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<td>Project Title: TREATMENT OF NAÎVE AND FLUDARABINE-REFRACTORY CLL IN THE TCL-1 MOUSE MODEL WITH IPI-926 AND OTHER HEDGEHOG PATHWAY INHIBITORS</td>
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**Introduction**

1. Introduction

Recent publications have - both - raised the issue and challenged the significance of the hedgehog (Hh) pathway's role in B-Cell malignancies such as Chronic Lymphocytic Leukemia (CLL).

Research on the Hh pathway and its inhibition has two totally distinct beginnings - one of this two different starting points was the result of a thoroughly planned scientific experiment in developmental biology resulting in the discovery of the Hh gene as a factor in body patterning in drosophila (1), the other a rather random one, just an observation by farmers in Idaho in the 1950ies, bothered by high frequency of misshaped newborn sheep in their herds - which was finally related to the ewe's ingestion of Veratrum californicum, a lily containing cyclopamine which was recognized as a Hh pathway inhibitor, a SMOOTHEND(SMO)-inhibitor specifically (2-5). The connection to cancer research was eventually established by the observation of pathogenetically relevant mutations of PATCHED gene (a Hh pathway component) in familial and sporadic basal cell carcinoma (6, 7) as well as in
medulloblastoma (8) – in vitro testing revealed a potential role of Hh pathway inhibition in different tumor entities (9) and there is no report of in-human activity in medulloblastoma (10). Meanwhile, the role of the Hh pathway in different solid tumor entities is subject to research.

The relevance of Hh signalling cascade (and its inhibition) in B-cell malignancies has been investigated by Dierks et al (11) – revealing that Hh signalling seems to be a part of the pro-survival interaction between stroma and malignant lymphoma cells. Cyclophosphamide inhibited lymphoma growth in vitro and in mice injected with lymphoma cells.

Further investigation regarding the specific significance of Hh pathway in CLL seems interesting, as pathogenesis of CLL is in particular dependent on microenvironmental factors (12).

A first report on in vitro testing of Hh activity in CLL was indeed promising, showing that cyclophosphamide could abrogate pro-survival stimuli of co-culture CML-D cells on patient-derived CLL cells (13). Unfortunately, those early promising in vitro data could not be reproduced by Desch et al (14), suggesting that the effect of SMO-inhibitor cyclophosphamide was rather unspecific and off-target. In addition, there was no consistent evidence of any up-regulation of Hh pathway components, questioning its significance in CLL.

The early promising data mentioned above, led us to investigate whether this in vitro data could be transferred to a in vivo CLL mouse model. In addition to cyclophosphamide, we had another Hh inhibitor available for in vivo testing, IPI-926, a compound with approx. 8-fold potency regarding SMO-inhibition.

On the basis of its involvement in human T-PLL and some B-cell neoplasias, Johnson et al (15) developed a transgenic mouse strain, where the TCL1-oncogene is set under control of B-Cell specific promotor/enhancer sequences, resulting in TCL1-overexpression in the B-cells. After a variable latency period (median 8 months), this TCL1 transgenic mouse strain develops a disease, that immunophenotypically and phenotypically resembles human CLL.

The very similarity of the disease in TCL1-mice and in humans has its challenges as well – disease heterogeneity regarding latency, stage and disease course hampers interpretation of results if only small numbers of mice are tested. We therefore planned to investigate IPI-926 in a NOD-SCID mouse cohort, that was transplanted with splenocytes of diseased TCL1-mice. This would also allow, to test IPI-926 in a disease stage with low tumor-burden – as in a similar setting, promising data have been published regarding IPI-926 treated NOD-SCID mice injected with a precursor B-acute lymphoblastic leukaemia cell line (16).

While implementing our in vivo testing, the above mentioned, more challenging data on in vitro efficacy of Hh inhibition in CLL had been published (14) – we therefore started to investigate in vitro efficacy of IPI-926 in patient-derived CLL cells as well.

**Methods and Results**

### 2. Methods and Results

#### 2.1. Test Cohort 1 of TCL1-mice: Technical Feasibility and Screening for Hh pathway activity

**2.1.1. Aim:**
- test technical feasibility of and gain experience in oral gavaging
- establish reproducible techniques in blood collection and analyses
- establish mRNA-extraction of peripheral animal blood and its further processing
- screen (roughly) for Hh pathway activity

**2.1.2. Methods:**
- Test Cohort 1 with 6 mice (3 TCL1 positive, 3 TCL1 negative);
- IPI-926 40mg/kgKG po daily;
- blood collection and mRNA-extraction on d1 and d3;
2.1.3. Results and Conclusions for next phase:
- oral gavage difficult, high mortality after 7 days (presumably due to gavage) → necesssary to improve gavage technique (with help of infinity personal);
- further refinement in terms of reproducibility of WBC analyses in peripheral blood necessary
- qualitative mRNA-expression analyses show reduced BCL2-expression on d3 after start of IPI-926 application in the two TCL-1 positive mice evaluable on d3 (see example below); these results need to be confirmed in quantitative RT-PCR;

Mouse 1 (m1) tcl-1 positive:

