Prevention of Vitamin A Teratogenesis by Phytol or Phytanic Acid
Results from Reduced Metabolism of Retinol to the Teratogenic Metabolite, All-trans-retinoic Acid

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Previous studies in our laboratory showed a synergistic interaction of synthetic ligands selective for the retinoid receptors RAR and RXR in regard to teratogenic effects produced in mice (M. M. Elmazar et al., 2001, Toxicol. Appl. Pharmacol. 170, 2–9). In the present study the influence of phytol and phytanic acid (a RXR-selective ligand) on the teratogenicity of retinol and the RAR-selective ligand all-trans-retinoic acid was investigated by coadministration experiments on day 8.25 of gestation in NMRI mice. Phytol and phytanic acid, noneffective when administered alone, did not potentiate the teratogenicity induced by retinol or all-trans-retinoic acid. On the contrary, phytol and phytanic acid greatly reduced retinol-induced teratogenic effects (ear anotia, tail defects, exencephaly). The effect of phytol on all-trans-retinoic acid teratogenesis was limited (only resorptions and tail defects were reduced). Pharmacokinetic studies in nonpregnant animals revealed that phytol coadministration with retinol reduced plasma levels of retinol and retinyl esters, and drastically reduced the levels of the teratogenic retinol metabolite, all-trans-retinoic acid. Phytanic acid also reduced the oxidative metabolism and teratogenic effects of retinol. These results indicate that phytol and phytanic acid did not synergize with retinol and all-trans-retinoic acid in our mouse teratogenesis model. Instead, phytol and phytanic acid effectively blocked the teratogenic effects of retinol by drastically reducing the metabolic production of all-trans-retinoic acid. Phytol and phytanic acid may be useful for the prevention of vitamin A teratogenicity.

Key Words: vitamin A; retinol; retinoic acid; phytol; phytanic acid; teratogenicity prevention; metabolism; retinoid receptors.

The oxidative metabolite of retinol (vitamin A alcohol), all-trans-retinoic acid, is essential for cell growth and differentiation, reproduction, and embryonic development (Gudas, 1994; Maden, 1994; Nau et al., 1994). Two known families of nuclear receptors are thought to be the key players in mediating the effects of retinoids (Chambon, 1993; Lohnes et al., 1995; Mangelsdorf et al., 1994): the RAR gene family (including the RARα, RARβ, and RARγ subtypes and their isoforms) as well as the RXR gene family (including the RXRα, RXRβ, and RXRγ subtypes and their isoforms). These receptors are part of the steroid/thyroid hormone receptor superfamily and function as ligand-activated transcription factors controlling the expression of a number of responsive genes. The effect of all-trans-retinoic acid is mediated by its binding to and transactivating of the RXR, while another retinoic acid isomer, 9-cis-retinoic acid, may bind and transactivate the RXR as well as the RAR (Allenby et al., 1993; Levin et al., 1992). It is well established that retinoids and their receptors are involved in normal and abnormal embryonic development (Chambon, 1993). Both all-trans-retinoic acid and retinoid receptors are present in embryonic tissues in a specific spatial and temporal distribution (Dolle et al., 1990; Durston et al., 1989; Ruberte et al., 1991, 1993; Scott et al., 1994; Thaller and Eichele, 1987; Yamagata et al., 1994). It was demonstrated that all-trans-retinoic acid (Fig. 1) as well as its natural precursor retinol are teratogenic in a wide variety of species, including mice (Nau et al., 1994). Additionally, blocking the oxidative metabolism of retinol to all-trans-retinoic acid by administration of an alcohol dehydrogenase inhibitor (4-methylpyrazole) significantly lowered the teratogenic response of retinol in mice (Collins et al., 1992).

