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ORIGINAL ARTICLE

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All-*trans*-retinoic acid modulation of drug-metabolizing enzyme activities: investigation with selective metabolic drug probes

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Abstract *Purpose*: All-*trans*-retinoic acid (ATRA) is a retinoid analogue that has been shown to be effective in acute promyelocytic leukemia. It is currently being investigated for efficacy in the treatment and prevention of various types of cancer. One of the factors limiting its use is the observed increase in ATRA clearance and elimination which occurs shortly after treatment is started, leading to reduced levels of drug in the body and loss of effectiveness. ATRA efficacy may be enhanced if this autoinduction of metabolism can be overcome, for example through the inhibition of the activity of the induced specific metabolizing enzyme(s). This requires the identification of this induced enzyme(s) and development of approaches to selectively inhibit its activity. *Methods*: In the course of a phase II evaluation of ATRA in prostate cancer, we investigated the activities of five specific cytochrome P450 (CYP) (CYPs 1A2, 2C19, 2D6, 2E1 and 3A4) and N-acetyltransferase enzymes using a newly developed five-drug cocktail involving caffeine, mephenytoin, debrisoquine, chlorzoxazone and dapsone respectively. Enzyme activities were assessed in 17 patients with hormone-refractory prostate cancer before the initiation of ATRA therapy, after 14 days of continuous ATRA administration and 7 days after cessation of drug therapy. *Results*: After 14 days of ATRA therapy, the activities of CYP2E1 (chlorzoxazone hydroxylase) and N-acetyltransferase (in fast acetylators only) were increased by 83% and 29% ($P < 0.05$), respectively. Both activities returned to baseline by 7 days after cessation of therapy and the profiles were similar to the changes seen in the clearance of ATRA itself. There were no changes in the activities of any of the other enzymes investigated. *Conclusion*: This study shows that ATRA selectively modulates the activities of specific metabolizing enzymes and that this approach may be useful in identifying enzymes that can be explored in an attempt to mitigate ATRA autoinduction through selective modulation of enzyme activities. Further investigations are required to determine whether the elevated enzymes are also responsible for the increased clearance and elimination of ATRA.

Key words All-*trans*-retinoic acid · Drug metabolism · Selective modulation · Enzyme activities · Selective drug probes

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Introduction

All-*trans*-retinoic acid (ATRA) is a retinoid analogue [29] which is effective in vitro against several tumor cell lines [3, 6, 18]. ATRA has shown substantial activity in inducing remission in acute promyelocytic leukemia

(APL), a unique subtype of leukemia characterized by a translocation resulting in fusion of the *RARα* gene on chromosome 17 with the *PML* gene on chromosome 15 [4, 5, 14, 38]. We have reported a single case in which ATRA induced a partial response in a man with advanced hormone-refractory prostate cancer [32]. ATRA is currently being evaluated in more detail in phase I and phase II studies for efficacy in both the treatment and prevention of malignancies.

While ATRA is effective in inducing remission in APL, recurrence is often seen and the results of phase II trials in other cancers have been disappointing [17, 22, 23, 33]. Administration of ATRA is associated with the rapid enhancement of its own clearance which leads to a striking decrease in plasma levels and tissue exposure for a given dose [1, 22, 23, 30, 33]. It has been suggested that recurrence of APL, despite continued ATRA therapy, is a consequence of enhanced clearance and reduced ATRA exposure [22, 23]. Some groups have reported that the baseline clearance of ATRA is considerably greater among individuals with lung, prostate and head and neck cancers than that seen among patients with APL [26]. The lack or loss of effectiveness of ATRA in human phase II trials of different cancers may, therefore, reflect inadequate or insufficient drug delivery. This suggests that it may be possible to improve ATRA efficacy by reducing its enhanced metabolism to increase drug delivery.

The mechanism underlying the enhanced clearance of ATRA is not clearly understood; evidence suggests that it is a result, at least in part, of induction of the activity of metabolizing enzymes responsible for ATRA metabolism and elimination [22, 23, 33]. Though the specific enzymes involved are not known, possible candidates are the cytochrome P450 (CYP) group of enzymes [19, 27, 28, 34–36, 41, 42]. This is supported by reports that the use of known inhibitors of these metabolizing enzymes are able to restore levels of ATRA to pre-chronic dosing levels [26].

