Corrections

PLANT BIOLOGY. For the article “Bacterial volatiles promote growth in Arabidopsis,” by Choong-Min Ryu, Mohamed A. Farag, Chia-Hui Hu, Munagala S. Reddy, Han-Xun Wei, Paul W. Paré, and Joseph W. Kloepper, which appeared in issue 8, April 15, 2003, of Proc. Natl. Acad. Sci. USA (100, 4927–4932; First Published April 8, 2003; 10.1073/pnas.0730845100), the authors note the following. Due to a printer’s error on page 4930, bars 3 through 7 of histogram A of Fig. 4 were incorrectly labeled. The corrected figure and a corrected legend appear below.

![Corrected Figure](https://example.com/corrections/fig4_corrected.png)

**Fig. 4.** Growth promotion of *A. thaliana* ecotype Col-0 with exposure to extracted bacterial volatiles from growth-promoting (GB03 and IN937a) and nongrowth-promoting (DHSa) bacteria and synthetic 2,3-butanediol (A) and exposure to volatiles released from *B. subtilis* WT (168) and mutant strains defective in the production of 2,3-butanediol (BSIP1173 and BSIP1174) (B). Different letters indicate significant differences between treatments according to least significant difference at *P* = 0.05. A dose–response curve with synthetic 2,3-butanediol in the presence of *A. thaliana* seedlings confirmed the efficacy of this volatile bacterial metabolite in promoting plant growth. The level of exogenous 2,3-butanediol (2 ng) that was observed to trigger optimal plant growth promotion was lower than those collected from the GB03 or IN937a strains over the 24-h collection interval (1–5 µg), and it may be at least in part due to a high initial release of the synthetic 2,3-butanediol when introduced into the I-plates containing the Arabidopsis seedlings, as compared to the more even emissions of VOCs generated from the bacterial strains.

DEVELOPMENTAL BIOLOGY. For the article “A genetic model for a central (septum transversum) congenital diaphragmatic hernia in mice lacking *Slit3*,” by Wenlin Yuan, Yi Rao, Randal P. Babiuk, John Greer, Jane Y. Wu, and David M. Ornitz, which appeared in issue 9, April 29, 2003, of Proc. Natl. Acad. Sci. USA (100, 5217–5222; First Published April 17, 2003; 10.1073/pnas.0730709100), the authors note the following correction. The name John Greer should have appeared as John J. Greer. The online version has been corrected. The corrected author list appears below.

Wenlin Yuan, Yi Rao, Randal P. Babiuk, John J. Greer, Jane Y. Wu, and David M. Ornitz

DEVELOPMENTAL BIOLOGY. For the article “Identification of a small molecule inhibitor of the hedgehog signaling pathway: Effects on basal cell carcinoma-like lesions,” by Juliet A. Williams, Oivin M. Guicherit, Beatrice I. Zaharian, Yin Xu, Ling Chai, Hynek Wichterle, Charlene Kon, Christine Gatchalian, Jeffery A. Porter, Lee L. Rubin, and Frank Y. Wang, which appeared in issue 8, April 15, 2003, of Proc. Natl. Acad. Sci. USA (100, 4616–4621; First Published April 4, 2003; 10.1073/pnas.0732813100), the authors request that Roel Nusse, Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, be added to the list of authors between Christine Gatchalian and Jeffery A. Porter. The corrected author line appears below.

Juliet A. Williams, Oivin M. Guicherit, Beatrice I. Zaharian, Yin Xu, Ling Chai, Hynek Wichterle, Charlene Kon, Christine Gatchalian, Roel Nusse, Jeffery A. Porter, Lee L. Rubin, and Frank Y. Wang
Identification of a small molecule inhibitor of the hedgehog signaling pathway: Effects on basal cell carcinoma-like lesions


*Curis Incorporated, 61 Moulton Street, Cambridge, MA 02138; ‡Hammer Health Sciences Center, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, 10th Floor, New York, NY 10032; and §Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305

Edited by Anthony P. Mahowald, University of Chicago, Chicago, IL, and approved February 12, 2003 (received for review May 10, 2002)

