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35. Expression plasmids contained the complete coding region of the indicated gene fused to the *Drosophila actin 5C* promoter in pAct (*18*). Reporter plasmids were as follows: *per-luc*, a 4.2-kb fragment of the *period* promoter fused to firefly luciferase (*32*); *tim-luc*, a 6.5-kb Bam HI–Sal I fragment of the *timeless* gene corresponding to the region upstream of the start of translation fused to firefly luciferase; and *CME-lacZ*, six *Toll* site 4 CMEs fused to *lacZ* (*18*). As a control for transfection efficiency, we used a plasmid containing the *actin 5C* promoter fused to *Renilla* luciferase in pRL-null (Promega). S2 cells in 12-well plates were transfected with Lipofectin (Gibco BRL) according to the manufacturer's recommendations. Each transfection contained 0.1 to 0.2 μg of each expression plasmid, 0.2 to 0.4 μg of reporter, 0.001 μg of control plasmid, and pAct to keep the amount of DNA per transfection constant. Cells were harvested 48 hours after transfection, and enzyme activity was measured with the Luciferase Assay, Dual-Luciferase Reporter Assay, and β-Galactosidase Enzyme Assay systems (Promega). For each sample, reporter activity was normalized to *Renilla* luc activity. Reporter activity was plotted relative to activity when cotransfected with empty expression vector pAct. Values are the mean ± SEM of two to four replicate experiments.
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39. Each multimer reporter consisted of four 18-bp elements containing the E box from either the *per* or *tim* promoter. Sequences shown in Fig. 2C are from the sense strand of the *per* promoter and the antisense strand of the *tim* promoter. To construct the reporters, the following synthetic oligonucleotides were annealed and inserted into a pGL3 vector (Promega) containing the *hsp70* basal promoter: wild-type *per* multimer, 5′-GTACCGCCGCTCACGTGGCGAACC CTCGAGCCGCTCACGTGGCGAA-3′, 5′- CCCGC GAGCCGCTCACGTGGCGAACAT TAATGCCGCT CACGTGGCGAACC-3′, 5′-CTCGCGGGTTCGCC ACGTGAGCGGCTCGAGGGTTCGCCACGTGA GCGGC-3′, 5′-TCGAGGTTCGCCACGTGAGCG GCATTAATGTTCGCCACGTGAGCGG-3′; mutant *per* multimer, 5′-GTACCGCCGCTCAGCTGGCGA ACCCTCGAGCCGCTCAGCTGGCGAA-3′, 5′- CC CGCGAGCCGCTCAGCTGGCGAACATTAATGCC GCTCAGCTGGCGAACC-3′, 5′-CTCGCGGGTT CGCCAGCTGAGCGGCTCGAGGGTTCGCCAG CTGAGCGGCG-3′, 5′-TCGAGGTTCGCCAGCTG AGCGGCATTAATGTTCGCCAGCTGAGCGG-3′; wild-type *tim* multimer, 5′-GTACCTGATTACACGT-GAGCCGAAGAGATTGATTACACGTGAGCCG-3′, 5′-AGGCGGTTGATTACACGTGAGCCGAAC-TAGTTGATTACACGTGAGCCGAC-3′, 5′-AACCG CCTCGGCTCACGTGTAATCAATCTCTTCGGC TCACGTGTAATCAG-3′, 5′ -TCGAGTCGGCTC ACGTGTAATCAACTAGTTCGGCTCACGTGTA ATC-3′; mutant *tim* multimer, 5′-GTACCTGA TTACAGCTGAGCCGAAGAGATTGATTACAGCTG AGCCG-3′, 5′- AGGCGGTTGATTACAGCTGAG CCGAACTAGTTGATTACAGCTGAGCCGAC-3′, 5′-AACCGCCTCGGCTCAGCTGTAATCAATCTCTT CGGCTCAGCTGTAATCAG-3′, 5′ -TCGAGTCGGC TCAGCTGTAATCAACTAGTTCGGCTCAGCTGTAA TC-3′. Replicate experiments were performed, and a representative data set is shown.
40. Two-hybrid assays were carried out on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) plates as described (*33*). For these assays, we used LEXA-dCLOCK(1–496) as bait, from which most of the COOH-terminal region was removed.
41. The indicated DNA fragments from the *per* gene clock control region were ligated into pBgl-lacZ, a one-hybrid reporter plasmid (*25*), and one-hybrid yeast reporter strains were constructed by insertion of recombinant pBgl-*lacZ* reporter plasmids into the *ura3* locus of YPH 499 (*34*). The negative control strain was constructed identically with nonrecombinant pBgl-*lacZ*. One-hybrid reporter strains were transformed with the indicated expression plasmids, and transformants were patched onto X-Gal plates (*33*) for detection of β-galactosidase (β-Gal) activity. The 21-bp fragment is part of the 69-bp circadian-regulated region (*10*) and contained the E-box with 8 bp of 5′ and 7 bp of 3′ flanking sequences. For the 21-bp fragment with the mutated E-box, the E-box sequence was scrambled by means of a random number table. E-box mutant: 5′-ATTCGC.
42. Plasmids expressing *per* and *tim* from the *actin 5C* promoter in pAct were constructed. Transfections and assays were performed as described (*35*) with minor modifications. For each well, expression plasmids were used at 0.001 μg for dCLOCK and 0.01 μg for PER and TIM. An additional plasmid with the *hsp70* promoter driving *lacZ* was used at 0.1 μg per well to control for transfection efficiency. For each sample, activity was normalized to β-Gal activity. For each reporter, activity was normalized to activity when transfected with pAct. The value for dCLOCK-activated wild-type reporter was set to one. Replicate experiments were performed, and a representative data set is shown.
43. We thank M. Myers, L. Saez, and M. Young for the *tim* sequence and clones; T. L. Schwartz for the head cDNA library; J. Park and J. Hall for in situ chromosomal mapping of *dclock*; R. Stanewsky, S. Crews, and P. Hardin for various clones; J. Kreps, R. Raman, and C. Andersson for their generous help; and A. McLachlan and P. Ghazal for helpful discussions. This work was supported by National Institutes of Mental Health grant MH-51573 and the NSF Center for Biological Timing (S.A.K.), the Pew Foundation (fellowship to M.F.C.), and an NSF and McKnight Scholars Award (C.J.W.). J.S.T. is an Investigator of the Howard Hughes Medical Institute.