Studies at the molecular level led to the presumption that heterodimerization of a RAR/ligand complex with an RXR is required for efficient DNA binding and transactivation of target genes responsible for teratogenic effects (Durand et al., 1992; Zhang and Pfahl, 1993). To further dissect the complex pattern of retinoid induced developmental effects, possible roles of specific RAR subtypes were investigated by using synthetic retinoids that specifically bind to and transactivate individual RARs (Elmazar et al., 1996). It was shown that administration of the RARα-selective ligand Am580 to pregnant mice produced the most severe defects, including spina bifida, micrognathia, and tail defects. On the other hand, the RARγ agonist CD437 evoked a different spectrum of malformations, like exencephaly and cleft palate. These results revealed that some
teratogenic effects might be mediated by RARα-RXR heterodimers, while others are a result of the formation of RARγ-RXR heterodimers (Elmazar et al., 1997, 2001). The unselective, natural ligand all-trans-retinoic acid produced tail defects as well as exencephaly when given on day 8 of gestation (Elmazar et al., 1996; Elmazar and Nau, 1998). Although ligand binding to the RXR is not a prerequisite for the formation of RAR-RXR heterodimers, it was recently shown that coadministration of the synthetic RXR agonists AGN191701 or LG1069 to pregnant mice potentiated the teratogenic effects of the selective RARα ligand Am580 (Elmazar et al., 1997; Elmazar and Nau, 1998) as well as the unselective natural ligand all-trans-retinoic acid and its precursor retinol, respectively (Elmazar and Nau, 1998). Single administration of the RXR agonist alone did not produce any teratogenic effect (Elmazar et al., 1997, 2001; Elmazar and Nau, 1998).

Besides 9-cis-retinoic acid, phytanic acid (Fig. 1) was identified as another natural ligand and transactivator of RXRs (LeMotte et al., 1996). This compound is a branched chain fatty acid and an oxidation product of phytol (Fig. 1), which is part of the chlorophyll molecule in fruits and vegetables (Steinberg, 1995). It was demonstrated that the precursor phytol is bioactivated to phytic acid in several species (Hansen et al., 1966; Klenk and Kremer, 1965; Mize et al., 1966; Steinberg et al., 1966; Stoffel and Kahlke, 1965). In particular, phytic acid itself occurs in substantial amounts in adipose tissues of ruminants because chlorophyll is efficiently degraded by ruminal bacteria, and the released phytol is absorbed and subsequently oxidized to phytic acid (Avigan, 1966). Therefore, high amounts of phytic acid can be found in dairy products such as milk and butter. Phytic acid is also present in human blood in μM concentrations (Steinberg, 1995; Verhoeven et al., 1998). Extremely high concentrations (mM levels) can be found in some disease states such as Refsum’s disease or Zellweger syndrome, where dysfunction of phytic acid α-oxidation leads to an accumulation of phytic acid in human blood and tissues (Steinberg, 1995; Verhoeven et al., 1998).

Interestingly, patients with these disorders displayed similar symptoms as described for vitamin A deficiency or hypervitaminosis A, such as retinitis pigmentosa and ichthyosis (Kaufman, 1998; Stützgen, 1982; Van Soest et al., 1999).

Simultaneous administration of phytic acid and Am580 to pregnant mice led to a substantial potentiation of Am580-induced malformations, namely micrognathia (29 to 98%) and tail defects (7 to 98%). Although spina bifida did not occur when phytic acid or Am580 were given alone, coadministration produced 51% of this type of malformation (Elmazar and Nau, 1998).

The present experiment, therefore, was designed to investigate whether the natural RXR ligand phytic acid and its precursor, phytol, would interact with the natural RAR ligand all-trans-retinoic acid or its precursor retinol. To study metabolic interactions of these structurally similar lipophilic compounds, plasma pharmacokinetics in nonpregnant mice were additionally investigated. The bioactivation of retinol to all-trans-retinoic acid and its further metabolism to the phase I metabolites all-trans-4-oxo-retinoic acid and all-trans-4-hydroxy-retinoic acid as well as its phase II metabolite all-trans-RAG (all-trans-retinoyl-β-D-glucuronide) was particularly analyzed.

**MATERIALS AND METHODS**

**Laboratory precautions.** Treatment of the animals, collection of biological samples, and laboratory procedures were performed in dark rooms under dim yellow light to prevent isomerization of the test materials.