The link between basal cell carcinoma (BCC) and aberrant activation of the Hedgehog (Hh) signaling pathway has been well established in humans and in mouse models. Here we report the development of assays, including two novel in vitro BCC models, which allowed us to screen for Hh inhibitors and test their validity as potential treatments for BCC. We identified a novel small molecule Hh inhibitor (CUR61414) that can block elevated Hh signaling activity resulting from oncogenic mutations in Patched-1. Moreover, CUR61414 can suppress proliferation and induce apoptosis of basaloid nests in the BCC model systems, whereas having no effect on normal skin cells. These findings directly demonstrate that the use of Hh inhibitors could be a valid therapeutic approach for treating BCC.

*This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BCC, basal cell carcinoma; Hh, Hedgehog; Shh, Sonic hedgehog; Patch-1, patched-1; Smo, smoothened; CK, cytokeratin; H&E, hematoxylin and eosin; ShhOCT, lipid-modified octyl moiety coupled to its N terminus (ShhOCT) was used; this lipid-modified protein has G2/M phases of their cell cycle, and prolonged their replicative capacity (18).

All of this information suggests that compounds that are able to suppress aberrantly activated Hh signaling may cause growth arrest of BCC cells and may provide an effective chemotherapeutic for BCC. Previously, a cell-based system had been used to show that the plant-derived Hh inhibitor cyclopamine can block the oncogenic activation of the Hh signaling pathway and decrease proliferation of cultured mouse fibroblasts (e.g., NIH 3T3 cells) (19). However, these studies were limited, being based on the action of inhibitors on cell lines. The effect of antagonizing Hh signaling on human BCC lesions has not been reported, largely because of the difficulty in culturing BCC-derived materials.

Here we report several assay systems for the identification of synthetic small molecule inhibitors of the Hh signaling pathway and validation of the effectiveness of these inhibitors in the treatment of BCC-like lesions. To achieve the latter, two novel in vitro BCC culture systems have been developed: one in which mouse embryonic skin punches were treated with a highly active form of Hh protein to induce basaloid nests (reminiscent of basal islands in BCC) and another in which adult mouse skin explants containing BCC-like lesions, derived from Patch-1+/− heterozygous mice after long-term UV irradiation, were maintained in culture. A potent small molecule Hh inhibitor (CUR61414) arrested proliferation of basal cells within the BCC-like lesions and induced them to undergo apoptosis leading to complete regression of the lesions, without affecting neighboring skin cells. Combined with recent results on the effects of cyclopamine on medulloblastoma growth (20), our data suggest that inhibitors of the Hh pathway may be effective chemotherapeutics to treat cancers that arise from mutations in that pathway.

Materials and Methods

Screening for Hh Inhibitors. C3H.10T1/2 cells (American Type Culture Collection) were grown in DMEM/10% FBS, with penicillin/streptomycin/glutamine. The Hh-responsive (Gli3Luciferase) reporter construct (a gift from Brigid Hogan, Vanderbilt University, Nashville, TN) was transfected into the 10T1/2 cells, and stable clones were selected in 1 µg/ml Geneticin (GIBCO/BRL, Grand Island, NY). In these studies, a modified form of Shh that has an octyl moiety coupled to its N terminus (ShhOCT) was used; this lipid-modified protein has...

†J.A.W. and O.M.G. contributed equally to this work.

*To whom correspondence should be addressed. E-mail: lrubin@curis.com.
shown to be much more potent in cell-based assays (21). The clone (s12) showing the strongest induction of luciferase activity (8- to 10-fold) on addition of ShhOCT was selected for use. To identify novel inhibitors, various collections of small molecules were screened in this assay. For this purpose, s12 cells were plated in 96-well plates at 20,000–30,000 cells per well and grown for 24 h before compound addition, in DMEM/10% FBS (with penicillin/streptomycin/glutamine). Next, the plates were changed to medium with 0.5% FBS, and compounds (2–5 μM each) were added in the presence of 0.5 μg/ml ShhOCT. After 24 h, plates were assayed for luciferase activity with the LucLite kit (Packard, Meriden, CT). To ensure specificity for the Hh signaling pathway, compounds were subjected to a simian virus 40 (SV40)-luciferase counterscreen in which the luciferase activity was constitutively present, independent of Hh signaling (data not shown). Only those compounds that showed inhibition of the Hh-induced, and not the SV40-dependent, luciferase activity were further tested.

