

studies carried out in models that test compounds by direct inoculation of small xenograft tumours, it is not appropriate to compare these data with results obtained from supermodels in which large tumour volumes were challenged by physiological, systemic drug delivery. The question remains which model is more predictive of clinical outcome? Like the fashion industry, I think I will place my money on the supermodels.

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 The author was previously an unpaid consultant with Genentech (who are developing a SMO inhibitor to treat cancer) on the HH pathway.

Tom Curran in Children's Hospital of Philadelphia, Philadelphia, PA, USA
 Tel: +1 267 426 2819; Fax: +1 267 426 2791
 E-mail: currant@email.chop.edu
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On hedgehogs and human cancer

Ariel Ruiz i Altaba and Frédéric Varnat thank the editor for inviting a response to Dr. Curran's letter, which questions the specificity of cyclopamine and the xenografts used in our recent work on human sporadic colon carcinomas (CCs) (Varnat et al, 2009). To respond, in detail, we address five issues.

First, a key issue is when and where is the pathway active in order to know if effects of HH-GLI antagonists can be specific. We and others established that *GLI1* transcription is, so far, the only reliable and general HEDGEHOG-GLI (HH-GLI) pathway activation marker (e.g. Lee et al, 1997), and that it is expressed in many kinds of sporadic human tumours. Antisense ribonucleic acid (RNA) *in situ* hybridizations on fresh patient-derived tissues have unambiguously shown the tumour cells themselves, not the surrounding stroma, express *GLI1* and other HH-GLI pathway components in basal cell carcinomas, melanomas, gliomas, medulloblastomas, prostate and colon cancers (Clement et al, 2007; Dahmane et al, 1997; Sanchez et al, 2004; Stecca et al, 2007; Varnat et al, 2009). Recent *in situ* hybridization images of pancreatic cancer are undecisive (see Lauth et al, 2010). In cases

where antibodies to pathway components have been used, strong labelling is found in the tumour cells themselves (e.g. Varnat et al, 2009; see also www.proteinatlas.org). So far there is no evidence of exclusive pathway activity in human stromal cells. In contrast, mice display paracrine Hh signalling in several organs but the use of human xenografts to determine mode of action appears inappropriate as these are chimeras. The epithelial tumor cells of human carcinomas are thus main targets of HH-GLI antagonists.

Second, is cyclopamine specific? The plant alkaloid cyclopamine, discovered by R. Keeler over 40 years ago has been extensively used (with over 400 entries in PubMed) to specifically block HH signalling. Cyclopamine induces cyclopia in newborns from mothers of several species that eat the producing plant *Veratrum californicum*, the mouse *Shh* KO mimics this phenotype and the labs of Roelink and Beachy showed that cyclopamine acts on cells that receive the *Shh* signal by blocking Smoothed (Smo; SMOH in humans) a key transducer of Hh signals (e.g. Chen et al, 2002).

Cyclopamine can block HH-GLI signalling in human sporadic tumour cells

in vitro, specifically decreasing *GLI1* messenger RNA (mRNA) levels, at concentrations ranging from 1 to 10 μ M showing dose-dependent effects, modulated by serum levels. The best tests for specificity rely on mimicry by targeting SMOH with RNA interference (RNAi), the use of insensitive SMOH mutants and pathway epistatic analyses. Targeting SMOH through lentivector-mediated short hairpin RNA (shRNA) silencing mimics the effects of cyclopamine in multiple human tumour cells (Clement et al, 2007; Stecca et al, 2007; Varnat et al, 2009). The effects of 1–10 μ M cyclopamine in CCs are rescued by expressing *GLI1* or inhibiting suppressor of fused, positive and negative pathway elements, respectively, that act downstream of SMOH (Varnat et al, 2009; Frédéric Varnat and Ariel Ruiz i Altaba in preparation). *SMOH* RNAi is also rescued by *GLI1* expression in gliomas and CCs (Clement et al 2007; Varnat et al, 2009). Furthermore, expression of a constitutively active form of Smo (SmoA1) renders cells insensitive to 5 μ M cyclopamine (Kim et al, 2010), and expression of N-Myc, a *GLI* target, in cerebellar cells rescues the effects of cyclopamine (Kessler et al, 2009). Claims that treatments with 1–10 μ M cyclopamine are universally toxic or non-specific are thus unfounded.