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Teratogen-Mediated Inhibition of Target Tissue Response to *Shh* Signaling

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Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis have been studied for more than 30 years as potent teratogens capable of inducing cyclopia and other birth defects. Here, it is shown that these compounds specifically block the Sonic hedgehog (Shh) signaling pathway. These teratogens did not prevent the sterol modification of Shh during autoprocessing but rather inhibited the response of target tissues to Shh, possibly acting through the sterol sensing domain within the Patched protein regulator of Shh response.

A striking aspect of *Shh* function is its role in developmental patterning of the head and brain, as revealed in $Shh^{-/-}$ mouse embryos by the occurrence of severe holoprosencephaly (HPE) (*1*). HPE is characterized by development of the prosencephalic derivatives as a single undivided vesicle that consists of the fused remnants of the dorsal telencephalic lobes, with an undivided eye field and an absence of ventral forebrain structures such as the optic stalks, the optic chiasm, and the pituitary (*1*, *2*). Externally, severe HPE is characterized by an absence of midline facial structures and development of a proboscis consisting of fused nasal chambers at a location overlying a cyclopic eye. Loss-of-function mutations at the human *Shh* locus are associated with a milder and more variable form of HPE that is inherited in autosomal dominant fashion, indicative of haploinsufficiency at the human *Shh* locus (*3*).

Hedgehog (Hh) proteins undergo an intramolecular autoprocessing reaction that

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entails internal cleavage and covalent addition of cholesterol to generate the mature signaling molecule (*4*–*6*). Given this critical role for cholesterol modification in the biogenesis of Hh proteins, it is noteworthy that certain perturbations of cholesterol homeostasis cause HPE. For example, HPE is induced in rat pups exposed during gestation to distal inhibitors of cholesterol biosynthesis such as triparanol, AY9944, or BM15.766 (*7*–*9*). Milder forms of HPE are observed in 5% of patients with Smith-Lemli-Opitz Syndrome, which is thought to be caused by a defect late in the cholesterol biosynthetic pathway (*10*). HPE is also observed in mouse embryos deficient in megalin, a member of the low-density lipoprotein (LDL) receptor family that is expressed in embryonic neuroectoderm and binds and internalizes LDL (*11*). Finally, HPE is induced in lambs born to pregnant ewes that consume *Veratrum californicum*, and the teratogenic effects of this plant have been traced to the alkaloids cyclopamine and jervine (*12*). These two closely related compounds resemble cholesterol in structure, and jervine acts as a distal inhibitor of cholesterol biosynthesis (*13*).