Chick Explant Assay. Intermediate (“naive”) regions of embryonic chick neural tube (stage 10–11) were dissected, embedded in collagen, and cultured as described before (22–24). These explants (n = 6) were then treated with vehicle alone, ShhOCT, or ShhOCT + test compound. After 22 h, explants were fixed and stained with antibodies against Pax7 and Nkx2.5.

Ptc-h Null Assay. To generate a “Ptc-h Null” cell line, several Ptc+/- (Ptc-null) mouse embryonic stem (ES) cell clones were isolated from a parental Ptc+/- (Ptc-heterozygote) ES cell line in the presence of G418 (1 mg/ml). One of the clones was injected into nude mice to generate teratomas. Teratomas were harvested 4 weeks later and dissociated into single cell suspensions for culturing. A spontaneously immortalized and fibroblast-like (Ptc-null) cell line, Tera-28, was established from the culture. For compound testing, Tera-28 cells were grown in DMEM with 10% FBS and incubated with the compound (1–5 μM) for 72 h. Total RNA was then isolated from the cells (RNA Isolation kit from Qiagen, Valencia, CA) for detecting Gli-1, β-actin, and GAPDH transcripts by conventional RT-PCR and TaqMan analysis (Applied Biosystems, Foster City, CA). The β-actin (for gel analysis) and GAPDH (for TaqMan) transcripts were used for normalizing PCR results for Gli-1. PCR products from conventional RT-PCR were analyzed by gel electrophoresis on 1.5% agarose gels.

Embryonic Skin Punch Assay. Mouse embryos, from Ptc+/-;LacZ mice, were collected and killed at late gestation (embryonic day 17.5) and their skins excised. Circular punches (4 mm in diameter) were placed in a collagen-coated Transwell (BIOCOAT Cell Culture Insert, Becton Dickinson Labware, Bedford, MA) and cultured at the air–liquid interface, with the epidermis side facing up. The culture medium contained 5% FBS in DMEM/F12 (3:1) with added epidermal growth factor, insulin, and hydrocortisone. To induce formation of basoloid nests, punches were treated with different concentrations of CUR61414 in vehicle. One of the four punches from each set was then processed for detailed analysis of cell proliferation and apoptosis. For this purpose, punches were fixed, embedded and sectioned. The sections were then stained with an anti-PCNA antibody to measure the amount of proliferation, and the terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) assay was used on adjacent sections to quantify the degree of apoptosis. Each section contained ~8–14 individual lesions. Lesions toward the edges of the sections were excluded from all calculations. For each lesion, the total number of cells was measured, as was the number of either PCNA+ (proliferating) or TUNEL+ (apoptotic) cells. Mitotic and apoptotic indices were then calculated as percent of total cells and averaged across the 15 individual punches for each condition.

Effect of CUR61414 on the UV-Induced Lesions. Skin punches (4 mm) were generated from UV-irradiated Ptc+/-;LacZ animals and cultured. After treatment with vehicle or CUR61414, sections were fixed and stained with X-gal. The number of basoloid lesions (recognized by their blue color) in each punch, regardless of size, was then counted by a blinded observer. Histological analysis showed that individual lesions contained ~50 and 300 cells (data not shown). Three experiments, each containing four separate skin punches, were analyzed.

Supporting Information. Additional materials and methods are described in Supporting Text, which is published as supporting information on the PNAS web site, www.pnas.org.
CUR61414 did not block other developmentally regulated signaling in a well-established Hh assay. We also determined that CUR61414 in the Hh-reporter assay; comparison with jervine. (Vehicle (Veh., 0.5% DMSO), jervine (jerv., 5 μM), and CUR61414 (CUR, 1 μM) in the presence of vehicle (Veh., 0.5% DMSO), jervine (jerv., 5 μM), and CUR61414 (CUR, 1 μM) analyzed by RT-PCR (β-actin is the internal control).

