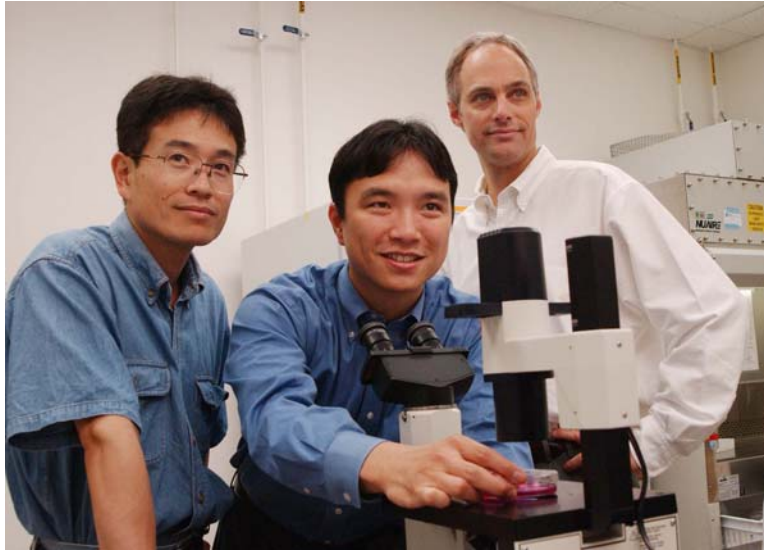


RESEARCHERS UNCOVER BIOCHEMICAL CONNECTION BETWEEN HIGH-FAT DIETS AND INCREASED COLON-CANCER RISK



DALLAS – May 17, 2002 – Researchers at **UT Southwestern Medical Center** at Dallas have uncovered what could be a **key clue** in tracing the **connection** between **high-fat diets** and **increased colon-cancer risk**.

Their findings, published in today's edition of **Science**, reveal that the body's natural mechanisms aren't built to handle **lithocholic acid**, a toxic byproduct of **dietary fat**, in the volume generated by **high-fat diets**.

Dr. David Mangelsdorf, professor of pharmacology and investigator in the **Howard Hughes Medical Institute (HHMI)** at **UT Southwestern**, said observational evidence established a strong association between **high-fat diets** and **colorectal cancer**, but **scientists could not explain the biological and biochemical mechanisms that formed the link**.

"The rate of colorectal cancer is much higher in the United States - where a **high-fat diet** is common - than in Japan, where people don't eat a lot of fat and **colorectal cancer** is almost nonexistent. **But no one has understood why that is,**" he said.

The new findings show that at least part of the answer lies in the body's inability to cope with large amounts of **lithocholic acid**, produced when the body processes **cholesterol**. The body produces **bile acids** when it breaks down **cholesterol, part and parcel of dietary fat**. Those bile acids go to the **small intestine** and are broken down into **secondary bile acids**, one of which is **lithocholic acid**.

Most **secondary bile acids** circulate to the **liver**, but only a little bit of **lithocholic acid** does so. Much of it remains in the **small intestine**, then moves into the **colon, or large intestine**.

"**Lithocholic acid** is highly toxic, and it builds up in a high-fat diet," Mangelsdorf said. "We don't know how it causes **cancer**; but it is known to cause **cancer** in mice, and people with **colon cancer** have high concentrations of it."

Scientists knew that a certain **receptor** controlled the small amount of **lithocholic acid** in the **liver**. **Receptors** are proteins that bind to certain substances to help the body absorb or get rid of them. The **lithocholic acid-controlling receptor** also is present in the **colon**. But there isn't enough of it to cope with large volumes of **lithocholic acid**.

However, the **lithocholic acid-controlling receptor** is similar in structure to another receptor, which binds to **vitamin D** to help the body absorb **calcium**. Mangelsdorf's team wondered if the **vitamin D receptor** might also help eliminate **lithocholic acid**.