**Animals.** Female mice (NMRI: Harlan-Winkelmann, Borchsen, Germany, 29–35 g) were mated between 0600 and 0900 h. The animals with vaginal plugs were separated, and the first 24 h after conception were designated gestational day 0 (GD 0). The animals were allowed food (Altromin, 1324 diet, Lage, Germany) and water *ad libitum* and kept under controlled conditions of room temperature (21 ± 1°C), relative humidity (55 ± 5%), and a 12-h light/dark cycle, with light between 1000 and 2200 h.

**Chemicals.** Phytol (3,7,11,15-tetramethyl-hexadec-2-ene-1-ol), phytic acid (3,7,11,15-tetramethyl-hexadecanoic acid), all-trans-retinoic acid, retinol,
and cremophor EL were purchased from Sigma. 14-Hydroxy-4,14-retinoic acid or phytol EL were purchased from Sigma (Deisenhofen, Germany).

6 Tail defects (%) 0 0 13 (22) 13 (20) 0

Ear anotia (%) 0 0 7 (12) 15 (23) 3 (4) 19 (21) 1 (1, 3)

Spina bifida (%) 0 0 1 (2) 1 (1.5) 1 (1.4) 0 0 0

Note. Teratogenic effects of oral phytanic acid (PA; 100 mg/kg), phytol (POH; 500 mg/kg), all-trans-retinoic acid (RA; 20 mg/kg) or retinol (ROL; 50 mg/kg) alone or in combination with RA or POH to NMRI mice on gestational day 8.25.

a All substances were dissolved in Cremophor EL (25% w/v in distilled water) with a dose volume of 5 ml/kg.

b p < 0.05 compared with RA using Fisher’s exact test.

c p < 0.05 compared with ROH using Fisher’s exact test.

d Significantly higher compared to ROH using Student’s t-test (p < 0.01).

and cremophor EL were purchased from Sigma (Deisenhofen, Germany). Unless otherwise indicated, retinoid standards for high performance liquid chromatography (HPLC) were purchased from Sigma. 14-Hydroxy-4,14-retinol and anhydroretinol were generous gifts from Dr. F. Derguini (Memorial Sloan-Kettering Cancer Center, New York, NY). 4-Oxo- and 4-hydroxyretinoic acid isomers were provided by Hoffmann-La Roche (Basel, Switzerland, and Nutley, NJ). Retinyl esters (except retinyl palmitate) and all-trans-retinoyl-β-D-glucuronide (RAG) were synthesized in our laboratory while standard all-trans-retinol and all-trans-retinyl-β-D-glucuronide (RAG) were synthesized in our laboratory while additional RAG was provided by Drs. A. A. Barua and J. A. Olson (Ames, IA). Methanol and isopropanol were of HPLC gradient-grade and obtained from Roth (Karlsruhe, Germany). Ethanol and ammonium acetate was purchased from Merck (Darmstadt, Germany). β-Glucuronidase from E. coli (EC 3.2.1.31) was obtained from Boehringer Mannheim (Germany).

**Drug administration.** Groups of mice were given a single po administration of either phytic acid (100 mg/kg), phytol (500 mg/kg), all-trans-retinoic acid (20 mg/kg), or retinol (50 mg/kg) by gastric intubation on GD 8.25. For combination experiments, animals were given all-trans-retinoic acid or retinol simultaneously (in the same syringe) with either phytic acid or phytol. Each agent was suspended in 5% cremophor EL in distilled water in concentrations so that each animal was administered 5 ml/kg. For pharmacokinetic investigations nonpregnant mice were treated as described for pregnant animals.

**Fetal examination.** On GD 18, the pregnant animals were sacrificed by cervical dislocation. Implantation sites, resorptions, and live fetuses and resorptions were counted. Live fetuses were weighed individually and examined for external malformations. The results of the combination experiments were compared with the corresponding all-trans-retinoic acid or retinol group using two-tailed unpaired Student’s t-test (fetal weight) or Fisher’s exact test (malformations). All calculations were carried out using GraphPad InStat-2 Software (Jandel Co., San Raffael, CA).