![CUR61414](image)

Fig. 1. Identification of a small molecule Hh inhibitor. (A) Schematic representation of the chemical structure of CUR61414. (B) Inhibitory activity of CUR61414 in the Hh-reporter assay; comparison with jervine. (C) CUR61414 reverses Hh-induced expression of neural markers (Pax7, Nkx2.2) in the chick neural plate explant assay. (D Left) Response of the Hh reporter cells (s12) and Ptc-Null cells (Tera-28) to SHH in the presence of vehicle (Veh., 0.5% DMSO), jervine (jerv., 5 μM), and CUR61414 (CUR, 1 μM).

The ability of CUR61414 to act as a true hedgehog inhibitor was confirmed by using a chick neural plate explant assay (22, 23). Neural progenitors in the developing chick neural tube, as they differentiate in response to a gradient of Hh protein, undergo a specific sequence of expression of the transcription factors Pax7 and Nkx2.2. Pax7 increases at very low Hh concentrations, but, as the concentration of Hh is elevated, Pax7 is then down-regulated, whereas Nkx2.2 is up-regulated (23, 26, 27). The expression patterns of these markers were examined in neural tube explants treated with Hh protein and with various concentrations of CUR61414. Although it was slightly less potent on chicken than on mammalian cells, CUR61414 was able to suppress changes in both markers in a dose-dependent fashion (Fig. 1C). Interestingly, the pattern of suppression appeared to be the inverse of the pattern of induction: Nkx2.2 decreased at higher inhibitor concentrations, whereas Pax7 first increased then decreased as Hh activity was progressively inhibited (Fig. 1C). These results confirm that CUR61414 can inhibit Hh signaling in a well-established Hh assay. We also determined that CUR61414 did not block other developmentally regulated signaling pathways, like those for BMP and Wnt (see Figs. 5 and 6, which are published as supporting information on the PNAS web site).

Recently published work (23, 28, 29) has demonstrated that CUR61414, jervine, and certain other classes of small molecule Hh antagonists bind directly to Smo, a G protein-coupled receptor. Therefore, it was also important to show that CUR61414 does not have significant inhibitory effects on other G protein-coupled receptors. This was established in a series of radioligand binding assays in which the Hh inhibitor was shown not to affect ligand binding for a variety of other receptors of this class (Table 3, which is published as supporting information on the PNAS web site). In sum, this work demonstrates that CUR61414 is a specific Hh pathway inhibitor.

**CUR61414 Blocks Hh Signaling in Ptc-Null Cells.** Because most mutations associated with the Hh signaling pathway in human BCC lesions arise in *PTCH1*, we decided to test the efficacy of CUR61414 on cell lines carrying inactive Ptc-1. As expected, this compound, which acts downstream of Ptc-1, strongly inhibited Hh signaling in these cells (Fig. 1D). Similar results were observed with quantitative RT-PCR (TaqMan) analysis (Fig. 7, which is published as supporting information on the PNAS web site).

**An in Vitro BCC Model System.** Having shown that CUR61414 can prevent the activation of the Hh signaling pathway in cells that have a mutation commonly observed in BCC, we wanted to test its activity of these inhibitors in the context of normal skin and BCC-like skin lesions. For this, we set out to develop an *in vitro* BCC model system.

It had been shown previously that basaloid nests develop in CK14-Shh transgenic mice and in human skin in which Shh is overproduced in basal keratinocytes (14, 30). Building on these observations, we established a skin culture system in which punches from mouse embryonic skin, derived from Ptc-1<sup>−/−</sup> heterozygote mice, were maintained *in vitro* in the presence of recombinant Shh protein. Because *PTCH1* is a target of Hh signaling, the *Ptc-1-lacZ* transgene allowed us to monitor Hh signaling by following β-galactosidase activity. Shh addition to the embryonic skin caused strong activation of its signaling pathway (Fig. 2A and B). Histological analysis of the Hh-treated skin showed large nodular clusters of basal cells (basaloid nests), absent in vehicle treated samples, and reminiscent of the basal cell islands observed in BCC (Fig. 2D and E). These nodular clusters appeared as soon as 4 days after protein treatment (data not shown), but only developed fully after 6 days. Intense X-gal stain, reflecting elevated Hh signaling, appeared restricted to these nodular clusters (Fig. 2E), except for staining associated with some basal keratinocytes and hair follicles, also known to be sites of Hh signaling.