These results suggest that an approach that can be used to increase levels of ATRA in the body is to block or inhibit the activity of the particular enzyme(s) whose activity is elevated with chronic ATRA administration. The strategy is to prevent or reduce the level of induction of ATRA metabolism and elimination in order to maintain sustained high levels at a constant dose. The achievement of this objective requires that the specific metabolizing enzyme(s) involved be identified so that selective rather than general inhibition of enzyme activity can be used. This information can be obtained by evaluating the activities of several metabolizing enzymes before and after several days of ATRA therapy to identify those whose activities are induced and thus may be implicated in ATRA metabolism. To this end, we have serially evaluated the activity of six drug-metabolizing enzymes during the course of a phase II trial of ATRA in patients with hormone-refractory prostate cancer [33]. This study utilized a "cocktail" approach that simultaneously assesses the activities of multiple specific drug-metabolizing enzymes (five CYPs and N-acetyltransferase, NAT) [8]. The goal was to obtain information, from the cocktail study, to reveal enzymes whose activities are elevated after 14 days of ATRA therapy. These enzymes may then be the focus of novel approaches to optimize the use of ATRA through an inhibitory modulation of their activities. The activities were assessed before and after chronic ATRA as well as 1 week after the end of therapy. We report here the effect of ATRA on the activities of these specific enzymes.

Materials and methods

The selection, description and characteristics of the patient population have previously been reported [33]. Briefly, 17 patients with recurrent, histologically diagnosed adenocarcinoma of the prostate and manifestations of progressive disease despite initial endocrine therapy, were enrolled in the study. Patients were required to have adequate hepatic function (bilirubin ≤ 2 mg/dl, SGOT/SGPT more than four times normal), renal (creatinine ≤ 2 mg/dl) and bone marrow function (WBC $\geq 3500/mm^3$, platelets $> 100,000/mm^3$). They were treated for 14 days with oral ATRA (50 mg/m^2, three times daily) supplied as opaque gelatin 10 mg capsules by the Investigational Drug Branch of the National Cancer Institute. Therapy was interrupted for 7 days and treatment resumed on day 21 giving a 14 days on/7 days off dosing schedule. The pharmacokinetics of ATRA were assessed with the first oral dose, after 14 days of therapy and again on day 21, 7 days after cessation of therapy [33].

The activities of five specific CYPs and NAT were investigated with the cocktail of five drugs shown in Table 1. The enzymes were chosen based on the availability of simple methods for assessment of their activities in vivo and to cover a broad range of activities of enzymes that are known to be important in drug metabolism in humans. Though their specific involvement in ATRA metabolism had not been clearly demonstrated, the fact that, together, their activities represented the metabolism of more than 90% of drugs in therapeutic use, offered a good chance of identifying enzyme(s) whose activities were altered. The activities were measured on three occasions: before the first dose of ATRA, after 14 days of daily treatment with ATRA and 7 days after cessation of ATRA therapy. The enzymes whose activities were measured and the drug probes used are shown in Table 1. Each of the drugs has been shown to be selectively metabolized by the respective enzymes and phenotypic methods and measures have been developed to assess the activities of the enzymes in vivo. The drugs were given by simultaneous oral administrations of single low doses. This approach has previously been validated for its ability to independently measure the activities of the respective enzymes [8]. Blood samples (30 ml) were obtained before and at 4 and 8 h after the drug cocktail administration. Voided urine was collected for 8 h into a container containing 1 g of ascorbic acid as preservative for dapsone N-hydroxylamine following cocktail administration. The volume was measured and an aliquot was saved; plasma and urine samples were stored at −30 °C until analysis.

Drugs and metabolites in plasma and urine were quantified by their respective specific methods: high-performance liquid chromatography for the determination of caffeine and paraxanthine [9], 4′-hydroxymephenytoin [39], debrisoquine and 4-hydroxydebrisoquine [7], chlorzoxazone and 6-hydroxychlorzoxazone [31], dapsone and N-hydroxydapsone in urine and dapsone and monoacetyldapsone in plasma [20]; and chiral gas chromatography for the determination of enantiomers of mephenytoin [40]. Appropriate controls determined that none of these determinations was affected by ATRA. The respective phenotypic measures, which reflect enzyme activity (Table 1), were calculated from the quantified drugs and metabolites and used to determine the effects of ATRA on the activities of specific enzymes. The data were

Table 1 Drug probes and phenotypic measures used to estimate the activities of specific drug-metabolizing enzymes