**Pharmacokinetic studies.** All-trans-retinoic acid or retinol was given alone or in combination with either phytic acid or phytol to groups of nonpregnant mice (n = 3 per group and time point). Single blood samples of approximately 100–150 μl were taken in heparinized capillary tubes from the retro-orbital sinus under brief ether anesthesia. Plasma was prepared by centrifugation of the blood for 10 min at 4°C and 1500 x g and stored at −80°C until analysis. Time intervals for blood collection were 0.5, 1, 2, and 4 h after administration of all-trans-retinoic acid or simultaneously with phytic acid or phytol, and 2, 4, 8, and 12 h after administration of retinol alone or simultaneously with phytanic acid or phytol. Blood samples from untreated mice (n = 5) were also taken for determination of endogenous retinoid levels. Maximum concentrations (Cmax) were the observed values, and area under the concentration-time curve (AUC) values were calculated using the linear trapezoidal rule. Mean comparisons of concentration data were done using one-way ANOVA followed by Dunnett post hoc test; p values < 0.05 were considered significant.

**HPLC analysis.** Plasma samples were extracted with a 3-fold volume of isopropanol and further submitted to solid-phase extraction according to a previously described method (Collins et al., 1992). A modification of the HPLC method described by Eckhoff and Nau (1990) was used for determination of plasma retinoids. This method used a linear gradient formed from 57.5% methanol and 42.5% aqueous 60 mM ammonium acetate (initial composition) to 95% methanol and 5% ammonium acetate over 11 min. To also determine retinol and retinyl esters in a single chromatographic run, methanol percentage was increased to 100% at 11.2 min and further maintained at this level until 25 min (Tzimas et al., 1994). The starting conditions of the gradient were reached again at 26 min. Detection was performed by simultaneous monitoring of the UV absorbance of the eluate at 340 and 356 nm using a Shimadzu SPD 10 AV detector (Kyoto, Japan). Due to the limited volume obtained from blood samples, the sample weight was 25 μl instead of 100 μl as described for the original method (Collins et al., 1992). Therefore, the detection limit of retinoids was as follows: 9-cis-retinoic acid, all-trans-4-oxo-retinoic acid and 13-cis-4-oxo-retinoic acid, 2.8 ng/ml; 13-cis-retinoic acid and all-trans-retinoic acid, 2 ng/ml; RAG, 4 ng/ml; 14-HRR, 5 ng/ml; retinyl esters and anhydroretinol, 20 ng/ml. Peak eluates of putative retinoid glucuronides were collected, evaporated to dryness, redissolved in buffer, subjected to hydrolysis by β-glucuronidase, after which the purified retinoids were rechromatographed. The procedure was described in detail by Sass et al. (1994).

**RESULTS**

Teratogenic effects of phytic acid or phytol given alone. A single dose of phytic acid (100 mg/kg) or phytol (500 mg/kg) given orally on GD 8.25 led to resorptions of 20 and 3%, respectively (Table 1). Fetal weights were 1.18 ± 0.13 g (phytanic acid) and 1.26 ± 0.13 g (phytol), respectively. Live
fetuses showed no visible malformations. When compared with the historical control values of 4% resorptions and fetal weight of 1.260.13 g of this strain of mice (Elmazar et al., 1997), the phytanic acid group had a higher resorption rate while the phytol group had a higher fetal weight.

Teratogenic effects of all-trans-retinoic acid alone or coadministered with phytanic acid or phytol. All-trans-retinoic acid, given at 20 mg/kg orally on GD 8.25, produced a high number of resorptions (RS, 44%). External abnormalities of live fetuses included ear anotia (EA, 12%), tail defects (TD, 22%), and exencephaly (EX, 17%). Spina bifida (SB, 2%) occurred only in 1 case, while micrognathia (MG) was not observed at all. Fetal weight was 1.18 ± 0.13 g (Fig. 2).

