

Induction of tumor-derived hedgehog ligand by chemotherapy



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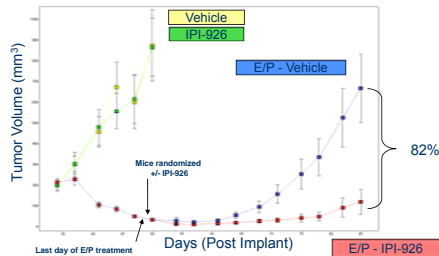
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Abstract # 323

ABSTRACT

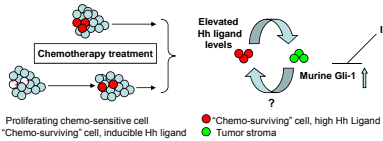
The activity of the small molecule Hedgehog (Hh) pathway inhibitor (IPI-926) has been reported in LX22 xenografts – a primary model of human small cell lung cancer (SCLC) (Travaglione et al., AACR 2008). In these studies, daily oral administration of IPI-926 resulted in a substantial tumor growth delay after debulking the tumor with etoposide and carboplatin (EP) chemotherapy. These data suggested that the Hh pathway is important in SCLC regrowth post chemotherapy, however, the mechanism of action for IPI-926 was unclear. Follow up studies in the LX22 model were designed to examine Hh pathway modulation by IPI-926 post EP treatment. Hh ligand, specifically Indian Hh, is up-regulated in the tumor cells following chemotherapy, as measured by RT-PCR. Further studies demonstrate that stromal derived murine Gli-1 was induced in response to tumor derived ligand. Murine Gli-1 expression remained elevated compared to the expression level in naive tumors for at least 14 days post the cessation of EP treatment and was fully inhibited by IPI-926. Up regulation of tumor-derived Hh ligand post-chemotherapy may confer upon the surviving cell population a dependency upon the Hh pathway that is important for tumor recurrence. These findings are consistent with the observed paracrine cross-talk between the tumor and the surrounding stroma previously shown to be important for Hh signaling. Induction of Hh ligand post chemotherapy was also studied in bladder and ovarian cancer tumor models and the underlying mechanism explored. *In vivo* mice bearing UMUC3 bladder cancer xenografts were treated with 100 mg/kg Gemcitabine 2 times/week for 2 weeks. Tumors from these treated mice had increased IHH expression similar to that observed in the LX22 model. In addition, *in vitro* studies showed that in UMUC3 cells exposed to either doxorubicin or gemcitabine for 12-24 hours, all 3 Hh ligands (Sonic, Indian and Desert) are up-regulated. To determine if cellular stresses other than chemotherapy up-regulate ligand expression, UMUC3 cells were exposed *in vitro* to various stressors including hypoxia. Compared to normoxic controls, Sonic Hh ligand expression was increased under hypoxic conditions. In summary, multiple tumor types show up-regulation of Hh ligands post chemotherapy and other cellular stressors, suggesting that a surviving sub-population is dependent upon the Hh pathway and thus susceptible to Hh pathway inhibition. Elevated Hh ligand expression could impart a survival advantage to a subpopulation of tumor cells, promoting their tumorigenic properties following chemotherapy. Taken together, these results provide strong rationale for testing Hedgehog inhibitors in clinical indications such as SCLC, bladder, or ovarian cancer that are initially chemo-responsive but eventually relapse.

SCLC *in vivo* efficacy

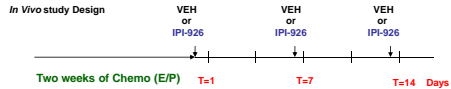


When the LX22 xenografts reached 200mm³ they were randomized into four groups. The Vehicle group grew rapidly and IPI-926 as a single agent (40mg/kg, QD) had no effect. However, the mice treated with SOC agents etoposide/carboplatin responded rapidly with observable regression while on treatment. At the end of chemotherapy, the mice were either treated with vehicle or IPI-926 (40mg/kg, QD). The vehicle treated group re-grow, while the IPI-926 now showed anti-tumor activity, and substantially inhibited the re-growth of these tumors.

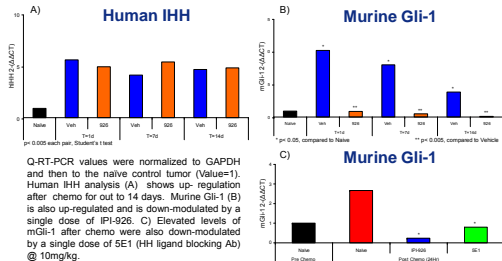
Chemotherapy induced Hedgehog (Hh) ligand



Induced Hh ligand leads to murine Gli-1 response

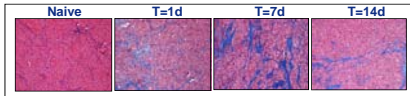


Following treatment with SOC agents etoposide/carboplatin, a single dose of IPI-926 (40mg/kg) was given 24 hours prior to LX22 tumor collection at the various time points including: 1, 7 or 14 days post the cessation of chemo (N= 4 per group). Tumors were collected for Q-RT-PCR analysis and histological evaluation.