The Hh-induced basaloid nests showed various features observed in human BCC lesions and in mouse BCC models (14, 15, 31). First, these nodular structures were observed with quantitative RT-PCR (TaqMan) analysis (Fig. 3, which is published as supporting information on the PNAS web site). Recently published work (23, 28, 29) has demonstrated that CUR61414, jervine, and certain other classes of small molecule Hh antagonists bind directly to Smo, a G protein-coupled receptor. Therefore, it was also important to show that CUR61414 does not have significant inhibitory effects on other G protein-coupled receptors. This was established in a series of radioligand binding assays in which the Hh inhibitor was shown not to affect ligand binding for a variety of other receptors of this class (Table 3, which is published as supporting information on the PNAS web site). In sum, this work demonstrates that CUR61414 is a specific Hh pathway inhibitor.

**CUR61414 Blocks Hh Signaling in Ptc-Null Cells.** Because most mutations associated with the Hh signaling pathway in human BCC lesions arise in *PTCH1*, we decided to test the efficacy of CUR61414 on cell lines carrying inactive Ptc-1. As expected, this compound, which acts downstream of Ptc-1, strongly inhibited Hh signaling in these cells (Fig. 1D). Similar results were observed with quantitative RT-PCR (TaqMan) analysis (Fig. 7, which is published as supporting information on the PNAS web site).

**An in Vitro BCC Model System.** Having shown that CUR61414 can prevent the activation of the Hh signaling pathway in cells that have a mutation commonly observed in BCC, we wanted to test its activity of these inhibitors in the context of normal skin and BCC-like skin lesions. For this, we set out to develop an *in vitro* BCC model system.

It had been shown previously that basaloid nests develop in CK14-Shh transgenic mice and in human skin in which Shh is overproduced in basal keratinocytes (14, 30). Building on these observations, we established a skin culture system in which punches from mouse embryonic skin, derived from Ptc-1<sup>−/−</sup> heterozygote mice, were maintained *in vitro* in the presence of recombinant Shh protein. Because *PTCH1* is a target of Hh signaling, the *Ptc-1-lacZ* transgene allowed us to monitor Hh signaling by following β-galactosidase activity. Shh addition to the embryonic skin caused strong activation of its signaling pathway (Fig. 2A and B). Histological analysis of the Hh-treated skin showed large nodular clusters of basal cells (basaloid nests), absent in vehicle treated samples, and reminiscent of the basal cell islands observed in BCC (Fig. 2D and E). These nodular clusters appeared as soon as 4 days after protein treatment (data not shown), but only developed fully after 6 days. Intense X-gal stain, reflecting elevated Hh signaling, appeared restricted to these nodular clusters (Fig. 2E), except for staining associated with some basal keratinocytes and hair follicles, also known to be sites of Hh signaling.

The Hh-induced basaloid nests showed various features observed in human BCC lesions and in mouse BCC models (14, 15, 31). First, these nodular structures were observed with quantitative RT-PCR (TaqMan) analysis (Fig. 3, which is published as supporting information on the PNAS web site).
CUR61414 Blocks the Formation and Induces the Regression of Basaloid Lesions. When added with Shh to embryonic skin punches, CUR61414 blocked Hh-induced signaling in a dose-dependent manner (Fig. 2B and C and Fig. 8, which is published as supporting information on the PNAS web site). This was seen in decreased β-galactosidase activity and in decreased levels of Gli-1 message (data not shown). Inhibition of Hh signaling led to complete prevention of lesion formation. This was confirmed by careful histological examination of the cultured skin (Fig. 2F).