Coadministration of phytanic acid led to a nonsignificant reduction of resorptions to 35%. External abnormalities (23%) as well as exencephaly (20%) were slightly, but not significantly increased, while the incidence of tail defects remained comparable (20%). Spina bifida and micrognathia were found in 1 case each. Fetal weight (1.17 ± 0.13 g) was unaffected.

Coadministration of phytol decreased the number of resorptions significantly (p < 0.05). External abnormalities (4%) and exencephaly (14%) were slightly, but not significantly reduced. On the other hand, tail defects were not observed in this group. Spina bifida and micrognathia were found in 1 case each. Fetal weight (1.18 ± 0.12 g) was unaffected.

Teratogenic effects of retinol alone or coadministered with phytanic acid or phytol. Administration of retinol (50 mg/kg orally on GD 8.25) resulted in a high number of resorptions (39%). Malformations seen in live fetuses included ear anotia (21%) and exencephaly (28%). Tail defects (1%) occurred in only 1 case, while spina bifida and micrognathia was not observed at all. Fetal weight was 1.23 ± 0.14 g (Fig. 3).

Coadministration of phytanic acid with retinol decreased the number of resorptions significantly to 18% (p < 0.05). Also in this case there were no external malformations, with the exception of 1 case of ear anotia. Fetal weight (1.23 ± 0.10 g) was unaffected.

Coadministration of phytol with retinol led to a highly significant reduction of resorptions to 5% (p < 0.001). There were no external malformations. Fetal weight (1.29 ± 0.13 g) was significantly higher (p < 0.01) compared with the retinol group.

Endogenous retinoids in mouse plasma. Retinol (180.1 ± 26.4 ng/ml), retinyl palmitate/oleate (116.4 ± 41.4 ng/ml) and retinyl stearate (52.1 ± 24.6 ng/ml) were detected in plasma of untreated, nonpregnant mice (n = 5). Additionally, retinyl linoleate (16.8 ± 4.1 ng/ml) was found in 2 plasma samples.

Plasma pharmacokinetics of all-trans-retinoic acid and its metabolites in nonpregnant mice. Figure 4 displays a characteristic chromatogram of a plasma sample taken 1 h after dosing with all-trans-retinoic acid. All-trans-retinoic acid (7), 9-cis-retinoic acid (6), and 13-cis-retinoic acid (5) were identified by coelution with authentic retinoids. Additionally, the phase I metabolites all-trans-4-hydroxy-retinoic acid (2) and all-trans-4-oxo-retinoic acid (1) as well as the phase II metabolites 13-cis-RAG (3) and all-trans-RAG (4) were found (Table 2). The identification of glucuronide metabolites was confirmed by treatment of peak eluates with β-glucuronidase and subsequent detection of the retinoic acid isomers. The occurrence of all-trans-4-hydroxy-retinoic acid was further substantiated by LC-MS-MS (data not shown).

Plasma kinetics of all-trans-retinoic acid after dosing with all-trans-retinoic acid either alone or simultaneously with phytanic acid or phytol are shown in Figure 5. After treatment with
all-trans-retinoic acid alone, plasma levels of all-trans-retinoic acid increased within 1 h up to 5020 ± 3644 ng/ml and then declined at 4 h to half of the C_{max}. Simultaneous administration of phytanic acid with all-trans-retinoic acid led to lower C_{max} values of all-trans-retinoic acid (4297 ± 1513 ng/ml; Table 2), although the difference was not significant. However, in comparison to the dosing with all-trans-retinoic acid alone, elimination of all-trans-retinoic acid was faster. A similar kinetic behavior of all-trans-retinoic acid could be observed after coadministration of all-trans-retinoic acid with phytol. C_{max} values were even lower (3333 ± 1019 ng/ml).

In Table 2, C_{max} as well as AUC_{0–4 h} values of selected retinoid metabolites are compared. Although C_{max} and AUC values were lower after coadministration of all-trans-retinoic acid with either phytanic acid or phytol, a significant difference could only be observed for the C_{max} of all-trans-4-oxo-retinoic acid in both combination groups, and of all-trans-4-hydroxy-retinoic acid in the group given retinoic acid and phytanic acid.