Enzyme	Drug (dose)	Phenotypic measure [reference]
CYP1A2	Caffeine (100 mg)	Paraxanthine/caffeine 8-h plasma ratio [10]
CYP2C19	Racemic mephenytoin (100 mg)	*R*/*S* urinary enantiomeric ratio 0–8-h urinary recovery of 4-hydroxymephenytoin [39]
CYP2D6	Debrisoquine (10 mg)	Debrisoquine recovery ratio (DBRR)[a] $= \frac{\text{OHDB}}{\text{OHDB} + \text{DB}}$ [15]
CYP2E1	Chlorzoxazone (250 mg)	6-Hydroxychlorzoxazone/chlorzoxazone 4-h plasma ratio (Frye et al., unpublished observation)
CYP3A4	Dapsone (100 mg)	Dapsone recovery ratio (DPRR)[a] $= \frac{\text{OHDDS}}{\text{OHDDS} + \text{DDS}}$ [21]
N-Acetyltransferase	Dapsone (100 mg)	Monoacetyldapsone/dapsone 8-h plasma ratio [20]

[a] Where OHDB, DB, OHDDS and DDS represent the 0–8 h urinary recoveries of 4-hydroxydebrisoquine, debrisoquine, N-hydroxydapsone and dapsone, respectively

compared by nonparametric ANOVA via the Mack-Skillings test and with the level of significance set at $P \leq 0.05$.

Genotypic classification of each subject was determined for *CYP2D6*, *CYP2E1* and NAT (*NAT2**) by restriction fragment length polymorphism (RFLP) and allele-specific polymerase chain reaction (PCR)-based methods. Briefly, DNA was extracted from peripheral blood mononuclear cells (PBMC), which were separated at the time of collection and stored at −80 °C, by incubating 2–5 µl of cells with 20 µl Gene Releaser (Bioventure, Murfreesboro, Tenn.) according to the manufacturer's instructions, followed by amplification. For *CYP2D6* and *CYP2E1* genotyping, PCR amplification was followed by product digestion with appropriate restriction endonucleases. *Pal*I was used to screen for the *CYP2D6* variant B genotype as described by Gough et al. [12]. With PBMC DNA isolated by the Gene Releaser protocol, a 410-bp portion of the *CYP2E1* polymorphic site yielded fragments of 120 and 290 bp following digestion with *Pst*I which were resolved by polyacrylamide gel electrophoresis [16]. The allele-specific PCR approach for *NAT2** genotyping identified several allelic variants [37]. Specific primers for the wildtype (NAT2*4) and mutant allele M1 which recognize both NAT2*5A, NAT2*5B, M2 which recognizes both NAT2*6A, NAT2*6B, and M3 which recognizes both NAT2*7A and NAT2*7B were used in separate PCR reactions as described [2] using gene-released genomic DNA from PBMC. The presence or absence of a band for each mutant allele was determined by polyacrylamide gel electrophoresis.

Results

We have previously reported that, in this patient group with hormone-refractory prostate cancer, ATRA has a high oral clearance with wide intersubject variation at baseline [33]. Following 2 weeks of therapy, ATRA pharmacokinetics changed in a pattern consistent with an induction of its metabolism resulting in increased apparent oral clearance (Fig. 1), reduced maximum plasma concentration, reduced area under the plasma concentration-time curve, and prolonged time to achieve the maximum concentration [33].

Evaluation of the phenotypic measures of all enzymes assessed revealed differences in their response to ATRA

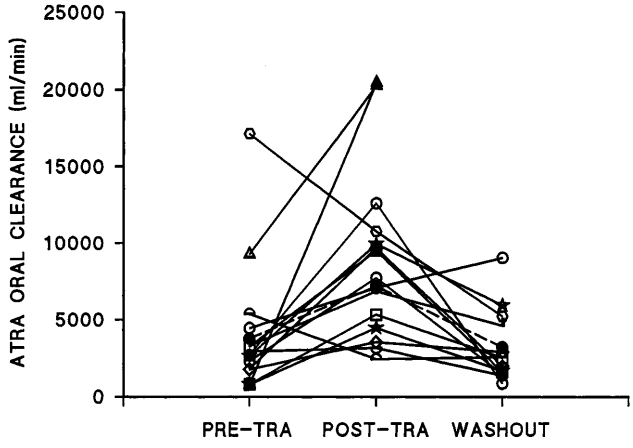

Fig. 1 Oral clearance of ATRA before and after chronic treatment with ATRA for 14 days and also after a washout interval of 7 days (n = 13–17, ●–● mean of data)