Next, we examined the effect of CUR61414 on basaloid nests that were preestablished by 6 days of skin punch treatment with Shh (Fig. 3A). The punches were exposed to either vehicle or CUR61414 in the continued presence of Shh for an additional 4 days. Histological analysis showed that CUR61414 significantly decreased the size and number of basaloid structures so that they became barely detectable (Fig. 3B). Importantly, this compound did not appear to affect normal cells in the epidermis and dermis (Fig. 3B). Accordingly, the expression profiles of normal epidermal markers appeared unchanged in compound-treated skin (Fig. 9, which is published as supporting information on the PNAS web site).

To investigate how CUR61414 induced regression of the basaloid nests, skin punches with established lesions were exposed to this compound for only 48 h to determine effects on proliferation and apoptosis of cells inside the nests and in the remaining normal areas of skin. Proliferation in the basaloid nests was significantly reduced after CUR61414 treatment (Fig. 3C and D; Table 1), whereas the proliferation of normal basal keratinocytes appeared unaffected (Fig. 3D). In addition, the number of apoptotic nuclei within those structures was significantly elevated in the compound-treated samples (Fig. 3E and F; Table 1). Again, hardly any apoptotic cells were observed outside of the basaloid nests (Fig. 3F). The caspase inhibitor zVAD blocked CUR61414-induced cell death in these lesions, but did not affect CUR61414’s inhibition of proliferation in the basaloid nests (data not shown). These data suggest that specific inhibition of proliferation and growth arrest followed by apoptosis resulted in disappearance of the treated BCC-like basaloid lesions.

CUR61414 Shrinks UV-Induced Basaloid Lesion in Adult Mouse Skin. Finally, we wanted to test the efficacy of the Hh inhibitor against basaloid lesions induced when Ptc 

April 15, 2003

Table 1. CUR61414 blocks proliferation and induces apoptosis in basaloid lesions in mouse embryonic skin punches

<table>
<thead>
<tr>
<th>Treatment of skin punches</th>
<th>Mitotic index, %*</th>
<th>Apoptotic index, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>35.63 ± 5.38</td>
<td>4.83 ± 0.92</td>
</tr>
<tr>
<td>CUR61414 (1 μM)</td>
<td>10.46 ± 4.13</td>
<td>31.75 ± 4.17</td>
</tr>
<tr>
<td>CUR61414 (5 μM)</td>
<td>7.71 ± 2.63</td>
<td>34.33 ± 6.71</td>
</tr>
</tbody>
</table>

Skin punches were first treated with ShhOCT followed by treatment with [ShhOCT + Vehicle] or [ShhOCT + CUR61414] for another 48 h. The average number of cells per lesion was 24 (100%).

*Percent PCNA-labeled cells per lesion.
†Percent TUNEL-labeled cells per lesion.
‡0.1–0.5% DMSO.
D). Treatment with CUR61414 caused regression of these lesions, as observed by both X-gal staining (Fig. 4 A and B; Table 2) and histological analysis (data not shown). Regression in the CUR61414-treated punches appeared to be the result of massive cell death, as a significant increase in apoptotic nuclei was observed in basaloid nests after treatment (Fig. 4 F–K). Again, no overt toxicity or noticeably increased apoptosis was observed in the skin surrounding the lesions (Fig. 4 E).

Table 2. CUR61414 induces regression of UV-induced basaloid lesions in skin from adult Ptc1/2-lacZ heterozygote transgenic mice

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Treatment of skin punches</th>
<th>Number of basaloid lesions$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>50.50 ± 5.30</td>
</tr>
<tr>
<td>2</td>
<td>CUR61414</td>
<td>15.75 ± 5.70</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>12.00 ± 1.41</td>
</tr>
<tr>
<td></td>
<td>CUR61414</td>
<td>4.00 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>20.25 ± 6.20</td>
</tr>
<tr>
<td></td>
<td>CUR61414</td>
<td>9.75 ± 3.60</td>
</tr>
</tbody>
</table>

*Four-millimeter skin punches were generated from the irradiated mice and cultured for 96 h in vitro, with vehicle (0.5% DMSO) or CUR61414 (5 μM).