Q-RT-PCR values were normalized to GAPDH and then to the naive control tumor (Value=1). Human IHH analysis (A) shows up-regulation after chemo for out to 14 days. Murine Gli-1 (B) is also up-regulated and is down-modulated by a single dose of IPI-926. C) Elevated levels of mGli-1 after chemo were also down-modulated by a single dose of SE1 (Hh ligand blocking Ab) @ 10mg/kg.

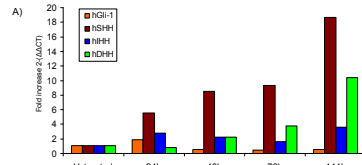
Masson Trichrome Stain



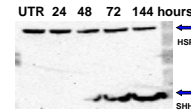
The naive LX22 tumor displays thin "rivers" of stroma (100x). The Masson Trichrome stain highlights the infiltration of stroma that occurs at time points 1, 7, and 14 days after chemotherapy (EP) treatment.

Multiple chemotherapies induce Hh ligand

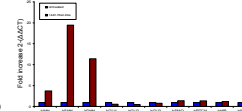
Induction of Hh ligand expression in UMUC-3 cells post Doxorubicin treatment



SHH protein levels

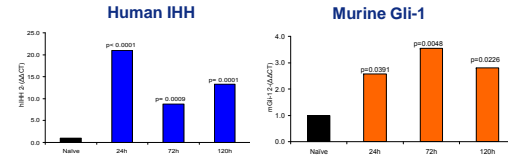


Analysis of various Hh pathway genes



UMUC-3 (bladder) cells were treated *in vitro* with 500nM Doxorubicin for 24 hours. A) Cells were collected at either 24, 48, 72 or 144 hours post dose for RT-PCR and Western analysis. A similar response to 200nM Gemcitabine treatment was also seen at both the RNA and protein level (data not shown). C) Analysis of various Hh pathway members at 144 hours post Doxorubicin, shows the induction is ligand specific and does not impact autocrine Hh pathway activity.

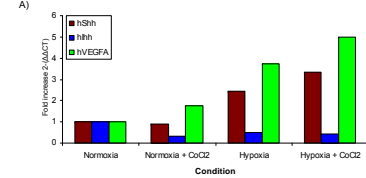
In Vivo induction of ligand in the UMUC3 bladder xenograft post Gemcitabine treatment



The UMUC3 bladder xenograft was treated with Gemcitabine @ 100mg/kg, i.p., QD3x4. After the last dose of Gemcitabine, tumors were collected at either 24, 72, or 120 hours, and processed for Q-RT-PCR analysis. A) Human IHH ligand and B) murine Gli-1 were up-regulated out to 120 hours after chemotherapy.

Stress induced Hh ligand up-regulation

Ligand is un-regulated in UMUC3 cells cultured for 16H in hypoxic conditions



Western blot analysis of HIF-1α

Multiple cellular stresses were tested *in vitro* on the UMUC3 cell line. In the above experiment, the UMUC3 cells were tested under normoxic (21%O₂/5%CO₂) or hypoxic (1%O₂/5%CO₂, BaH₂) conditions. Cobalt (CoCl₂) was added as a chemical mediator of hypoxia, as measured by stabilization of HIF-1α. VEGFA is a transcriptional target of HIF-1α, and was also measured to indicate a hypoxic state. At the 16hr time point, cells were collected for A) q-RT-PCR analysis of Hh ligand genes and B) western analysis of HIF-1α. Other cellular stresses tested were glucose deprivation, serum starvation, oxidative stress, low pH, and heat shock. Only glucose deprivation resulted in a similar increase in ligand expression, therefore ligand up-regulation was not a general response to all cellular stresses.

Summary/Conclusions

We have previously reported in a primary SCLC tumor model, the anti-tumor activity of IPI-926 only after cytoreduction by chemotherapy. We now show that after chemotherapy, mRNA levels of Hh ligand are elevated which leads to murine Gli-1 up-regulation. *In vivo*, the up-regulated murine Gli-1 levels are completely down-modulated by IPI-926. The phenomenon of tumor Hh ligand induction extends to multiple tumor types, chemotherapies, and certain cellular stresses, such as hypoxia. In conclusion:

- *In Vivo*, induction of tumor derived ligand by chemotherapy leads to murine stromal Gli-1 up-regulation and response to IPI-926.
- Multiple tumor types show up regulation of Hh ligand after chemotherapy or certain cellular stresses. Up regulation of ligand could provide a survival advantage to the cells dependent on the Hh pathway.
- These results provide a strong rationale for testing Hedgehog inhibitors in clinical indications such as SCLC, ovarian and bladder cancers that are initially chemo-responsive, but eventually relapse.