As seen in Fig. 1, B to E, exposure of chick embryos to jervine at the intermediate to definitive streak stage (*14*) induced external malformations characteristic of HPE, with a variable extent of loss of midline structures and consequent approximation and fusion of paired lateral structures such as the mandibular and maxillary processes as well as the optic vesicles and olfactory processes. We circumvented the inherent variability of these in ovo treatments by using an explant assay, which facilitates a more uniform application of these hydrophobic compounds (*15*). Medial neural plate with notochord attached was dissected from a region just rostral to Hensen's node (Fig. 1F), a level where the notochord expresses Shh (*16*) but the neural plate does not yet express floor plate cell (HNF3β) or motor neuron (Isl1) markers (*17*). Induction of these cell types depends on Shh signaling both in vivo and in vitro (*1*, *18*), and, as seen in Fig. 1G, both HNF3β and Isl1 were induced within the explanted neural plate tissue after a 40-hour incubation. Induction of HNF3β and Isl1 was

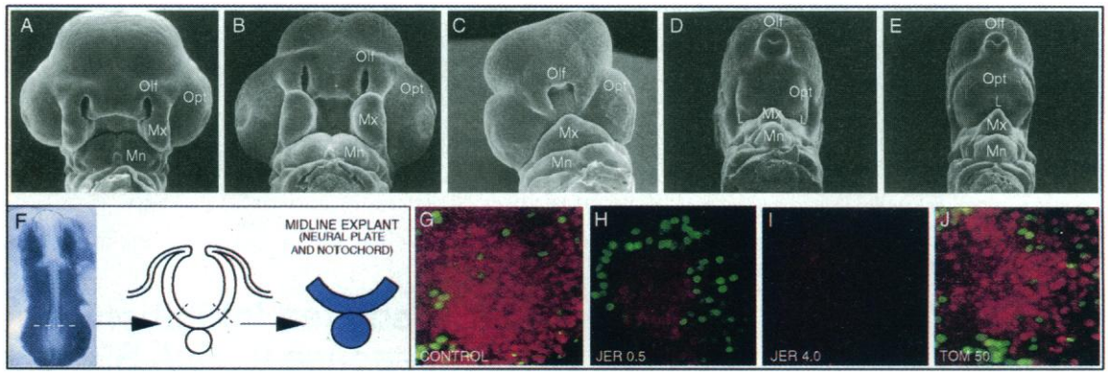
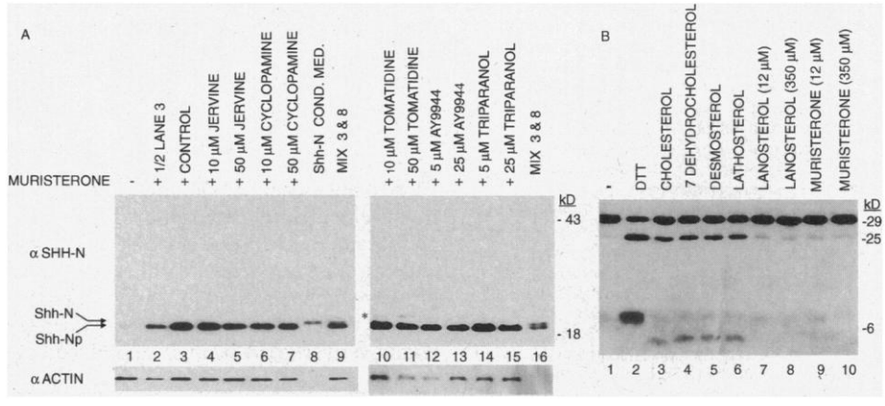


Fig. 1. Jervine induces holoprosencephaly and blocks endogenous *Shh* signaling. (**A**) Scanning electron micrograph of external facial features of an untreated embryo. (**B** to **E**) Embryos were exposed to 10 μM jervine (*14*) with variable loss of midline tissue and resulting fusion of the paired, lateral olfactory processes (Olf), optic vesicles (Opt), and maxillary (Mx) and mandibular (Mn) processes. Complete fusion of the optic vesicles and lenses (L) results in true cyclopia (E). (**F**) Midline tissue was removed from stage 9 to 10 chick embryos at a level just rostral to Hensen's node (white dashed line) and further dissected (black dashed lines) to yield an explant containing an endogenous source of *Shh* signal (notochord) and a responsive tissue (neural plate ectoderm) (*15*). (**G**) After 2 days of culture in a collagen gel matrix, expression of floor plate cell [HNF3β, rhodamine (red)] and motor neuron [Isl1, fluorescein isothiocyanate (FITC) (green)] markers is induced in untreated neural ectoderm or (**J**) in explants treated with the nonteratogenic alkaloid tomatidine (TOM) (50 μM). (**H**) Intermediate doses of jervine (JER) (0.5 μM) block induction of HNF3β, while permitting induction of Isl1 (see text). (**I**) Higher doses of jervine (4.0 μM) fully inhibit HNF3β and Isl1 induction.