†Average number of lesions across four skin punches.

Discussion

A substantial body of information suggests that the Hh signaling pathway, a key regulator of embryonic development, is involved in the initiation of BCC (32, 33), the most common cancer in the western world. The data consistently support the view that prolonged and uncontrolled stimulation of this pathway promotes basal cell proliferation and formation of the generally nonmetastatic basaloid nests that define BCC. Thus, we were interested in testing whether potent small molecule inhibitors of this pathway could be used to treat this particular type of cancer, either by causing tumor regression or, at least, by preventing tumor recurrence or growth of new tumors. That this might indeed occur is supported by a recent publication by Berman et al. (20), showing that cyclopamine can inhibit the growth of medulloblastoma cells whose growth is stimulated by activation of the Hh signaling pathway.

To accomplish this, we set up a screen for inhibitors that act downstream of Ptc-1, the protein most commonly mutated in this disease. We identified several different chemical classes of inhibitors, each of which strongly inhibits Hh signaling in all test systems to which they have been applied to date. CUR61414 is a synthetic inhibitor that specifically binds to and antagonizes the activity of Smo in the Hh signaling pathway (24). Similarly, in a recent study by Chen et al. (28) several other classes of small synthetic modulators of Hh signaling were presented that bind to Smo.

CUR61414 has physiochemical properties (e.g., size, ease of synthesis, solubility, etc.) that make it a desirable candidate for drug development. In addition, CUR61414 has certain pharmacological properties not described in this paper, such as prolonged inactivation of the Hh pathway after a single application and lack of observable toxicity in preclinical testing that also make it a suitable drug candidate.

To validate the Hh inhibitors in a more BCC-like setting, we set up two novel skin punch culture systems. One is based on treating embryonic skin punches for several days with Shh protein, thereby providing a tissue culture model similar in most regards to the transgenic mouse model described by Oro et al. (14). This culture system is easy to set up, and BCC-like basaloid nests, with characteristic morphology and markers, form in a few days. Compounds can be readily tested under these circumstances. When administered together with Hh protein, Hh antagonists were able to block the formation of the lesions, but had no effect on normal keratinocytes orstromal cells in the skin. Hh inhibitors were also tested for their ability to cause regression of preexisting lesions. CUR61414 was able to decrease the rate of proliferation of cells within the tumors significantly without affecting proliferation outside of the lesions. Correspondingly, CUR61414 increased the rate of apoptosis of tumor cells without causing an obvious increase in apoptosis of normal skin cells.
Additionally, no nonspecific toxicity was seen even after more than a week of treatment with CUR61414. This finding suggests that CUR61414 acts as an anti-proliferative agent affecting specifically cells in which proliferation is driven by activation of the Hh pathway (as in the basaloid lesions). These cells, prevented from dividing, die by apoptosis. However, normal skin cells, where proliferation is not dependent on Hh signaling, are not affected by the presence of CUR61414. These effects are quite different from what would be observed if skin punches were treated with an anti-mitotic agent, such as 5-flourouracil.

Although the embryonic skin punch assay offers many advantages for testing compounds in vitro, the Hh-induced basaloid lesions are unlikely to have accumulated additional mutations commonly found in human BCC, notably p53 and Ras (11, 34, 35). Some of those mutations, however, have been found in a mouse BCC model, as described by Aszterbaum et al. (15). *Pch*-i mice were subjected to long periods of UV irradiation, resulting in small, but typical, BCCs throughout their skin. To rule out the possibility that the mutations residing outside the Hh signaling pathway might influence the efficacy of the Hh inhibitor, skin punches from the UV-irradiated mice were placed in culture under conditions in which secondary mutations have accumulated.

To confirm that these compounds are effective when other nonspecific effects on normal skin cells. Future work will be needed to confirm that these compounds are effective when applied to lesions in vivo.