While Sasai et al (2006) find that HH signalling is repressed in medulloblastoma cells *in vitro*, several labs have proven activity in these cells (e.g. Eberhart, Gulino, Watkins). Indeed, results from over 35 labs (including those of Beachy, Dierks, Ingham, Nusslein-Volhard, Matsui, Melton, Reya, Robbins, Roussel, Scott, Tabin and Watkins) on species ranging from humans to fish support the specificity of this drug.

Third, is our conclusion that HH-GLI signalling is essential in human sporadic CCs based solely on data with cyclopamine? No. We have provided parallel lines of evidence in favour of a key role of HH-GLI in tumour cells using RNAi: independent, 21-nucleotide siRNAs to *GLI1* block prostate cancer cell proliferation and lentivector-encoded shRNAs to *SMOH* or expression of *GLI3R* block melanoma, glioma and CC cancer proliferation, promoting apoptosis (Clement et al, 2007; Sanchez et al, 2004; Stecca

et al, 2007; Varnat et al, 2009). Conversely, the HH-GLI pathway can be further enhanced by shRNAs to *PTCH1*, an inhibitor of SMOH and by increasing GLI1 levels in CCs (Varnat et al, 2009). We show that *in vitro* and *in vivo* these actions are mutually and epistatically rescued. Injection of cyclopamine into GLI1-overexpressing tumours had no effect (Varnat et al, 2009), and the growth of cell line and primary CCs in xenografts was abolished by knock-down of SMOH but rescued by co-expression of GLI1 (Varnat et al, 2009). The detailed results shown with a large number of controls in Varnat et al (2009) have solved the controversy on the role of HH-GLI signalling in CCs in favour of an absolute requirement.

Fourth, in what concerns the CC and melanoma xenografts we have used we note that cyclopamine could eradicate tumours of 10 mm³ (Stecca et al, 2007; Varnat et al, 2009), but also ten times larger (100 mm³; Stecca et al, 2007). Moreover, when these tumours were fully 'cured' and cyclopamine treatment stopped, they recurred aggressively. We had to treat for 20 additional days to prevent all recurrences, as assessed even 1 year after cessation of treatment, and kill any remaining tumour stem cells (Stecca et al, 2007; Varnat et al, 2009). The animals so treated did not show any signs of non-specific damage.

It is important to note that systemic intraperitoneally delivered cyclopamine also blocks metastatic growth in the lungs of mice injected intravenously with human melanoma cells (Stecca et al, 2007). Here, cyclopamine treatment was

started 2 weeks after injection, to make sure that the cells had time to seed the target tissues. No side effects were observed. Critically, similar results were obtained by blocking HH-GLI signalling with cell-autonomous *shSMOH* in CCs (Varnat et al 2009), and conversely, the number and size of metastases were enhanced by knockdown of *PTCH1* or increased expression of GLI1.

These *in vivo* assays provide critical insights into the role of HH-GLI signalling in human cancer, whether they fall into suggested regimens by the USA National Cancer Institute, pharma or not.

Fifth, in parallel with xenografts, cyclopamine treatment is effective against endogenous tumours in mouse genetic cancer models. For instance, systemic cyclopamine treatment prevented medulloblastoma growth in *Ptch1*^{+/-}; *p53*^{-/-} mice (Sanchez and Ruiz i Altaba, 2005), and the growth *in vitro* and *in vivo* of *Tyr*->*NRAS*^{Q61K}; *Ink4A*^{-/-} sporadic melanomas, which express *Gli1*, was inhibited by cyclopamine treatment (Stecca et al, 2007). In addition, mice lacking *Apc* develop intestinal adenomas, whereas those lacking *Apc* and *Smo* simultaneously (*Apc*^{fl/fl}; *Smo*^{fl/fl}; *CreTam*) do not, and the effect of deleting *Smo* is mimicked by systemic cyclopamine treatment (Varnat et al, 2010). Finally, other groups have shown that cyclopamine treatment and deletion of *Smo* also yield similar results in mouse liquid tumours.

Taken together, the data fully support the specificity of cyclopamine under the conditions used and the essential and intrinsic role of HH-GLI signalling in a

plethora of human tumours, including CCs as described in Varnat et al. More generally, the lack of effective antimetastatic drugs begs for new imaginative, detailed and rigorous approaches, whether these are fashionable or not.

The authors declare that they have no conflict of interest.

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A. Ruiz i Altaba is a consultant of Phistem. He has no financial interest in cyclopamine. We apologize for the omission of many important references that could not be included due to space restrictions.

A. Ruiz i Altaba and F. Varnat in Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland
 E-mail: Ariel.RuizAltaba@unige.ch
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