The researchers discovered that the **vitamin D receptor** actually plays a major role in eliminating **lithocholic acid**. Like the receptor that works in the liver, the **vitamin D receptor** binds to **lithocholic acid**, then binds to a **specific gene**, called **CYP3A** that triggers production of an enzyme that breaks down the toxic acid. Those findings were made using **assays**, which are small, flat panels used to study genetic activity outside living organisms.

Next, the researchers used tissue cultures to show that the process is replicated in living cells. Then, the team fed **vitamin D** and **lithocholic acid** to mouse models. The **lithocholic acid** activated the animals' **CYP3A genes**, as well as other genes that the **vitamin D receptor** is known to bind to after binding with **vitamin D**.

"It turned out that in vivo, the **vitamin D receptor** appeared to play a large role in breaking down **lithocholic acid**," Mangelsdorf said.

While the research identifies a possible target for helping the body eliminate excess **lithocholic acid**, exploiting the research might not be so simple. Taking extra **vitamin D** would stimulate more activity in the **vitamin D** receptors, but that also would cause the body to absorb more **calcium**. Ingesting too much **vitamin D** can lead to **hypercalcemia**, a toxic condition that occurs with excessive calcium buildup.

Mangelsdorf said the body's natural **lithocholic acid-response mechanism** simply wasn't built to handle the amount of fat in the modern American diet.

"Our bodies can handle slight changes in **lithocholic acid** that come from a **normal diet**, but not a **high-fat diet**," he said. "**The current American diet can provide more fat on a daily basis than a human being was ever meant to handle.**"

Dr. Makoto Makishima, a former research associate in the **HHMI** at **UT Southwestern**, was lead author of the study. Other **UT Southwestern** researchers who contributed were Timothy T. Lu, an M.D./Ph.D. student in pharmacology, and Dr. Hideharu Domoto, a postdoctoral fellow in pharmacology.

Other institutions contributing to the study were the **Salk Institute for Biological Studies** and the **University of Arizona College of Medicine**. The study was supported by the **Howard Hughes Medical Institute**, the **National Institutes of Health**, the **Robert A. Welch Foundation** and the **Human Frontier Science Program**.

Vitamin D Receptor As an Intestinal Bile Acid Sensor

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The vitamin D receptor (VDR) mediates the effects of the calcemic hormone $1\alpha,25$ -dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. We show that VDR also functions as a receptor for the secondary bile acid lithocholic acid (LCA), which is hepatotoxic and a potential enteric carcinogen. VDR is an order of magnitude more sensitive to LCA and its metabolites than are other nuclear receptors. Activation of VDR by LCA or vitamin D induced expression in vivo of CYP3A, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine. These studies offer a mechanism that may explain the proposed protective effects of vitamin D and its receptor against colon cancer.

A contributing factor to the deleterious effects of a high-fat diet is an associated increase in the excretion of fecal bile acids (1), the most toxic of which is the secondary bile acid LCA (Fig. 1A). Unlike the primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA), LCA is poorly reabsorbed into enterohepatic circulation and passes into the colon. At high concentrations, LCA induces DNA strand breaks, forms DNA ad-

ducts, and inhibits DNA repair enzymes (1–3). LCA can also promote colon cancer in animals (4), and its concentration is higher than other secondary bile acids in patients with colorectal cancer (5).

In contrast to the positive correlation among high-fat diets, LCA, and colon cancer, dietary intake of vitamin D and calcium is related to a reduced incidence of colorectal cancer (6). Furthermore, vitamin D supplementation inhibits colon carcinogenesis induced by either high-fat diets or intrarectal instillation of LCA (7, 8). One route for LCA elimination is through its catabolism by the enterohepatic cytochrome P450, CYP3A, a putative target gene of vitamin D (9, 10). Expression of CYP3A in the liver is regulated by the nuclear xenobiotic and pregnane X receptor (PXR, also called SXR), which can be activated by high concentrations (≥ 100 μ M) of LCA (11, 12). Primary bile acids (in particular, CDCA and CA) are also ligands for the farnesoid X receptor, FXR (13, 14). However, neither PXR nor FXR responds to vitamin D, and LCA-induced expression of CYP3A is still present in PXR-null animals.