**Plasma pharmacokinetics of retinol and its metabolites in nonpregnant mice.** After dosing with retinol, the dominant retinoid metabolites in plasma were retinol itself as well as the retinyl esters retinyl palmitate/oleate (not separable with our HPLC method), retinyl stearate, and retinyl linoleate (Fig. 6 and Table 3). An oxidative metabolism of retinol was demonstrated by the occurrence of retinoic acid isomers (all-trans-, 13-cis-, and 9-cis-retinoic acid) and further metabolism led to the formation of all-trans-4-oxo-retinoic acid and all-trans-RAG. Additionally, the retro-retinoids anhydroretinol and 14-hydroxy-4,14-retro-retinol were detected (data not shown).

### Table 2

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>C_{max} (ng/ml) ± SD</th>
<th>AUC_{0–4 h} (ng · h/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans-RA</td>
<td>5020 ± 3644</td>
<td>15473 ± 4893</td>
</tr>
<tr>
<td>All-trans-4-oxy-RA</td>
<td>449.7 ± 1.9</td>
<td>805.4 ± 142.0</td>
</tr>
<tr>
<td>All-trans-4-hydroxy-RA</td>
<td>157.7 ± 28.0</td>
<td>318.2 ± 75.8</td>
</tr>
<tr>
<td>All-trans-RAG</td>
<td>414.0 ± 160.5</td>
<td>1334 ± 607</td>
</tr>
</tbody>
</table>

**Note.** Pharmacokinetic parameters of all-trans-retinoic acid (RA) and its metabolites in plasma of nonpregnant NMRI mice after administration of RA alone or simultaneously with phytanic acid (PA) or phytol (POH) (*n* = 3 per group).

*{Maximum concentration (observed values).}

*{Area under the concentration-time curve for the indicated time interval.}

*p* < 0.05, **p* < 0.01, lower compared to RA (one-way ANOVA followed by Dunnett test).
Plasma kinetics of retinol are shown in Figure 6A. After dosing with retinol alone, plasma levels of retinol increased within 2 h to 10-fold ($C_{\text{max}} = 2045 \pm 609$ ng/ml) and then slowly declined until 12 h to levels 1.5-fold above endogenous concentrations. Kinetics of retinol were similar after coadministration with phytanic acid, although peak concentrations were slightly lower ($C_{\text{max}} = 1648 \pm 177$ ng/ml). The simultaneous administration of phytol with retinol led to a substantial influence on the kinetics of retinol, since concentrations increased only 2.5-fold until 2 h, remained in this range until 8 h, and subsequently increased slightly to reach its $C_{\text{max}}$ at 12 h ($C_{\text{max}} = 519.0 \pm 167.1$ ng/ml, $p < 0.01$). Furthermore, as shown in Figure 6B and Table 3, the oxidative metabolism of retinol to all-trans-retinoic acid was inhibited by coadministration with either phytanic acid or phytol. Coadministration with phytanic acid reduced the plasma AUC of all-trans-retinoic acid to 1/3 ($p < 0.05$), while simultaneous dosing with phytol almost completely blocked the formation of all-trans-retinoic acid ($p < 0.01$). In this group, very low levels of all-trans-retinoic acid could be identified only at 2 h in 1 sample. Additionally, neither all-trans-4-oxo-retinoic acid nor all-trans-RAG was detected in these samples. The $C_{\text{max}}$ of retinyl palmitate/oleate were also significantly reduced in both combination groups.