administration. The activity of CYP2E1, as measured by the ratio of 6-hydroxychlorzoxazone to chlorzoxazone in 4-h plasma samples, exhibited wide intersubject variation at baseline. After 14 days of ATRA treatment, this ratio still exhibited wide intersubject variation but the value had increased substantially (83%) from baseline (pre-ATRA; $P < 0.05$; Fig. 2). This change in ratio suggests more efficient conversion of parent drug to metabolite after chronic ATRA treatment than before treatment. The ratio returned to baseline by 7 days after discontinuation of ATRA therapy (Fig. 2). There was a significant positive relationship between the induced apparent oral clearance of ATRA and the induced ratio of 6-hydroxychlorzoxazone to chlorzoxazone (day 14 measures; $P < 0.05$; Fig. 3). However, there was no correlation between their respective baseline measures, nor the absolute or percentage changes in these measures (data not shown).

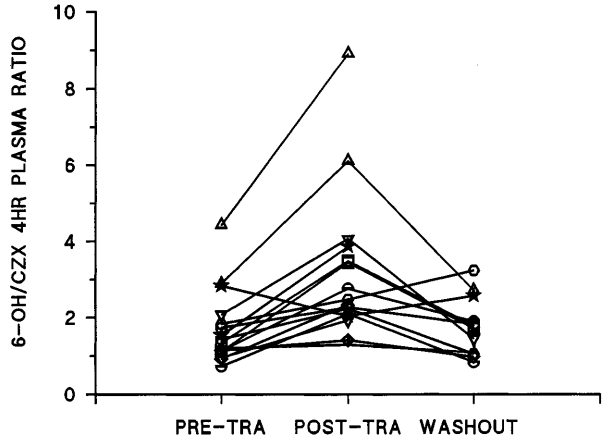

Fig. 2 4-Hour plasma ratio of 6-hydroxychlorzoxazone to chlorzoxazone before and after chronic treatment for 14 days with ATRA and after a washout interval of 7 days (n = 12–17)

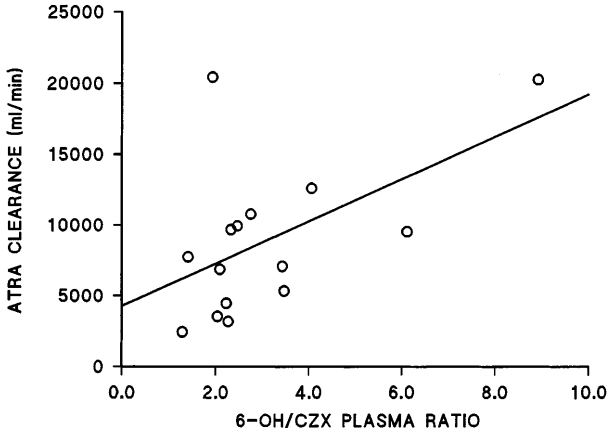

Fig. 3 Relationship between the clearance of ATRA and 4-hour plasma ratio of 6-hydroxychlorzoxazone to chlorzoxazone after 14 days of chronic treatment with ATRA in patients with hormone-refractory prostate cancer (n = 15, r = 0.54, $P < 0.05$)

The NAT activity as measured by the 8-h plasma ratio of monoacetyldapsone to dapsone also changed during ATRA therapy. However, this was dependent on the individual acetyltransferase phenotype. In fast acetylators (n = 6), the acetylation ratio was increased (29%) after 14 days of ATRA treatment ($P < 0.05$). In a profile similar to that seen with changes in ATRA clearance (Fig. 1), the increased ratio returned towards baseline after 7 days off therapy (Fig. 4). There was no

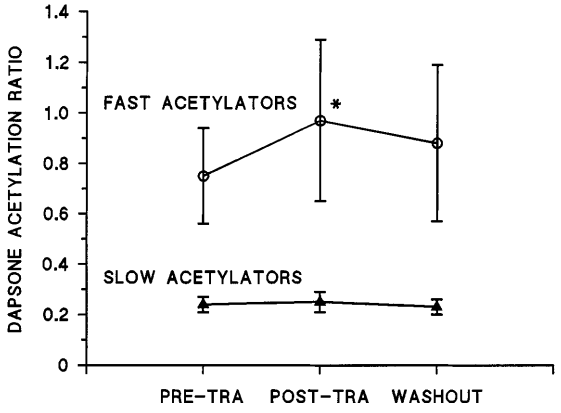

Fig. 4 Dapsone acetylation ratio (mean ± SD) in fast (n = 6) and slow (n = 11) acetylators before and after 14 days of chronic treatment with ATRA and after a 7-day washout (*$P < 0.05$)

correlation between the baseline and induced values of these measures nor between the baseline values and absolute or percentage changes. In slow acetylators, there was no change in the acetylation ratio measured before therapy compared to after 14 days of ATRA therapy or after 7 days cessation of therapy (Fig. 4).