This suggests another LCA-dependent pathway for inducing CYP3A expression (11).

To determine if bile acids could act on the vitamin D receptor (VDR) to induce CYP3A expression, we used a ligand-screening assay based on the ligand-induced interaction of a nuclear receptor with its coactivator (14). The receptor-interacting domain of the coactivator SRC-1 was fused to the DNA binding domain of the yeast transcription factor GAL4, and various nuclear receptors were fused to the transactivation domain of the herpes virus VP16 protein. Expression plasmids for GAL4-SRC-1 and VP16-nuclear receptor were transfected with a GAL4-responsive luciferase reporter plasmid into human embryonic kidney (HEK293) cells and examined for luciferase expression after LCA treatment. LCA (30 μ M) induced a ligand-dependent interaction between VDR and SRC-1 (Fig. 1B). As previously reported (14), LCA also activated FXR. However, no other nuclear receptors were activated by LCA (Fig. 1B) (15), including PXR, which required higher LCA concentrations (≥ 100 μ M) to be activated. To further investigate the ligand specificity of VDR and FXR, we tested various primary, secondary, and conjugated bile acids in this assay (Fig. 1C). We performed these experiments in the presence or absence of the ileal bile acid transporter (IBAT), because hydrophilic bile acids such as CA and conjugated bile acids require transport across cell membranes (16). As expected, treatment of cells with the vitamin D hormone $1,25(OH)_2D_3$ activated VDR but not FXR (Fig. 1C). Conversely, the primary bile acids CDCA, CA, and their conjugated metabolites were effective ligands for FXR but not VDR. FXR was also activated by the secondary bile acids, deoxycholic acid, LCA, and their conjugated metabolites (Fig. 1C). However, the only bile acids that activated VDR were LCA and its major metabolites 3-keto-LCA (Fig. 1A), glyco-LCA, and 6-ke-

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to-LCA (Fig. 1C, shaded area). The 6-keto metabolite of LCA is of further interest because it did not activate FXR. Hence, although VDR and FXR both serve as bile acid receptors, they have distinct specificity profiles.

We also compared the dosage dependency of bile acids to activate human VDR, FXR, and PXR. LCA and 3-keto-LCA activated human VDR with median effective concentration (EC_{50}) values of 8 μ M and 3 μ M, respectively (Fig. 1D). These bile acids also activated FXR, but effective concentrations were 2 to 3 times greater than those for VDR (Fig. 1E). Although CDCA was an FXR agonist (EC_{50} = 7 μ M), it

was not effective on VDR at any concentration. LCA and 3-keto-LCA were also equally effective at activating full-length mouse or human VDR [see below and (15)]. The xenobiotic receptor PXR also is activated by LCA (11, 12). However, compared to VDR, PXR required a concentration at least 10 times greater than that of either LCA (Fig. 1F) or 3-keto-LCA (15) for activation. Therefore, the concentration at which these bile acids activate VDR is below the pharmacologic range that activates FXR and PXR. These data suggest that VDR is a more sensitive receptor for bile acids than are FXR and PXR, specifically for LCA and its major metabolite 3-keto-LCA.

To demonstrate that bile acids directly bind VDR as ligands, we performed a competitive binding assay using [3 H]1,25(OH) $_2$ D $_3$ and increasing concentrations of candidate bile acids (Fig. 2). Both LCA and 3-keto-LCA competed effectively with [3 H]1,25(OH) $_2$ D $_3$ for binding to VDR [inhibition constant (K_i) = 29 \pm 6 μ M and 8 \pm 3 μ M, respectively], with 3-keto-LCA exhibiting an affinity that is 3.5 times greater than that of LCA. A fluorescence polarization assay also showed that LCA, but not other bile acids, induced association with the coactivator peptide (fig. S1).