Finally, as the involvement of the signaling pathways that regulate embryonic development (e.g., Hedgehog, Wnt, transforming growth factor β; refs. 36–38), in the control of tumor growth becomes clearer, the convergence of developmental biology and oncology will undoubtedly be an area of increasing focus. It will be important to determine whether it is possible to cause regression of tumors in which secondary mutations have accumulated by inhibiting the primary pathways involved in their initiation. This would represent a truly novel way of treating certain types of cancer.

We thank John Lydon and Ricky Sanchez for histology assistance, Jean Flanagan, Sarah Leiker, and JoAnn Wicker for technical help, David Bumcrot for valuable discussions, and Doug Barker and Jane LaLonde for editorial assistance.

<table>
<thead>
<tr>
<th>No.</th>
<th>Receptor</th>
<th>Subtypes</th>
<th>Species</th>
<th>No.</th>
<th>Receptor</th>
<th>Subtypes</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adenosine</td>
<td>A₁,A₂,A₃</td>
<td>H</td>
<td>16</td>
<td>Melanocortin</td>
<td>MC4</td>
<td>H</td>
</tr>
<tr>
<td>2</td>
<td>Adrenergic</td>
<td>α₁A,α₂A,α₁B,α₂B,β₁,β₂</td>
<td>H, R</td>
<td>17</td>
<td>Melatonin</td>
<td>M₁, M₂, M₃, M₄</td>
<td>Ch</td>
</tr>
<tr>
<td>3</td>
<td>Angiotensin</td>
<td>AT₁,AT₂</td>
<td>H</td>
<td>18</td>
<td>Muscarinic</td>
<td>M₁₂,M₂₂,M₃₂,M₄₂</td>
<td>H</td>
</tr>
<tr>
<td>4</td>
<td>Bombesin</td>
<td>R</td>
<td></td>
<td>19</td>
<td>Neuropeptide</td>
<td>Y₁,Y₂</td>
<td>H</td>
</tr>
<tr>
<td>5</td>
<td>Bradykinin</td>
<td>B₁</td>
<td>H</td>
<td>20</td>
<td>Neurotensin</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Calcitonin</td>
<td>H</td>
<td></td>
<td>21</td>
<td>Opiate</td>
<td>δ-κ-μ</td>
<td>H</td>
</tr>
<tr>
<td>7</td>
<td>Cannabinoid</td>
<td>CB₁,CB₂</td>
<td>H</td>
<td>22</td>
<td>Platelet Activating Factor (PAF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Chemokine</td>
<td>CCR5-CXCR1-CXCR2</td>
<td>H</td>
<td>23</td>
<td>Purinergic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Cholecystokinin</td>
<td>CCK₄, CCK₅</td>
<td>H</td>
<td>24</td>
<td>Serotonin 5-HT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Dopamine</td>
<td>D₁,D₂,D₃,D₄,D₅</td>
<td>H</td>
<td>25</td>
<td>Tachykinin</td>
<td>NK₁,NK₂,NK₃</td>
<td>H</td>
</tr>
<tr>
<td>11</td>
<td>Endothelin</td>
<td>ET₁,ET₂</td>
<td>H</td>
<td>26</td>
<td>Thromboxane</td>
<td>A₂(TXA₂)</td>
<td>Rb</td>
</tr>
<tr>
<td>12</td>
<td>GABA</td>
<td>Agonist Site</td>
<td>R</td>
<td>27</td>
<td>Thryrotropin Releasing Hormone (TRH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Galanin</td>
<td>H</td>
<td></td>
<td>28</td>
<td>Vasactive Intestinal Peptide (VIP)</td>
<td>VIP₁</td>
<td>H</td>
</tr>
<tr>
<td>14</td>
<td>Histamine</td>
<td>H₁,H₂,H₃</td>
<td>R, GP</td>
<td>29</td>
<td>Vasopressin</td>
<td>V₁</td>
<td>H</td>
</tr>
</tbody>
</table>

CUR61414 (3 µM) was incubated with the different GPCRs to determine the effect on binding of the natural ligands. H, human; Rb, rabbit; R, rat; M, mouse; Ch, chicken. Non-human subtypes are underlined. All IC50s were between ≤30% stimulation and ≤30% inhibition, and therefore not considered significant.