### DISCUSSION

Synergistic potentials of synthetic RXR ligands on RAR-mediated effects were found in reporter gene assays, in cell systems and, with respect to teratogenicity, in zebrafish, *Xenopus*, and murine embryos (Elmazar et al., 1997, 2001; Minucci et al., 1997). In P19 embryocarcinoma cells the synergistic action of an RXR ligand on the transcription of the RAR$\beta$ gene could only be observed when suboptimal concentrations of the RAR ligand were applied. Neither selective ligands for the RAR$\alpha$, RAR$\beta$, or RAR$\gamma$ nor those for the RXR were able to influence the expression of target genes in P19 or F9 embryocarcinoma cells when given alone at low concentrations (Roy et al., 1995). On the other hand, coadministered RAR and RXR

### TABLE 3

**Pharmacokinetic Parameters of Retinol and Its Metabolites**

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>$C_{\text{max}}$ (ng/ml) $\pm$ SD</th>
<th>$AUC_{0-4}$ (ng $\cdot$ h/ml) $\pm$ SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>ROH 2045 ± 609, ROH + POH 519.0 ± 167.1**, ROH + PA 1648 ± 177</td>
<td>ROH 12015 ± 2370, ROH + POH 5227 ± 1644*, ROH + PA 10028 ± 3407</td>
</tr>
<tr>
<td>Retinyl palmitate/oleate</td>
<td>ROH 14680 ± 3289, ROH + POH 4777 ± 985**, ROH + PA 8273 ± 2104*</td>
<td>ROH 70259 ± 25566, ROH + POH 30764 ± 7040, ROH + PA 48682 ± 8790</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td>ROH 1018 ± 311, ROH + POH 4.8 ± 4.0**, ROH + PA 508.0 ± 402.1</td>
<td>ROH 2924 ± 1000, ROH + POH 13.7 ± 8.0**, ROH + PA 1055 ± 834*</td>
</tr>
</tbody>
</table>

*Notes:*
- Pharmacokinetic parameters of retinol (ROH) and its metabolites in plasma of nonpregnant NMRI mice after administration of ROH alone or simultaneously with phytanic acid (PA) or phytol (POH) ($n = 3$ per group).
- $C_{\text{max}}$ — Maximum concentration (observed values).
- $AUC_{0-4}$ — Area under the concentration-time curve for the indicated time interval.
- *In 2 of 3 animals, plasma levels of all-trans-RA were below limit of detection (< 2 ng/ml).**
- $p < 0.05$, $**p < 0.01$, lower compared to ROH (one-way ANOVA followed by Dunnett test).
ligands were able to affect the expression of target genes as well as cell differentiation (Roy et al., 1995). Synergistic actions of the natural RXR ligand phytanic acid or its precursor phytol on effects mediated by the natural RAR ligand all-trans-retinoic acid or its precursor retinol have not been reported up to now. However, it has been demonstrated that phytol acts synergistically on retinol mediated growth of murine leukemia cells (Suzuki et al., 1998), although the investigators did not indicate whether this effect was receptor mediated.

Recent results show that all-trans-retinoic acid-induced as well as retinol-induced teratogenicity in mice is potentiated by coadministration of the synthetic RXR ligand LG1069 (Elmazar and Nau, 1998). In the same manner, embryotoxic effects of the synthetic RARα ligand Am580 were potentiated by coadministration with phytic acid and its precursor phytol (Elmazar and Nau, 1998, unpublished observations). The results of the present study clearly demonstrate that embryotoxicity and teratogenicity of the natural RAR ligand all-trans-retinoic acid is not potentiated by the natural RXR ligand phytanic acid or by its precursor phytol. In contrast, coadministration of retinol, the precursor of all-trans-retinoic acid, with phytic acid or phytol led to a pronounced reduction of retinol-induced teratogenic effects.

Investigations on metabolism and pharmacokinetics revealed that phytic acid or phytol greatly decreases the formation of all-trans-retinoic acid from retinol. Furthermore, the oxidative metabolism of administered all-trans-retinoic acid to all-trans-4-oxo-retinoic acid was also decreased by phytic acid and phytol coadministration.