LCA is catabolized by CYP3A (11, 17), a putative target gene of vitamin D in the intestine (9, 10). To explore the role of LCA and VDR in the activation of CYP3A, we investigated the promoters of the mouse, rat, and human CYP3A genes for potential VDR-RXR heterodimer binding sites (Fig. 3A). All three gene promoters have direct repeats separated by three nucleotides (DR3 elements) similar to those found in other VDR target genes (18). In addition, the human CYP3A4 gene contains an everted repeat separated by six nucleotides (ER6) that has also been reported to be a VDR-RXR response element (10). Each of these elements bound to the VDR-RXR heterodimer (Fig. 3B), competed for the receptor heterodimer with high affinity (Fig. 3C), and was responsive to VDR-dependent transactivation by either LCA or 1,25(OH) $_2$ D $_3$ (Fig. 3D). It is noteworthy that these elements were previously reported to mediate the xenobiotic response of the PXR-RXR heterodimer in the liver (19). In a comparison to PXR, we found that all three CYP3A promoters were at least 10 times more sensitive to VDR when activated by LCA (15). The human CYP3A elements were the most sensitive and conferred the strongest

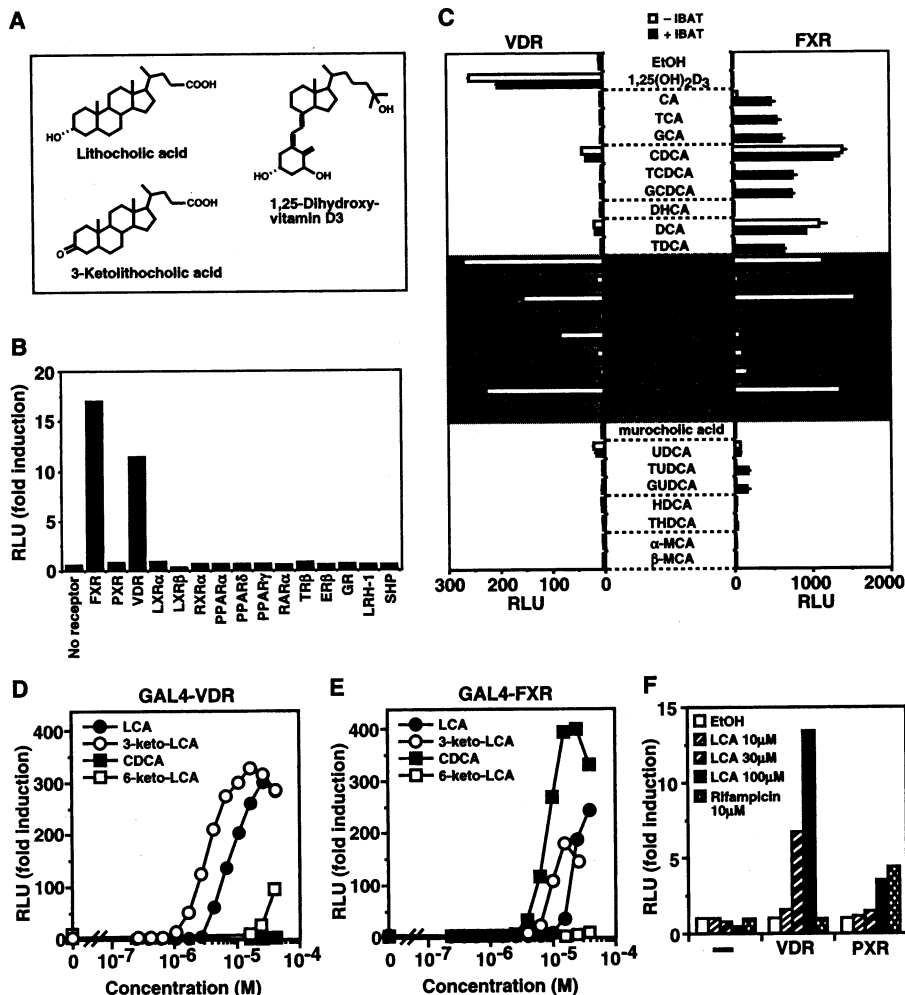


