# Effect of the Hedgehog-inhibitor cyclopamine on mice with osteosarcoma pulmonary metastases

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**Abstract.** Chemoresistant metastases of osteosarcoma in humans limit survival in approximately one third of patients. Furthermore, aggressive chemotherapy can lead to side effects and occurrence of secondary malignancies in long time survivors. Therefore, supplemental medical strategies are worthwhile. The well-directed manipulation of cancer-signaling-cascades is an appealing approach. Targeting of the Hedgehog-pathway in cancer has led to promising results *in vitro* as well as *in vivo* in a number of different tumor types. Recently, the impact of cyclopamine, which inhibits Hedgehog signaling by binding to the receptor Smoothened, was shown in different human osteosarcoma cell lines *in vitro* and *in vivo*. In the present study we examined the influence of cyclopamine on early pulmonary metastases *in vivo*. Murine osteosarcoma cells, OS-50, were injected into the lateral tail vein of young BALB/c mice. Treatment with subcutaneous cyclopamine injections began after three days. Two weeks later, the animals were sacrificed and the number of pulmonary metastases was counted. We could observe a trend towards decreased metastases in the cyclopamine group (~20%). On the other hand, remarkable side effects were caused by the cyclopamine/ethanol/triolein preparation (mainly skin ulcerations).

## Introduction

Osteosarcoma is the most frequent primary malignant bone tumor, occurring mostly in children. Although the majority of cases are chemosensitive to drug regimen containing adriamycin, cisplatin and to high-dose methotrexate, ~30-40% of patients are primarily refractory and possess a poor long term prognosis (1). Owing to the lack of alternative treatment options further therapeutic strategies need to be explored.

To reduce therapy side effects, especially in children, an appealing approach is the direct targeting of cancer signaling pathways. The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer (2). In osteosarcoma, the Hedgehog (Hh) pathway is aberrantly activated, and targeted inhibition of the Hedgehog receptor Smoothened has shown to induce growth arrest and apoptosis of osteosarcoma cells *in vitro* and *in vivo* (3,4).

At present, the prevalent agent for Smoothened inhibition is cyclopamine, a steroidal alkaloid isolated from the corn lilly (*Veratrum californicum*). Cyclopamine binds directly to Smoothened, thereby inhibiting the signal transduction to the nuclear target gene *GLI* (5). Inhibition of the Hh signaling pathway has also shown promising results in experimental settings for other Hh-dependent tumors, e.g. medulloblastoma, breast cancer, gastrointestinal tumors, prostate and skin cancers (6-13).

The Hh signaling pathway is known to regulate crucial mechanisms of proliferation and differentiation during embryonic development. Moreover, in adult tissues Hh signaling is one pathway that controls stem cell behavior and, as mentioned above, aberrant Hh signaling is implicated in a variety of tumor entities (14). Finally, Hh genes are deeply involved in skeletal development. In the Drosophila-embryo, the organs eye, wing and leg are patterned by Hh. In vertebrates, Indian Hedgehog (IHh), one of the three vertebrate homologues of Drosophila Hh, regulates the process of endochondral ossification. Moreover, it has a direct proliferating effect on chondrocytes (15,16).

Sonic Hedgehog protein (SHh) is the best-investigated vertebrate homologue of Drosophila Hh. In vertebrate embryos, SHh is expressed in the floor plate, the notochord and in the limb bud.

It has been shown that explanted presomitic chicken mesoderm differentiates into cartilage only when cultured in the presence of the SHh protein. In this differentiation pathway the expression of bone morphogenetic proteins seems to be a downstream-target of SHh action (17).

Subcutaneously implanted fibroblasts expressing SHh protein or IHh protein are able to induce cartilage formation followed by endochondral ossification in nude mice (18).