On the other hand, phytanic acid has also been demonstrated to be a ligand of the peroxisome proliferator-activated receptor α (PPARα; Ellinghaus et al., 1999; Wolfrum et al., 1999). Therefore, it might be possible that phytic acid acts as an RXR ligand in the presence of selective, synthetic RAR ligands, but as a PPARα ligand in presence of the nonselective, natural RAR ligand all-trans-retinoic acid. Additionally, 9-cis-retinoic acid was detected as a metabolite of all-trans-retinoic acid in plasma of mice. This retinoid was reported to be 200-fold more potent than phytic acid in mediating RXR-dependent transcriptional activity (Kitareewan et al., 1996). Since it was shown that 9-cis-retinoic acid can induce RXR-homodimerization, the presence of the RXR ligand 9-cis-retinoic acid may have limited RXR availability for RAR-RXR heterodimerization.

The most surprising and important result of the present investigation was that phytic acid, and in particular phytol, coadministration greatly reduced or completely abolished retinol-induced teratogenic effects. Pharmacokinetic studies clearly showed significantly reduced retinol Cmax and AUC values following coadministration of phytol (Fig. 6). Since it was shown in mice that phytol is absorbed via the lymphatic route (Baxter et al., 1967), an interaction in absorption and further transport of retinoids in chylomicrons appears likely. Phytic acid, and in particular phytol, coadministration addi-

tionally reduced the concentrations of the active ligand all-

trans-retinoic acid (Fig. 6B). Following coadministration of retinol and phytol, all-trans-retinoic acid formation was nearly undetectable and was reduced to half (p > 0.05) when phytic acid was given with retinol (Table 3). A steep dose-teratogenicity relationship for all-trans-retinoic acid is found in mice (Nau et al., 1994); therefore, a slight reduction in all-trans-retinoic acid Cmax is expected to be accompanied by a higher reduction in teratogenicity. All-trans-retinoic acid (AUC) was also significantly reduced in the group given retinol and phytanic acid. Embryotoxicity of all-trans-retinoic acid is correlated to AUC rather than to Cmax (Tzimas et al., 1997). Thus, metabolic interactions are mainly responsible for the reduction of retinol teratogenicity.

In vitro studies on metabolism of phytol showed that the bioconversion to phytic acid via the intermediate phytanic acid occurred in mitochondrial and microsomal fractions of rat livers, respectively (Muralidharan and Muralidharan, 1985, 1986). Cytosolic fractions had no activity. Furthermore, microsomal enzymes of the short chain alcohol dehydrogenase (SCAD) family were identified as retinol dehydrogenases in rat livers (Boerman and Napoli, 1995; Chai et al., 1995; Napoli, 1996; Posch et al., 1991). It remains to be investigated whether those enzymes that metabolize phytol or retinol might be identical. The oxidation of retinol to all-trans-retinoic acid via retinal as well as the 4-hydroxylation of all-trans-retinoic acid are known to be mediated by isoforms of P450 enzymes on endoplasmic reticulum (Roberts et al., 1979, 1980, 1992). The main degradation of phytic acid by α-oxidation seemed to be localized mainly in peroxisomes (Jansen et al., 1996; Pahan and Singh, 1993; Singh et al., 1993; Wanders et al., 1994), but activities were also found in microsomal fractions of human liver (Verhoeven et al., 1997). The α-oxidation of phytic acid, which is auto-inducible (Zomer et al., 2000), was shown to be inhibited by ketoconazole (Pahan et al., 1994) as well as by carbon monoxide (Muralidharan and Kishimoto, 1984), both known to be strong inhibitors of cytochrome P450 mediated metabolism. It was furthermore demonstrated that the cytochrome P450b5a, a prokaryotic P450 enzyme present in Bacillus megaterium ATCC14581 that resembles the isoforms of the P4504A family, was able to catalyze a phytic acid α-hydroxylation and was itself inducible by phytic acid (English et al., 1997). The results point to interactions of the metabolic pathways of retinoid and phytic acid/phytol metabolism.

Our results indicate a lack of synergism of the teratogenicity of retinol/retinoic acid, when coadministered with phytol/phy-
tanic acid. Instead, phytol and phytic acid effectively blocked the teratogenic activity induced by retinol via inhibition of the metabolism to all-trans-retinoic acid. Thus, phytol and phytic acid may be useful for the prevention of vitamin A teratogenesis.
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REFERENCES