Fig. 1. LCA and its metabolites are VDR agonists. (A) Structures of VDR agonists. (B) Receptor-specific activation by LCA. Various nuclear receptors were expressed in HEK293 cells and screened for activation by 30 μ M LCA with a mammalian two-hybrid GAL4-SRC-1 and VP16-receptor luciferase assay. (C) Ligand specificity of VDR- and FXR-activation by bile acids. VDR and FXR were screened for activation by 1,25(OH) $_2$ D $_3$ (0.1 μ M) or various bile acids (30 μ M), as in Fig. 1B. The screen was performed on transfected HEK293 cells expressing VDR or FXR in the presence (black bars) or absence (white bars) of IBAT to facilitate uptake of CA and the conjugated bile acids (16). The shaded area identifies LCA-specific metabolites. (D and E) Comparative dose response of human VDR and FXR to bile acids using a GAL4-receptor luciferase assay. (F) Comparative dose response of VDR and PXR to bile acids. Transfection assay was as in (D) and (E), except that full-length human VDR and PXR were expressed in monkey kidney CV1 cells with a luciferase reporter plasmid containing three copies of the human CYP3A4 ER6 element (see Fig. 3). Rifampicin was used as a positive control ligand for PXR. RLU, relative light units. Fold inductions by various ligands in (B), (D), (E), and (F) are relative to ethanol (EtOH) vehicle used as a control. See supporting online material for additional methods and bile acid abbreviation.

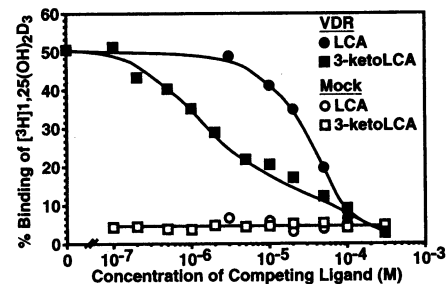
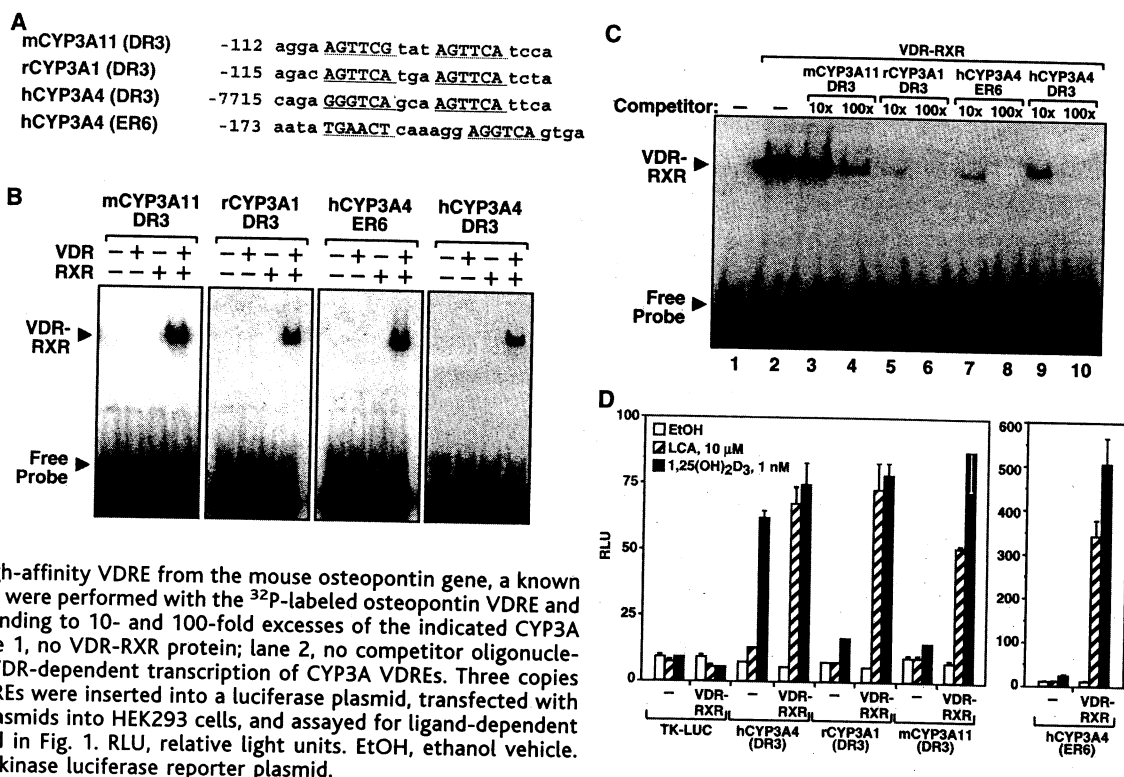


