels of *trans*-retinoic acid in the plasma after drug administration. Furthermore, doubling the *trans*-retinoic acid dose has been reported to be ineffective as a means of elevating the plasma *trans*-retinoic acid levels [2], although in some patients higher doses of *trans*-retinoic acid did re-induce clinical remissions [18].

In contrast to 13-*cis*-retinoic acid, the elimination of *trans*-retinoic acid in humans is rapid, with a half-life of less than 1 hr [1]. The only metabolite of *trans*-retinoic acid found in the plasma of humans on *trans*-retinoic acid therapy has been identified as 4-oxo-*trans*-retinoic acid, and 4-oxo-*trans*-retinoic acid-glucuronide has been identified as the primary urinary metabolite [1, 9, 10]. The plasma levels of 4-oxo-*trans*-retinoic acid did not increase under conditions in which *trans*-retinoic acid elimination was accelerated but the urinary excretion of 4-oxo-*trans*-retinoic acid-glucuronide was elevated 10-fold, suggesting that the oxidative reaction is the rate-limiting step in *trans*-retinoic acid metabolism [1]. Our *in vitro* studies measured the NADPH-dependent and the lipid hydroperoxide-mediated conversion of *trans*-retinoic acid to a more polar metabolite(s), including 4-oxo-

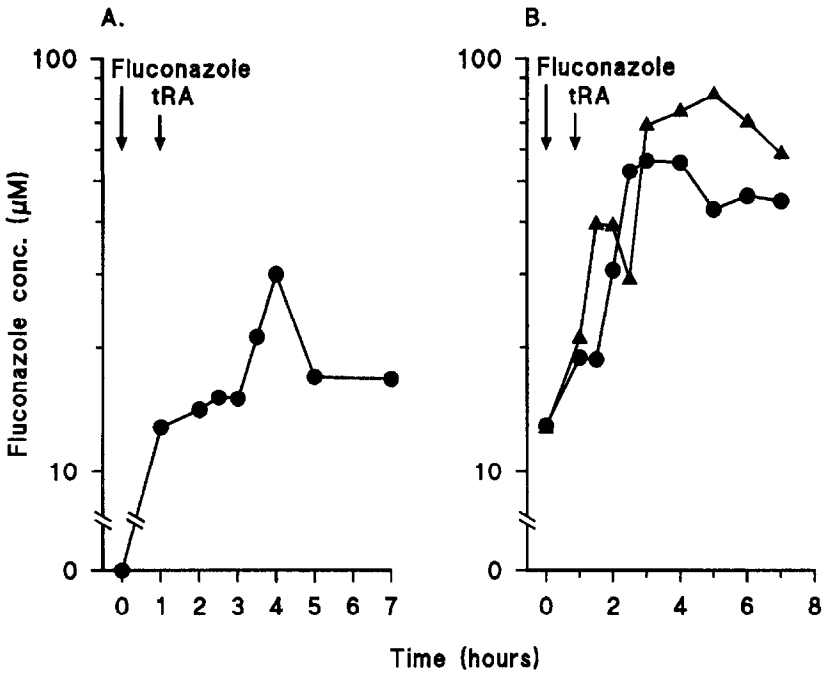

Fig. 4. Plasma levels of fluconazole in two patients with acute promyelocytic leukemia receiving *trans*-retinoic acid (tRA). Plasma levels of fluconazole were measured by HPLC, as described in Materials and Methods, at the indicated times after an oral dose of 200 mg. Sampling was done on day 13 (●) in patient 1 (panel A), and day 13 (●) and day 14 (▲) of daily oral *trans*-retinoic acid therapy in patient 2 (panel B), as described in the legend to Fig. 1. Fluconazole was administered 1 hr prior to the *trans*-retinoic acid, as indicated by the arrows.

trans-retinoic acid, using human microsomes, as a means of assessing the basis of the fluconazole effect observed *in vivo*.

Specific rodent, rabbit and human isoforms of cytochrome P450 that metabolize *trans*-retinoic acid have been identified [7, 20–22]. The activity of these isozymes was induced by chronic treatment with *trans*-retinoic acid and other P450 inducers, such as phenobarbital, in rodent and rabbit models [7, 21]. The induction with chronic *trans*-retinoic acid treatment appears to induce a selective elevation of those P450 activities involved in *trans*-retinoic acid metabolism [7]. Chronic *trans*-retinoic acid therapy has also been reported to induce the levels of cellular retinoic acid-binding proteins, cytoplasmic proteins that may selectively facilitate or restrict the biotransformation of *trans*-retinoic acid and its metabolites [8, 23–25]. The metabolism of *trans*-retinoic acid has been shown to be inhibited by the antifungal agents ketoconazole, clotrimazole, miconazole and liarozole, which act by inhibiting cytochrome P450-dependent enzymes [4, 5, 13, 14, 26, 27]. This further supports the role of oxidation of *trans*-retinoic acid as the primary metabolic pathway. Clinical trials have demonstrated that a single dose of ketoconazole and liarozole can completely or partially reverse the decline in *trans*-retinoic acid plasma levels that occurs with continued retinoid treatment, and our studies demonstrate a similar effect for fluconazole. In contrast to the acute effect of these modulating agents, repeated administration of ketoconazole for 2 weeks failed to maintain plasma *trans*-retinoic acid levels [4, 28, 29]; additional studies will be required to determine if the decline in plasma *trans*-retinoic acid that we observed with the third dose of fluconazole in one patient reflects a similar effect for that agent.

Oxidative metabolism of *trans*-retinoic acid can also occur via the P450 system utilizing lipid hydroperoxides through the "peroxide shunt" [22]. In agreement with an earlier report, substantially higher activity was observed in human microsomes with 13(*S*)-HpODE in place of NADPH. In contrast to the NADPH-mediated biotransformation, however, neither ketoconazole nor fluconazole inhibited the lipid hydroperoxide-dependent formation of *trans*-retinoic acid metabolites from *trans*-retinoic acid. It is unlikely, therefore, that this mechanism contributes to the observed *in vivo* effects of fluconazole and ketoconazole.

Fluconazole is a recently approved triazole antifungal drug that has a broad spectrum of systemic activity, including the treatment of mucocutaneous candidiasis and cryptococcal meningitis. It is less potent an inhibitor of cytochrome P450 monooxygenase activities in human microsomes than ketoconazole [27]. Evaluation of its pharmacologic profile, including pharmacokinetic disposition, side-effects and toxicities, suggests that it may be clinically superior to older imidazole antifungal agents [30]. Our demonstration of the capacity of fluconazole to reverse the self-induced acceleration of *trans*-retinoic acid metabolism provides a rationale for its further evaluation in combination with *trans*-retinoic acid in acute promyelocytic leukemia. Such studies would be of value in addressing the question of the role of altered *trans*-retinoic acid metabolism in the nearly universal development of clinical resistance to *trans*-retinoic acid in leukemia patients.

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