Assessment of the activities of the other specific enzymes (CYP1A2, CYP2C19, CYP2D6 and CYP3A4) revealed no changes associated with ATRA therapy; the measures before, after 14 days of ATRA and following a 7-day hiatus in ATRA therapy, were comparable and within the expected ranges for normal subjects (Table 2). For CYP2C19, two subjects were phenotypically poor metabolizers but the effect of ATRA in these subjects was not different from the others.

Genotyping analysis (Table 3) revealed that there were no CYP2D6 poor metabolizers among the subjects and this was in agreement with the phenotypic classification. One subject was found to have two copies of the *Pst*I variant alleles of *CYP2E1* but this appeared to be of no functional significance as the phenotypic measure in this subject was comparable to all the other subjects (baseline 4-h ratio of metabolite to parent drug of 1.43 compared with a mean of 1.73 ± 0.91 for all subjects). Five subjects had at least one NAT2* mutant allele and the predicted slow acetylator status agreed with the phenotypic classification. All other subjects were carriers of the wildtype or heterozygous genotype. Overall, there was more than 80% concordance between the genotypic and phenotypic classifications.

Table 2 Phenotypic measures of activities of specific metabolizing enzymes before and after 14 days chronic treatment with ATRA and after a 7-day washout (values are means ± SD)

Phenotypic measure	Pre-ATRA	After ATRA	Washout
Mephenytoin *R*:*S* ratio	4.36 ± 4.51	4.87 ± 7.95	5.09 ± 4.34
4-Hydroxymephenytoin urinary excretion (μmol)	75.7 ± 46.9	83.7 ± 45.5	85.4 ± 40.1
Dapsone recovery ratio	0.53 ± 0.12	0.53 ± 0.12	0.55 ± 0.13
Debrisoquine recovery ratio	0.68 ± 0.17	0.68 ± 0.17	0.66 ± 0.18
Paraxanthine/caffeine plasma ratio	0.75 ± 0.61	0.81 ± 0.55	0.76 ± 0.62

Table 3 Genotypic distribution of the 17 patients with hormone-refractory prostate cancer for specific drug-metabolizing enzymes. Values are number of patients (%). WT/HT represents wildtype and heterozygous genotypes, respectively

Enzyme	Fast/extensive metabolizer (normal, WT/HT genotype)	Slow/poor metabolizer (mutant)
CYP2D6	17 (100)	0
CYP2E1[a]	15 (88.2)	1 (5.9)
NAT2*[a]	6 (37.5)	10 (62.5)

[a] Data unavailable for one subject

Discussion

This study examined the activities of six specific drug-metabolizing enzymes before, during and after ATRA therapy to identify whether there were changes in activity which paralleled the increase in ATRA clearance. These enzymes were not chosen because of prior demonstration of their involvement in ATRA metabolism, but rather to represent as wide a range as possible of enzymes for which there are methods available to assess their activities in vivo. The rationale was that this would offer a good chance of identifying enzymes involved in drug metabolism in humans that ATRA modulates. The hope was that this group would also include those involved in its metabolism.

Changes in the activities of two enzymes, CYP2E1 and NAT (in fast acetylators only), were demonstrated. In each instance, the return towards baseline after the cessation of ATRA therapy strongly suggests that the increase associated with ATRA administration was a result of the administration of this drug. The association between the induced chlorzoxazone ratio and ATRA oral clearance while on ATRA therapy, suggests that a similar mechanism may regulate CYP2E1 activity and the enzymes responsible for ATRA clearance but does not indicate whether or not CYP2E1 is involved in ATRA metabolism. The fact that not all drug-metabolizing enzyme activities investigated changed with ATRA therapy indicates that the effect of ATRA on enzyme activities is selective and not a general effect on all drug-metabolizing activities. The concordance between the phenotype and genotype for many of the enzymes provides validation for the observed measures and suggests that they are not being unduly influenced by environmental factors. It also helps to validate the changes that were observed after ATRA therapy as being mainly a result of the treatment intervention and its effect on basal metabolizing activity.