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Constitutively activated Hh signaling due to a mutated parathyroid hormone-related protein (PTHrP) receptor can lead to enchondromatosis (Ollier and Mafucci diseases) and transgenic mice expressing the Hh transcriptional regulator GLI-2 develop enchondromatosis-like lesions (19). In our previous studies we demonstrated apoptosis of different human osteosarcoma cell lines *in vitro* under the influence of cyclopamine (4). Another recent study found that Hh inhibition slowed the growth of osteosarcoma xenografts in mice (3). Since pulmonary metastasis of chemoresistant osteosarcomas is one of the major reasons leading to death in affected patients, we now aimed to examine the impact of cyclopamine on early osteosarcoma pulmonary metastases in mice.

## Materials and methods

*Cell culture*. OS-50 cells were obtained from Professor Jörg Schmidt (Munich, Germany). This line was obtained from the primary culture of a spontaneous osteosarcoma which arose in the distal femur of an 894-day-old female Balb/c mouse (20). Cells were grown in DMEM (Dulbecco's modified Eagle's medium, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine and incubated at 37˚C in a 5% $CO_2$ water saturated atmosphere. Cyclopamine was purchased from LC Laboratories (Woburn, MA, USA). It was dissolved in ethanol, yielding a stock solution of 20 mg/ml. Tomatidine was obtained from Merck (Darmstadt, Germany).

*XTT assay*. Cells were plated at a density of $2.5x10^3$ cells/well in a 96-well plate. They were grown 24-72 h in the presence of cyclopamine or tomatidine, which has a similar structure to cyclopamine but lacks Hedgehog inhibition, at 50 $\mu$M in a medium containing 10% FBS. Medium with equivalent ethanol content but without cyclopamine served as an additional control. After 24, 48 and 72 h XTT was added and after 4 additional hours absorbance was measured in an ELISA plate reader with a wavelength of 450 nm and a reference wavelength of 650 nm.

To determine the cytotoxicity of cyclopamine at different concentrations, we seeded OS-50 cells as described above and treated them with various binary logarithmic concentrations from 0.25 to 500 $\mu$M. XTT was added after 72 h and absorption was measured 4 h later.

*Real-time RT-PCR*. Analysis of *GLI-1* expression in cyclopamine and mock treated OS-50 cells was achieved by real-time RT-PCR. RNA was prepared from the osteosarcoma cell line OS-50 24 h after treatment with 25 $\mu$M cyclopamine, tomatidine or a buffer. In brief, total-RNA was isolated using the RNeasy system (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality and amount of the RNA was determined using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE). Contaminating genomic DNA was removed by digestion with the RNase-free DNase kit following the manufacturer's protocol (Qiagen). Each 250 ng of RNA was reverse transcribed using the AffinityScript QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA), following the instructions of the manufacturer. Subsequently, the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on a Stratagene MX3005p QPCR system (Stratagene). The PCR was performed using the primer assay for mouse *Gli-1* (NM_010296, catalog number PPM41530E), purchased from SABioscience, Qiagen. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_008084, catalog number PPM02946E), considered as the reference gene, was measured. A melting curve analysis was applied to ensure the specificity of the PCR. Relative quantification of the mRNA levels of the target genes was determined using the comparative CT (threshold cycle values) method ($2^{-\Delta CT}$ method). The results were presented as fold change to GAPDH gene expression.

*Animal experiments*. Sixteen female 4-5-week-old BALB/c mice were obtained from Harlan Laboratories (The Netherlands). They were housed under standard conditions. After 2 days of acclimation the animals were injected with $5x10^5$ OS-50 cells into the lateral tail vein. Three days later, daily subcutaneous treatment with cyclopamine, solved in Triolein (Sigma, Germany) was initiated. Control animals were injected with Triolein/ethanol. Animals were weighed daily. Due to the serious side effects (skin ulcerations, scrubby coat) observed in preliminary studies we reduced the dosage and all animals received 2 days of 50 mg/kg cyclopamine and afterwards 25 mg/kg every other day. The cumulative dose was about 5 mg/animal.

The mice were sacrificed two weeks later. The lung specimens were examined for experimental pulmonary metastases. For identification of the metastases, the trachea was dissected free from tissue and transsected. Next, 2 ml of India ink solution was injected into the lungs by canulating the trachea until the lung lobes blowed up and stained dark. The lungs and trachea were resected en bloc and placed in Fekete's solution for 24 h. This solution permanently bleached the tumor foci on the lung surface white. The lobes of the mice lung were placed separately between two glass plates. Quantification of the lung metastases was performed in each group of mice by two blinded observers independently.