Fig. 2. Teratogens do not inhibit Shh autoprocessing. (**A**) Stably transfected HK293 cells containing an ecdysone-inducible Shh expression construct (lane 1, uninduced) were treated by addition of muristerone A (lanes 2 to 7 and 10 to 15) either alone (lanes 2 and 3) or in combination with jervine (lanes 4 and 5), cyclopamine (lanes 6 and 7), tomatidine (lanes 10 and 11), AY9944 (lanes 12 and 13), or triparanol (lanes 14 and 15) (*21*). Shh from control (lane 3) or drug-treated (lanes 4 to 7 and 10 to 15) cell lysates is efficiently processed with no detectable accumulation of precursor protein (M_r = 45 kD). The processed NH_2-terminal product (Shh-N_p) is cell associated and migrates faster than unprocessed Shh-N protein (lane 8) from the medium of cultured cells transfected with a construct carrying an open reading frame truncated after Gly^{198} (both Shh-N_p and Shh-N are loaded in lanes 9 and 16), indicating that Shh-N_p from treated cells likely carries a sterol adduct. The slower migrating species resulting from tomatidine treatment is ~1.9 kD larger, suggestive of a minor inhibition of signal sequence cleavage (see asterisk; lanes 10 and 11). Immunoblotted actin for each lane is shown as a loading control. (**B**) Coomassie blue–stained SDS-polyacrylamide gel showing in vitro autocleavage reactions of the bacterially expressed His_6Hh-C protein (~29 kD) incubated for 3 hours at 30°C in the absence of sterols (lane 1), with 50 mM dithiothreitol (DTT) (lane 2), 12 μM cholesterol (lane 3), 12 μM 7-dehydrocholesterol (lane 4), 12 μM desmosterol (lane 5), 12 μM lathosterol (lane 6), 12 and 350 μM lanosterol (lanes 7 and 8, respectively), and 12 and 350 μM muristerone (lanes 9 and 10, respectively). The 27-carbon cholesterol precursors (lanes 4 to 6) stimulate His_6Hh-C autoprocessing as efficiently as cholesterol (lane 3). Lanosterol (lanes 7 and 8) and muristerone (lanes 9 and 10) do not stimulate autoprocessing above background (lane 1). The NH_2-terminal product migrates as a ~7-kD species (lane 2) when generated in the presence of 50 mM DTT and as a ~5-kD species (lanes 3 to 6) with a sterol adduct.



inhibited by 4 μM jervine (Fig. 1I). At 0.5 μM, jervine still blocked expression of HNF3β, but expression of Isl1 was maintained or enhanced (Fig. 1H; see also Fig. 3). Partial and complete inhibition of Shh signaling was also obtained with increasing concentrations of AY9944, triparanol, and cyclopamine (*19*). In contrast, the structurally related but not teratogenic alkaloid tomatidine (*20*) did not block induction of HNF3β and Isl1, even at concentrations an order of magnitude higher than the inhibitory concentration of jervine (Fig. 1J).

To examine the potential effects of these teratogens on Shh processing, we used HK293 cells carrying a stably integrated construct for expression of Shh under ecdysone-inducible control (*21*). The Shh protein was efficiently processed (Fig. 2A, lanes 2 and 3), and addition of jervine, cyclopamine, tomatidine, AY9944, or triparanol during the 24-hour induction period did not diminish production of Shh-N_p, the processed NH_2-terminal product, or induce accumulation of unprocessed precursor [relative molecular mass (M_r) of 45 kD], even at doses 6- to 50-fold higher than those required to completely inhibit Shh signaling (Fig. 2A, lanes 4 to 7 and 10 to 15). All of the NH_2-terminal cleavage product generated in the presence of these compounds was detected in cell lysates, not in the culture medium, and had the same electrophoretic mobility as cholesterol-modified Shh-N_p (compare with lanes 8 and 9, Fig. 2A), consistent with the presence of a sterol adduct in the NH_2-terminal cleavage product. In addition, chick embryos treated with jervine after floor plate induction displayed normal apical localization of Shh protein within floor plate cells (*22*), indicative of normal secretion and sorting of intracellular Shh-N_p.

Because the plant alkaloids resemble cholesterol in structure, including the presence of a 3β hydroxyl, their effects were tested in a cholesterol-dependent in vitro autoprocessing reaction (*5*). None of these compounds could replace cholesterol or inhibit its stimulatory effect (*23*). In contrast, cholesterol could be replaced efficiently in the in vitro reaction by desmosterol and 7-dehydrocholesterol (Fig. 2B, lanes 4 and 5), the major precursors that accumulate in cells treated with triparanol and AY9944 (*9*). Other 27-carbon cholesterol precursors, including lathosterol (but not lanosterol, a 30-carbon cholesterol precursor), could participate in the reaction (Fig. 2B, lanes 6 to 8). These and other observations (*24*) suggest that many, possibly all, 27-carbon sterol intermediates in the biosynthetic pathway are potential adducts in the autoprocessing reaction, and this may account for the unimpaired efficiency of processing in the presence of distal synthesis inhibitors.