Fig. 2. LCA and 3-keto-LCA directly bind VDR in vitro. Human VDR was expressed in monkey kidney COS-7 cells, labeled with [3 H]1,25(OH) $_2$ D $_3$, and used for competitive binding assays. Results obtained with VDR-containing lysates (closed symbols) or mock lysates transfected with RXR (open symbols) are shown. Data points are representative of three independent experiments using LCA and 3-keto-LCA as competitors. Similar experiments using CDCA, CA, muricholic acid (MCA), and hydoxycholeic acid (HDCA) showed no competitive binding. See supporting online material for materials and methods.

Fig. 3. CYP3A genes are LCA-dependent VDR target genes. (A) VDR response element (VDRE) sequences from mouse (m), rat (r), and human (h) CYP3A genes. The numbers indicate positions in the gene promoter relative to the transcription start site. (B) VDR-RXR heterodimers bind to CYP3A VDREs. In vitro synthesized RXR and VDR were used in electrophoretic mobility band shift assays (24) with [³²P]-oligonucleotides shown in (A). The arrowheads depict DNA bound VDR-RXR and free probe. (C) CYP3A VDREs compete for VDR-RXR binding to the high-affinity VDRE from the mouse osteopontin gene, a known VDR target (18). Band shifts were performed with the ³²P-labeled osteopontin VDRE and analyzed for competitive binding to 10- and 100-fold excesses of the indicated CYP3A VDREs (lanes 3 to 10). Lane 1, no VDR-RXR protein; lane 2, no competitor oligonucleotide. (D) LCA stimulates VDR-dependent transcription of CYP3A VDREs. Three copies of the indicated CYP3A VDREs were inserted into a luciferase plasmid, transfected with VDR and RXR expression plasmids into HEK293 cells, and assayed for ligand-dependent transactivation as described in Fig. 1. RLU, relative light units. EtOH, ethanol vehicle. TK-LUC, control thymidine kinase luciferase reporter plasmid.



response (Fig. 3D). These data further suggest that VDR mediates LCA-dependent induction of CYP3A gene expression.

To confirm the effects of VDR on induction of CYP3A expression in vivo, the transactivation of the CYP3A11 gene was determined after treating mice with agonists for VDR, FXR, or PXR. For these experiments, we used 1 α -hydroxyvitamin D₃ (1 α (OH)D₃) (8) and EB1089 (20) as synthetic VDR agonists, pregnenolone-16 α -carbonitrile (PCN) as a PXR-selective agonist (19), and LCA as a panagonist for all three receptors. CYP3A11 mRNA expression in the intestine was increased in response to both VDR- and PXR-specific ligands, as well to LCA. In contrast, the VDR-specific target gene calbindin 9K (18) was activated by LCA and the VDR-selective agonists 1 α (OH)D₃ and EB1089, but not by PCN, indicating that LCA can function as a VDR agonist in vivo. Likewise, the FXR target gene, ileal bile acid binding protein (14), was transactivated by LCA, but not by VDR or PXR selective ligands. None of the compounds altered the expression of VDR (Fig. 4A). To demonstrate that LCA- and VDR-dependent activation in vivo does not require PXR, the expression of CYP3A11 was examined in PXR^{-/-} mice and PXR^{+/-} control mice (Fig. 4, B and C). As expected (11, 12), CYP3A11 mRNA expression in response to PCN was eliminated in the liver and intestine of PXR-null mice. However, CYP3A11 mRNA expression was still induced by both the VDR-selective ligands and LCA. This dem-

onstrates that VDR can function as an LCA sensor in vivo, resulting in increased expression of CYP3A.