*Immunohistochemistry*. Metastases visible before ink injection were excised (one animal each for cyclopamine treatment and control) and placed into zinc-formalin. After paraffin-embedding, they were cut into 5 $\mu$m sections. Next they were deparaffinized and antigen retrieval was performed by heating the slides in citrate buffer for 15 min. $H_2O_2$ was added to block endogenous peroxidase. The slides were then incubated overnight at 4˚C with the primary antibody (anti-mouse Ki-67, DAKO, M7249). As a secondary antibody we used HRP-conjugated anti-rat-polymer for 30 min (Histofine, 414311F). For visualization AEC (3-amino-9-ethylcarbazole) peroxidase substrate solution was added. Finally we performed counterstaining with hematoxylin. The Ki-67 index was determined by counting all cells and positive cells in 10 HPF.

## Results

To test the general sensitivity of OS-50 cells to Hedgehog (Hh)-inhibition (which was not previously described) we performed the classic XTT assay. In the first experiment, OS-50 cells were cultured with different concentrations of cyclopamine in medium containing 10% FBS. Three days

Table I. Effect of tomatidine and cyclopamine on the viability of OS-50 cells.

| | 24 hours | | 48 hours | |
|---|---|---|---|---|
| Treatment (25 $\mu$M) | OD | Growth inhibition (%) | OD | Growth inhibition (%) |
| Control | 0.36±0.01 | 0 | 0.66±0.06 | 0 |
| Tomatidine | 0.35±0.02 | 0.9 | 0.54±0.07 | 18.5[a] |
| Cyclopamine | 0.29±0.01 | 18.5[a] | 0.32±0.03 | 51.6[a] |

OD, optical density in the XTT assay. Values are means ± standard deviation (n=8). They were related to OD of control to calculate growth inhibition. [a]Indicates significance compared to control (t-test, P<0.0001).

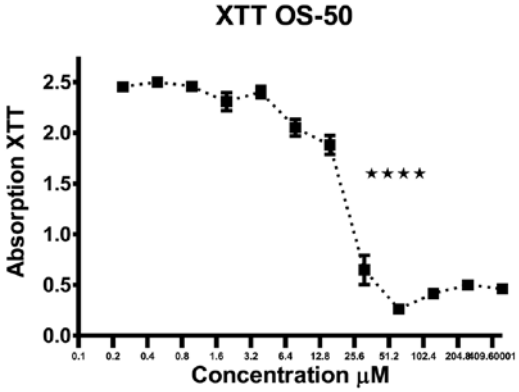


Figure 1. Viability assay of OS-50 cells upon cyclopamine treatment at different concentrations: OS-50 cells were treated with various concentrations of cyclopamine and absorbance at 450 nm was measured after 72 h of treatment and 4 h of XTT incubation. Absorbance is positively correlated with viability. Error bars represent standard error of the mean, asterix indicates significance (XY regression analysis, P<0.0001).

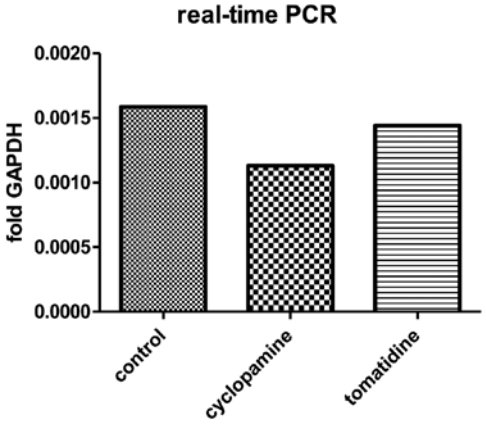


Figure 2. Results of real-time PCR (expression of *GLI-1*) after treatment of OS-50 cells with cyclopamine or tomatidine (25 $\mu$M).

later, the viability of cells was assessed. The highest reduction of viability, about 90%, was observable at a concentration of 62.5 $\mu$M. Between the range of 4 and 62.5 $\mu$M there was a continuously decreasing effect of cyclopamine on the viability of OS-50 cells. At higher concentrations we observed still decreased viability, but the effect was less pronounced (Fig. 1).