The mechanism involved in the elevation of the activity of CYP2E1 and NAT in this study is unclear. CYP2E1 is the alcohol-inducible form of CYP that has been implicated in the metabolism of many small molecular weight, carcinogenic compounds [13]. The characteristics of known substrates for CYP2E1 are different from those of ATRA and on the basis of structural characteristics alone, it would not be anticipated that ATRA would be metabolized by CYP2E1. A previous study has shown that metabolism of ATRA is ethanol-inducible in the rat [28], a finding consistent with a role for CYP2E1 in ATRA metabolism. The correlation between ATRA clearance and CYP2E1 phenotypic measure estimated after 14 days of ATRA therapy (Fig. 3) lends support to the suggestion of a role for CYP2E1 in ATRA metabolism. The lack of a correlation between the baseline values of these measures may be an indication that even if CYP2E1 is involved, it is not the only enzyme involved. The extent of its contribution to overall metabolism is unclear and may vary between baseline and induced state. Consistent with this suggestion is the observation that several enzymes are capable of metabolizing ATRA in in vitro studies using cells enriched in specific CYP enzymes including CYP1A1, CYP1A2, CYP2B6, CYP2D6, CYP2E1 and CYP3A4 [24]. Their relative contributions to the overall metabolism of ATRA in vivo are, however, also unknown.

The effect of ATRA on NAT is an unexpected finding. In humans, NAT exists in two different forms: NAT1 and NAT2. It has been previously suggested that NAT2 is the polymorphic form but recent evidence indicates that NAT1 also may be polymorphic [37]. The presence or absence of mutant genes results in slow and fast acetylator phenotypes, respectively. Thus, the fact that the enhancing effect of chronic ATRA therapy was exhibited only in fast acetylators suggests that ATRA affects only the isozyme form that is expressed in fast but not slow acetylators. This is contrary to a previous report that showed elevated acetylation of dapsone in both fast and slow acetylators after various chemotherapeutic regimens [25]. Though it was clearly demonstrated that change in NAT activity was caused by ATRA and that the pattern of change was similar to that in ATRA clearance, structural considerations suggest that it is unlikely that NAT is involved in ATRA metabolism. The mechanism of this interaction is unclear but may be related to the cooperativity between peroxisome proliferator activated receptor (PPAR) and retinoic acid receptor (RXR) that has been shown to activate the acylCoA gene [11]. The effect could thus be unrelated to the metabolism of ATRA itself. For this to be the operating mechanism, however, the availability of acetyl-CoA has to rate limit the extent of acetylation in fast but not slow acetylators such that the activation of the gene leads to increased expression of the enzyme. The resulting increased metabolic capacity will produce increased metabolism in fast but not slow acetylators and explain the differential effect between the two phenotypes. Alternatively, the sensitivity and response of fast and slow acetylators to the cooperativity between PPAR

and RXR, induced by ATRA, may be different. This observation is particularly important because it implies that potential interactions with substrates of this enzyme may occur only in a subset of individuals. To our knowledge, this report is the first to recognize selective regulation and/or modulation of different isoenzymes of NAT.

In summary, this study has demonstrated the utility of a cocktail approach in a phase II drug trial to investigate an autoinduction phenomenon with a potentially significant influence on the outcome of drug therapy. The approach achieved its objective of characterizing the selectivity of the effect of ATRA on activities of specific metabolizing enzymes. The study has shown that during chronic treatment, ATRA induces the activity of CYP2E1 and NAT (in fast acetylators only) and both activities return towards baseline within 7 days of cessation of therapy. By contrast, the activities of several other enzymes, namely CYP1A2, CYP2C19, CYP2D6 and CYP3A4, were unaffected. Whether the enzymes induced in this study are the ones that are involved in the increased metabolism and elimination of ATRA remains to be proven and this will have to be tested by determining whether selective inhibition of their activities can prevent the limiting autoinduction associated with ATRA therapy. This study provides a rationale for such studies. Also, it illustrates the utility of this cocktail approach as a strategy to study metabolic drug interactions and their selectivities especially, involving enzyme induction for which there is presently no suitable in vitro method.

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