To examine the possibility that teratogens affect the response of target tissues to Shh signaling, we used an intermediate neural plate explant (*15*) (Fig. 3A) that responds to recombinant Shh-N protein in a concentration-dependent manner (*18*). The dorsal marker Pax7 was repressed at low concentrations (2 nM, Fig. 3, B and C) (*25*), and the ventral markers Isl1 and HNF3β were induced at progressively higher concentrations (6.3 nM, Fig. 3D, and 25 nM, Fig. 3E) (*18*). These teratogens completely blocked the repression of Pax7 (at 2 nM Shh-N, Fig. 3, F to I) and the induction of Isl1 and HNF3β (at 25 nM Shh-N, Fig. 3, P

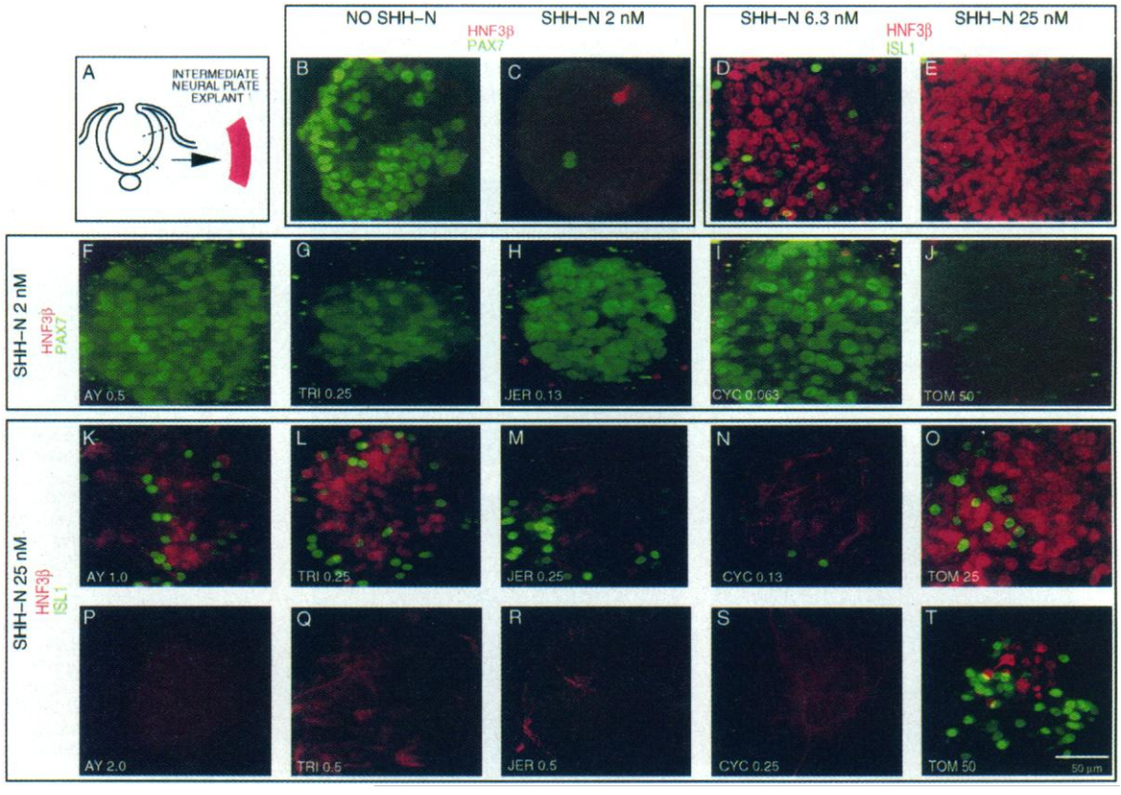


Fig. 3. Teratogens inhibit response of neural ectoderm to recombinant Shh-N protein. (**A**) Intermediate neural plate ectoderm, free of notochord and other tissues, was dissected as shown (dashed lines) from stage 9 to 10 chick embryos at a level just rostral to Hensen's node (see Fig. 1F). (**B**) Explanted intermediate neural plate tissue cultured in a collagen gel matrix for 20 hours expresses the dorsal marker Pax7 (FITC) but not the floor plate marker HNF3β (rhodamine). (**C**) Addition of recombinant, purified Shh-N at 2 nM suppresses Pax7 expression. (**D**) Markers of motor neuron (Isl1, FITC) and floor plate cell (HNF3β,rhodamine) fates are induced upon explant culture for 40 hours in the presence of 6.3 nM Shh-N. (**E**) At 25 nM Shh-N, HNF3β expression expands at the expense of Isl1 expression. The repression of Pax7 expression by 2 nM Shh-N is inhibited by (**F**) 0.5 μM AY9944 (AY), (**G**) 0.25 μM triparanol (TRI), (**H**) 0.13 μM jervine, and (**I**) 0.063 μM cyclopamine (CYC) but not by (**J**) 50 μM tomatidine. (**K** to **N**) Induction of HNF3β is reduced, whereas induction of Isl1 at 25 nM Shh-N is maintained or expanded at intermediate concentrations of AY9944 (1.0 μM) (K), triparanol (0.25 μM) (L), jervine (0.25 μM) (M), and cyclopamine (0.13 μM) (N). (**O**) Tomatidine at 25 nM displays a slight inhibitory effect with a decrease in HNF3β expression and an increase in the number of Isl1-expressing cells. (**P** to **S**) HNF3β and Isl1 induction is completely blocked at inhibitory doses twofold higher than those in (K) to (N). (**T**) Tomatidine at 50 μM markedly reduces HNF3β induction and enhances Isl1 induction.