To distinguish the effect of Hh-inhibition from the general cytotoxicity of steroidal alkaloids, we repeated the test and compared the effect of cyclopamine vs. the effect of tomatidine, a steroidal alkaloid similar to cyclopamine but lacking receptor activity. At a concentration of 25 $\mu$M we observed a decreased viability of 18.5 percent following tomatidine and 51.6 percent following cyclopamine treatment. The difference of the greater inhibition of cyclopamine is presumably caused by Hh-inhibition (Table I).

The results of the real-time polymerase chain reaction revealed indeed down-regulation of the Hh target gene *GLI-1*, which is a transcription factor that mediates the effect of Hh-signaling. Tomatidine, consistent with its lack of Hh receptor activity, could not demonstrate relevant down-regulation of *GLI-1* in relation to the control (medium with ethanol alone). However, the reduction of *GLI-1* expression after cyclopamine treatment was roughly only 30%, which is not as much as expected (Fig. 2).

To prove now if the cytotoxic action of cyclopamine on OS-50 cells *in vitro* could be replicated in an *in vivo* system, we injected $5x10^5$ OS-50 cells into the lateral tail vein of young BALB/c mice. Three days later, the treatment with subcutaneous cyclopamine in an ethanol/triolein preparation began. Due to the toxic effects of this preparation we had already reduced the dosage and the intervals of application. Nevertheless, almost all animals showed scrubby coat due to the treatment (also control group with only ethanol/triolein injection) and some developed more or less pronounced skin ulcerations at the application site. No animal died but some displayed lethargic appearance the second week. Retrospectively, those were the animals with the highest pulmonal metastasis rate. There was no apparent change of the weight between the groups before and after the treatment. Seventeen days after the inoculation of metastases all mice were sacrificed and the number of pulmonary metastases was determined. We observed only a trend toward decreased metastases in the cyclopamine group with a mean of 20 pulmonary lesions per animal. In the control group we counted a mean of 24.5 metastases per animal. However, the median for cyclopamine treated animals were 4 metastases and the median of the control animals were 21 metastases. But due to high variance of the values we could not state that the differences were significant (Figs. 3 and 4).

Finally, we determined the effects of cyclopamine treatment on the metastastatic proliferative activity by immunhistochem-

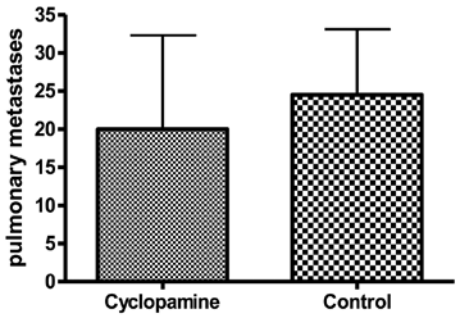


Figure 3. Result of the animal experiments (8 animals each group). Indicated are the numbers of pulmonary metastases and the error bars represent SEM. The differences are not significant.

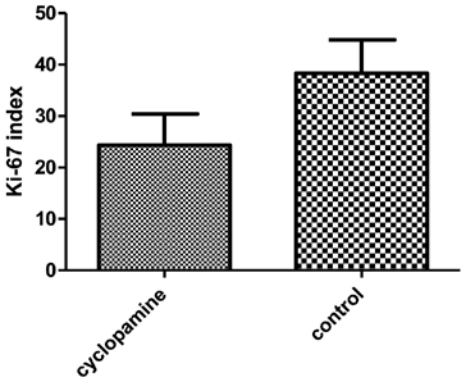


Figure 5. Percentage of immunoreactive metastastic cells after staining with Ki-67 antibody. Error bars represent standard error of the mean (t-test; P= 0.9456).