Taken together, these results point to VDR as a potential bile acid sensor in the enteric tract, where elevated concentrations of LCA may bind to VDR. This "adopted orphan" function of VDR (21) complements its endocrine role in the small intestine as a high-affinity [dissociation constant (K_d) = 0.1 to 1 nM] receptor for 1,25(OH)₂D₃ to promote calcium and phosphate absorption, which ensures proper mineralization of bone (18). The regulation of the LCA/VDR metabolic cascade is strikingly similar to that mediated by other evolutionarily related nuclear receptors [e.g., PXR, constitutive androstane receptor (CAR), FXR, and liver X receptor (LXR)] that function as lipid sensors and mediate detoxification of their ligands (21). By binding to VDR, both LCA and vitamin D may activate a feed-forward catabolic pathway that increases CYP3A expression and leads to the detoxification of LCA. These findings suggest a model to explain how the enteric system could protect itself from the potentially harmful effects of LCA and why vitamin D is protective against colon cancer under normal physiologic conditions. Protection provided by VDR activation may become compromised when the detoxification pathway is overwhelmed (e.g., by increased levels of LCA due to sustained high-fat diets) or under clinical conditions of vitamin D deficiency (e.g., rickets/osteomalacia). Consistent with this model, there is an epidemi-

ologic relation between the incidence of colon cancer and Western-style, high-fat diets (22), and the highest death rates from colon cancer occur in areas with a high prevalence of rickets (6). Furthermore, mice lacking VDR not only have rickets but also display enhanced cellular proliferation in the colon (23). Thus, this work should provide the impetus for further studies addressing the role of diet, bile acids, and vitamin D in colorectal cancer.

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25. Male mice (129Sv wild type, PXR^{+/-}, or PXR^{-/-}) were gavaged daily with vehicle (corn oil), 1.5 µg 1α(OH)D₃ (gift from M. Pechet, Research Institute for Medicine and Chemistry, Cambridge, MA), 1.5 µg EB1089 (gift from L. Binderup, Leo Pharmaceutical Products, Copenhagen), 1.5 mg PCN, or 8 mg LCA. Mice were killed, and mRNA from the intestine and liver were isolated for Northern blot analysis (24) using the indicated gene-specific cDNA probes.
26. We thank J. Repa, K. Gauthier, and N. Kalaany for assistance in animal experiments; C. Wu and A. Kowal for expert technical assistance; C. Haussler and members of the Mango lab for comments; and M. Brown, J. Goldstein, and D. W. Russell for critically reading the manuscript. D.J.M. and R.M.E. are investigators and M.M. is an associate of the Howard Hughes Medical Institute. T.T.L. is supported by a Pharmacological Sciences training grant from NIH. Funded by the Howard Hughes Medical Institute, an NIH Specialized Program of Research Excellence in lung cancer, the Robert A. Welch Foundation, the Human Frontier Science Program, and NIH grants (M.R.H.).

Supporting Online Material

(www.sciencemag.org/cgi/content/full/296/5571/1313/DC1) Material and Methods fig. S1

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Vitamin D Receptor As an Intestinal Bile Acid Sensor