to S). A complete inhibition of the response to 25 nM Shh-N required doses of teratogenic compounds twofold to fourfold higher than those required to completely block the 2 nM response. In addition, at a drug concentration half of that required for complete inhibition of 25 nM Shh-N treatment, Isl1 expression was retained or expanded (Fig. 3, K to N). Inhibition of the response to higher concentrations of Shh-N thus requires higher drug concentrations, and, at a fixed concentration of Shh-N, distinct degrees of pathway activation can be produced by distinct inhibitor concentrations. Tomatidine, in contrast, did not inhibit Pax7 repression (Fig. 3J) and only partially inhibited HNF3β and Isl1 induction (Fig. 3, O and T), even at concentrations 100- to 200-fold higher than those required for complete inhibition by jervine and cyclopamine.

In a test of the specificity of these compounds, explants from the ventral neural plate (Fig. 4A) responded to BMP7 protein by formation of neural crestlike migratory cells that express the HNK-1 surface antigen (*26*) (compare Fig. 4, B and C), even in the presence of jervine at 10 μM (Fig. 4D). This concentration is 20-fold higher than that required for a complete block of Shh-N signaling. Similar results were obtained with tomatidine and cyclopamine (*27*).

Given that these teratogens inhibit the response of target tissues to Shh signaling and that some of them affect distal cholesterol biosynthesis (*9*, *13*), it is noteworthy that the Patched (Ptc) protein, which controls the response to Shh signaling within responding tissues (*28*), contains a sterol sensing domain (SSD). The SSDs of two other proteins, hydroxymethylglutaryl coenzyme A (HMG CoA) reductase and SCAP (SREBP cleavage-activating protein), confer differential responses to high and low concentrations of intracellular sterols; a third SSD-containing protein, NPC1 (Niemann-Pick C1), is proposed to function in intracellular transport. The possibility thus emerges that these teratogens inhibit Shh signaling through the Ptc SSD, either through effects on Ptc protein stability, Ptc-dependent activation of downstream catalytic events, or Ptc-dependent effects on transport, as suggested by analogy to apparent SSD functions in HMG CoA reductase, SCAP, and NPC1, respectively (*29*).

The teratogenic effects of these compounds cannot simply be due to a reduction of cholesterol synthesis or due to the accumulation of an inhibitory sterol precursor because none of the explant responses to Shh-N were either blocked or restored by a potent proximal inhibitor of cholesterol biosynthesis (*30*). It is thus important to note that, in addition to its effects on distal cholesterol biosynthesis, AY9944 inhibits cholesterol esterification (*31*). This property is shared by a group of compounds termed class 2 transport inhibitors (*32*), which appear to act by reducing the flux of cholesterol and its sterol precursors from the plasma membrane (PM) to the endoplasmic reticulum (ER), thus preventing action of acyl-CoA cholesterol acyltransferase on exogenously delivered cholesterol and causing accumulation of cholesterol biosynthetic precursors. Class 2 sterol transport inhibitors appear to increase the activity of HMG CoA reductase and to stimulate SCAP activity (*33*). Given the ER localization of these two SSD-containing proteins, it is possible that disruption of sterol transport from PM to ER by class 2 compounds decreases sterol concentrations within intracellular compartments, despite normal or increased concentrations of cellular sterols overall. We have found that the other teratogens studied here also inhibit cholesterol esterification (*34*) and that other structurally dissimilar class 2 compounds inhibit Shh signaling in our explant assays (*35*). Because the Ptc protein is found at intracellular locations (*36*), a teratogen-induced defect in sterol transport could conceivably perturb Ptc function through its SSD. Further studies with these teratogenic compounds may help elucidate the mechanistic roles of Ptc and intracellular transport in the *Shh* signaling pathway.