Figure 4. Lungs of mice with pulmonary metastases of OS-50 cells. Left, lungs after cyclopamine treatment; Right, lungs after control treatment. Lungs were injected with ink and bleached with Fekete's solution afterwards.

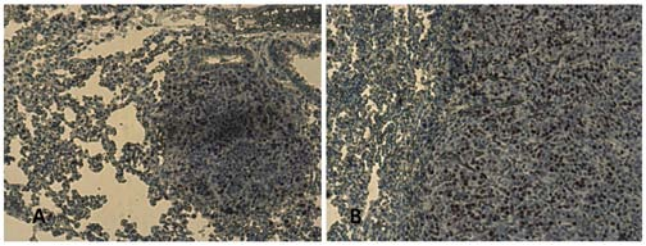


Figure 6. Immunohistochemistry with Ki-67 antibody and counterstaining with hematoxylin. Left image is showing a lung metastasis after cyclopamine treatment. Right image is showing a lung metastasis in a control animal.

istry with the Ki-67 antibody. We observed a distinct trend towards decreased Ki-67 immunoreactivity in the cyclopamine-treated group; however, the differences did not reach statistical significance (Figs. 5 and 6).

## Discussion

Osteosarcomas are often resistant to conventional chemotherapy. Therefore, additional therapeutic options are needed. Because of encouraging results of Hh inhibition in several distinct osteosarcoma cell lines *in vitro* we now aimed to examine the effects of Hh inhibition on osteosarcoma cells *in vivo*. We chose the pulmonary metastasis mouse model (via tail vein injection) since it is easily performable, reproducible and mimics the main clinical problem of osteosarcoma in humans: death by lung metastasis. Unfortunately, only few human cell lines are capable of producing lung metastasis in nude mice (21). In fact, the previously described animal model of the human osteosarcoma cell line U-2 OS in nude mice (22) did not furnish us with reproducible results. Therefore, we tried to establish pulmonary metastases with the murine osteosarcoma cell line OS-50 in BALB/c mice. This reproducibly led to numerous pulmonary metastases. However, *in vitro*, these cells were less sensitive to cyclopamine then several human osteosarcoma cell lines in our previous experiments (4). At neither dosage could we observe complete cell death. Also real-time PCR revealed only modest down-regulation of *GLI-1* expression, the target gene of the Hh-pathway. One possible explanation for this observation is the fact that in the current experiments we used higher concentrations of FBS (10% instead of 2.5%) which might have a cytoprotective effect. When we then examined the effects of cyclopamine on metastases in nude mice we soon observed serious side effects of the treatment with the reported dosage of 50 mg/kg/day. Almost all animals got a scrubby coat and some developed severe skin necroses.

The majority of previously published studies with this dosage in mice reported no side effects. Only one study reported observations similar to ours (23). We therefore had to reduce the dosage and applied about one third of the total cumulated amount of cyclopamine to each animal. Even with this dosage we observed side effects. The total number of pulmonary metastases in the cyclopamine-treated group tended to be lower than in the control group but this difference was not significant. A distinct effect of the cyclopamine treatment could generally not be observed. This finding is again concordant with the findings of Ecke *et al* (23), who treated mice bearing rhabdomyosarcoma xenografts with cyclopamine. The treatment with cyclopamine did not affect growth of those tumors *in vivo* in contrast to doxorubicin which had distinct effects. Furthermore, it was shown in a mouse model carrying medulloblastoma that by blocking the Hh pathway with a small molecule inhibitor at the highest dose, a complete eradication of the tumors was achieved (7).

Our main conclusion from this study is that cyclopamine as a subcutaneous preparation with trioleine and ethanol results

in many side effects which prevent it from achieving a therapeutic dose that can be used in sarcoma treatment. Therefore, future studies should involve other cyclopamine preparations (e.g. water-soluble cyclopamine derivatives) or small Hh inhibitors to prevent these side effects. Nevertheless, we can also not exclude that the cell line we used was particularly insensitive to Hh inhibition, as the results of real-time RT PCR revealed only modest down-regulation of the target gene *GLI-1*.

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