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Supplementary Material

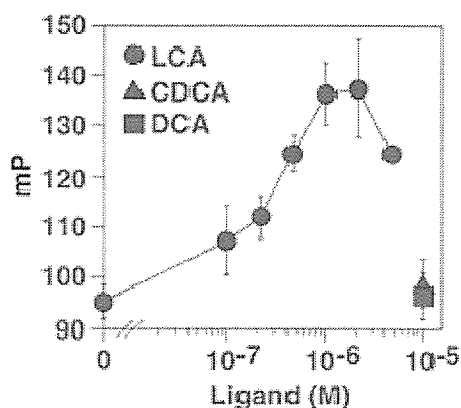
Materials and Methods

Luciferase cotransfection assays. Cotransfection assays were performed in HEK293 cells as described (1). In Fig. 1, B and C, cells were cotransfected with a GAL4-responsive luciferase reporter plasmid and cytomegalovirus-based (CMX) expression plasmids for GAL4-SRC-1 and VP16 chimeras containing the full-length mammalian nuclear receptors. Receptors tested were: FXR (farnesoid X receptor, NR1H4), PXR (pregnane X receptor, NR112), VDR (vitamin D receptor, NR1H1), LXR α (liver X receptor α , NR1H3), LXR β (liver X receptor β , NR1H2), RXR α (retinoid X receptor α , NR2B1), PPAR α (peroxisome proliferator activated receptor α , NR1C1), PPAR δ (peroxisome proliferator activated receptor δ , NR1C2), PPAR γ (peroxisome proliferator activated receptor γ , NR1C3), RAR α (retinoic acid receptor α , NR1B1), TR β (thyroid hormone receptor β , NR1A2), ER β (estrogen receptor β , NR3A2), GR (glucocorticoid receptor, NR3C1), LRH-1 (liver receptor homolog-1, NR5A2) or SHP (short heterodimer partner, NR0B2) (2). Other nuclear receptor tested but not shown include CAR (constitutively active receptor, NR1I3), HNF4 (hepatocyte nuclear receptor 4, NR2A1), and COUP-TFI (chicken ovalbumin upstream promoter transcription factor 1, NR2F1). CMX-VP16 alone was used as control (no receptor). In Fig. 1, D and E, cells were cotransfected with the GAL4-responsive luciferase reporter and expression vectors containing the GAL4 DNA-binding domain fused to the ligand-binding domain of human VDR (residues 90-427) or human FXR (residues 193-472). Transfection data were normalized to an internal β -galactosidase control and expressed as fold-induction or relative light units (RLU) from triplicate assays \pm SD. Bile acid (Sigma and Steraloids, Inc.) abbreviations: cholic acid, CA; taurocholic acid, TCA; glycocholic acid, GCA; chenodeoxycholic acid, CDCA; taurochenodeoxycholic acid, TCDCA; glycochenodeoxycholic acid, GCDCA; dehydrocholic acid, DHCA; deoxycholic acid, DCA; taurodeoxycholic acid, TDCA; lithocholic acid, LCA; tauroolithocholic acid, TLCA; glycolithocholic acid, GLCA; 5-sulfate-glycolithocholic acid, SGLCA; tauroursodeoxycholic acid, TUDCA; ursodeoxycholic acid, UDCA; glyoursodeoxycholic acid, GUDCA; hyodeoxycholic acid, HDCA; taurohyodeoxycholic acid, THDCA; muricholic acid, MCA.

Competitive ligand binding assay. Ligand binding was performed as described (3) using lysates from COS-7 cells transfected with expression plasmids for VDR or RXR α . Binding was performed overnight at 4°C in lysate buffer (3) with 0.71 nM (18 Ci/mmol) [3 H]1,25(OH) $_2$ D $_3$ (Amersham Pharmacia Biotech) and bile acid competitor. Unbound [3 H]1,25(OH) $_2$ D $_3$ was removed by adsorption to dextran-coated charcoal and the supernatant removed for scintillation counting. K_i values were calculated from a computer fit of competition curves from

triplicate assays \pm : SD.

Supplemental Figure 1. Fluorescence polarization assay of bile acids binding to VDR (5).



Supporting references and notes

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5. Fluorescence polarization assay. A fluorescein-labeled peptide (1 nM), which consists of LLRYLLDK (residues 748-755 of SRC-1), was incubated with purified glutathione-S-transferase-tagged human VDR (residues 90-427) and candidate ligands in 100 μ l of buffer (150 mM NaCl, 10 mM KPO₄, 2 mM CHAPS, 2 mM EDTA, 1 mM DTT at pH 7.3) in a 96-well plate on a shaker for 1 hr (4). Ligand-dependent recruitment of the coactivator peptide to VDR was then monitored as an increase in fluorescence polarization (mP) using an LJL analyst reader (LJL Biosystems). Means of triplicate data points \pm : SD are plotted.