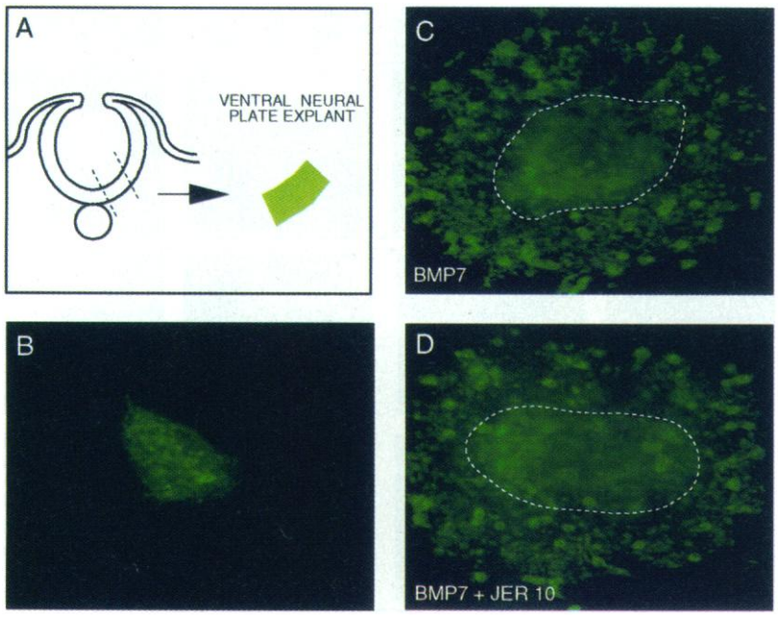


Fig. 4. Jervine does not inhibit the response of neural ectoderm to BMP7. (**A**) Ventral neural plate ectoderm was dissected as shown (dashed lines) from stage 9 to 10 chick embryos at a level just rostral to Hensen's node (see Fig. 1F). (**B**) Ventral neural plate explants cultured for 24 hours in a collagen gel matrix do not give rise to HNK-1–positive migratory cells unless BMP7 (100 ng/ml) is added (**C**). (**D**) Induction of migratory HNK-1–positive cells by BMP7 (100 ng/ml) is not inhibited by the presence of 10 μM jervine (C and D, explant borders outlined by white dashed lines) nor by the addition of the other plant-derived compounds (10 μM cyclopamine and 50 μM tomatidine) (*37*).

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14. Jervine [250 μl of a sonicated solution (1 mg/ml)] was applied to windowed, fertile chick eggs (White Leghorn, Chestertown, MD) at 14 hours of incubation. After 4 days of further incubation, the embryos were processed for scanning electron microscopy.
15. Embryos at Hamburger and Hamilton stage 9 to 10 (8 to 10 somites) were used for all explant assays [T. Yamada, S. L. Pfaff, T. Edlund, T. M. Jessell, *Cell* **73**, 673 (1993)]. AY9944 (Wyeth-Ayerst Research, Princeton, NJ), triparanol (Hoechst Marion Roussel, Cincinnati, OH), jervine, cyclopamine, or tomatidine (from W. Gaffield; all compounds were in 10 mM

stocks in 95% ethanol, except AY9944, which is water soluble), and purified Shh-N or BMP7 (from H. Reddi) were added at the initiation of the cultures. Control explants were cultured with the maximum ethanol concentration used for drug treatments. All of the explants were cultured for 40 to 48 hours except for assays of Pax7 repression, which were cultured for 20 to 22 hours. Primary antibodies used were HNF3β (K2), Isl1/Isl2 (40.2D6), Pax7 (from S. Morton and T. Jessell), and HNK-1/N-CAM (Sigma Biosciences).
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19. Induction of HNF3β and Isl1 in midline explants was fully inhibited with 4.0 μM AY9944, 1 μM triparanol, and 1 μM cyclopamine. Intermediate doses of AY9944 (0.5 μM), triparanol (0.25 μM), and cyclopamine (0.25 μM) blocked induction of HNF3β while permitting induction of Isl1.
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21. HK293 cells, stably transfected with *Shh* with the Ecdysone-Inducible Mammalian Expression System (Invitrogen), were cultured to 30 to 40% confluency and then changed to culture medium with 10% delipidated serum [K. M. Gibson *et al.*, *J. Lipid Res.* **31**, 515 (1990)] and insulin-transferrin-sodium selenite media supplement (Sigma). After 24 hours, the cells were induced to express *Shh* with 1 μM muristerone A (Invitrogen), and AY9944, triparanol, jervine, cyclopamine, or tomatidine was added [see (*15*)]. After an additional 24 hours, cell lysates were analyzed with 12% SDS-polyacrylamide gels.
22. Chick embryos were treated with ~10 μM jervine at stage 20 for 8, 16, 24, or 32 hours before fixation and stained with an antibody that recognizes Shh-N. No difference in the normal localization of Shh-N protein to the apical domain of floor plate cells or on the surfaces of commissural axons (*16*) was seen in treated embryos.
23. Cleavage of the His_6Hh-C protein was stimulated by 12 μM cholesterol, but this cleavage was neither stimulated nor inhibited by the addition of jervine, cyclopamine, or tomatidine, even at 350 μM.
24. Other sterols that participated in the reaction with similar efficiency to cholesterol are β-sitosterol, 5-androsten-3β-ol, and 7β-hydroxycholesterol. Sterols that participated with reduced efficiency are coprastan-3-ol, ergosterol, 4β-hydroxycholesterol, 19-hydroxycholesterol, 20α-hydroxycholesterol, 22(S)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 25-hydroxycholesterol. Epicholesterol, cholesterol acetate, α-ecdysone, 20-OH ecdysone, 3-keto-5-cholestene, and thiocholesterol (3(β)-thiol) were unable to participate in the reaction. The in vitro reaction thus requires an unhindered hydroxyl at the 3β position on a tetracyclic sterol nucleus, although neither the isooctyl side chain nor the number or positions of the double bonds in the sterol nucleus appear to affect autoprocessing critically.
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27. Jervine (10 μM), cyclopamine (10 μM), and tomatidine (50 μM) all failed to inhibit formation of migratory HNK-1–positive cells, either from ventral neural plate explants cultured with BMP7 (100 ng/ml) or from explanted dorsal neural plate with contiguous epidermal ectoderm (an endogenous source of BMP7).
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30. None of the explant responses to Shh-N were affected by addition of 25-hydroxycholesterol at 25 μM. 25-hydroxycholesterol is a potent inhibitor of HMG CoA reductase and at the concentrations used blocks de novo cholesterol synthesis in chick embryos and in cultured cell systems [S. C. Miller and G. Melnykovych, *J. Lipid Res.* **25**, 991 (1984); J. J. Bell, T. E. Sargeant, J. A. Watson, *J. Biol. Chem.* **251**, 1745 (1976); (*37*)]. The addition of 25-hydroxycholesterol to explant cultures did not reverse the inhibitory effects of any of the teratogenic compounds.
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35. The following class 2 sterol transport inhibitors at the given concentrations blocked the response of intermediate neural plate explants to 25 nM Shh-N, without affecting signaling by BMP7: 0.25 μM U 18666A, 50 μM chloroquine, 75 μM imipramine, and 20 μM progesterone.
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Inhibition of a *Mycobacterium tuberculosis* β-Ketoacyl ACP Synthase by Isoniazid

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Although isoniazid (isonicotinic acid hydrazide, INH) is widely used for the treatment of tuberculosis, its molecular target has remained elusive. In response to INH treatment, saturated hexacosanoic acid (C26:0) accumulated on a 12-kilodalton acyl carrier protein (AcpM) that normally carried mycolic acid precursors as long as C50. A protein species purified from INH-treated *Mycobacterium tuberculosis* was shown to consist of a covalent complex of INH, AcpM, and a β-ketoacyl acyl carrier protein synthase, KasA. Amino acid–altering mutations in the KasA protein were identified in INH-resistant patient isolates that lacked other mutations associated with resistance to this drug.

INH is a front-line drug of choice for the treatment of tuberculosis (*1*). Despite the apparent simplicity of its chemical structure, the mode of action of this drug is complex. INH is a prodrug that requires activation by the mycobacterial catalase-peroxidase enzyme (KatG) to an active form that then exerts a lethal effect on an intracellular target or targets (*2–4*). Because of physical and biochemical changes occurring coincident with INH toxicity, it has been proposed that the lethal effect lies in the biosynthetic pathway for mycolic acids (*2, 5–7*). The detrimental effect of INH on mycolic acid synthesis exactly parallels the time course of the loss of *Mycobacterium tuberculosis* viability and is accompanied by an accumulation of saturated hexacosanoic acid (C26:0), implicating this fatty acid as an intermediate in the biosynthetic pathway that produces mycolic acids (*8–11*).

The majority of INH-resistant clinical isolates become resistant by losing or altering KatG activity, not by mutation of the target of the activated prodrug (*12, 13*). Despite considerable effort, identification of the INH target in *M. tuberculosis* by genetic approaches has not been accomplished (*14*). However, a library of DNA fragments from a resistant strain of the fast-growing saprophyte *M. smegmatis* was used to isolate a putative target designated InhA (*15*). InhA is an NADH-dependent enoyl-[acyl carrier protein] (ACP) reductase with a chain-length specificity centering at 16 carbons (*16, 17*). Reconciling the catalytic function of InhA (reduction of an unsaturated fatty acid) with the observed biochemical correlate of toxicity (accumulation of a saturated fatty acid) has been extremely difficult. Furthermore, although this target is sufficient to induce resistance to 50 μg of INH per milliliter in *M. smegmatis*, the same constructs induce only low levels of resistance in *M. tuberculosis* (to 0.1 μg/ml, while KatG wild-type clinical isolates resistant to 1 to 2 μg/ml are commonly encountered) (*14*). Sequencing of clinical isolates

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