Expression of mRNA before and on d3 of IPI-926 application

![Expression of mRNA before and on d3 of IPI-926 application](image)

2.2. Test Cohort 2 of TCL-1-mice: Re-evaluation of meanwhile improved technical procedures, confirmation of potentially IPI-926 associated changes in mRNA-expression

2.2.1. Aim:
- confirm dramatically reduced gavage-associated mortality rate after training (by infinity personal)
- confirm improved WBC analyses; establish/confirm reproducible IgM+CD19+CD6+CD3- clone detection
- confirm potentially IPI-926 associated changes in BCL2 mRNA-expression seen in test cohort 1; screen again for changes in other Hh pathway components with refined blood collection and mRNA-extraction procedures
- collect survival data in a TCL-1 mouse population treated with IPI-926 +/- Fludarabine

2.2.2. Methods:
- 12 TCL-1 positive mice (cohort size might be extended after interim analyses), stratified for disease stage/parameters (age, WBC, Clone size, activity, clinically visible ascites/splenomegaly)
- Cohort divided in following treatment groups (3+ mice each):
  - vehicle (HPB/CD 5%) 40mg/kg/KG d1,3,5q7d
  - Fludarabine 34mg/kg/KG d1,3,5q14d
  - IPI-926 40mg/kg/KG d1,3,5q7d
  - Fludarabine/IPI-926 upfront combination (same doses/application intervals as above)
  - [Fludarabine upfront, IPI-926 maintenance after reduction of tumor load – group added, if cohort size extended]
- Endpoint assessment:
  - WBC count 2x/week, IgM+CD19+CD6+CD3- clone size 2x/week, weight 1x/week
  - mRNA-extraction (peripheral blood) 1x/week; RT-PCR (qualitative, then quantitative) regarding Hh components/targets (SMO, PTCH1, BCL2, CCND1, GL1-3)
2.2.3. Results:

- Cohort accrual stopped after including 9 mice
- Reasons for early termination of cohort:
  - No sign of gross activity of IPI-926 in treated mice (see below)
  - Heterogeneity of disease manifestation (predominantly leukemic vs splenomegalic), disease stage and course in TCL1-mice precludes proof of moderate (or minor) activity of IPI-926 in a cohort of reasonable sample size
  - Any potential effect of IPI-926 on the self-renewal compartment of TCL1-disease might be obscured in this cohort with in average high tumor burden
- No improvement of WBC in IPI-926-treated mice (and no relevant change in fludarabine cohorts either)

- No change in Clone Size:

- mRNA expression analysis of hedgehog genes: No consistent results; technical problems with quantitative RT-PCR might be the cause of this, however external re-evaluation (done by Infinity personal) neither did reveal any consistent deregulation of mRNA-expression
2.2.4. Conclusions for next step:
- In this Tc1-fg mice test cohort (#2), there is no sign of in vivo efficacy of IPI-926; however, the small sample size, an in average high tumor burden, and heterogeneity in disease stage might obscure any effect and have to be considered in interpretation.
- The above mentioned problems might be circumvented by the use of NOD/SCID mice (homogenous disease) and by a design, that is outlaided for smaller disease burden (cohort with preventive IPI-926, starting application immediately after Tc1-leukemia transplantation into NOD/SCID mice) and a sufficient number of mice.

2.3. Trial Cohort 1&2: Evaluation of IPI-926 in TCL1-transplanted NOD-SCID mice

2.3.1. Aim:
- Evaluate effects of IPI-926 on leukemic engraftment after Tc1-leukemia transplantation into NOD-SCID mice (trial cohort 1 - preventive/mrd-setting)
- Evaluate effects of IPI-926 on survival of leukemic mice (using advantageous homogeneity of disease presentation/course in NOD-SCID mice (with leukemia transplanted from TCL-1 mice) (trial cohort 2 - therapeutic/mrd-setting)

2.3.2. Methods:
- Trial Phase 1 [see figure below]:
  Two treatment groups (10-20 NOD/SCID mice per group):
  treatment starts immediately after Tc1-leukemia transplantation
  - Vehicle (HPBCD 5%) 40mg/kg/KG d1,3,5q7d
  - IPI-926 40mg/kg/KG d1,3,5q7d
  - Endpoint assessment (leukemic engraftment - time to leukemia onset):
    - WBC count 1x/week, IgM+CD19+CD5+CD3- clone size 2x/week, weight 1x/week

- Trial Phase 2 (to be conducted only if any sign of efficacy of IPI-926 in phase 1) [see figure below]:
  Two treatments groups (in total 10-20 NOD-SCID mice (with leukemia transplanted from leukemic TCL-1 mice))
  - Thorough observation of NOD-SCID mice regarding disease onset with regular WBC counts and assessment of clonality
  - Debubbling phase with fludarabine 34mg/kg/BW d1-d5q28d after disease onset (WBC >30G/l or clone size >30%)
  - If response to debubbling phase (WBC <30G/l), then 2 mrd-treatment groups (5-15 animals each group): Treatment starting after disease onset (WBC>30G/l or clone size >30%)
    - Vehicle (HPBCD 5%) 40mg/kg/KG d1,3,5q7d
    - IPI-926 40mg/kg/KG d1,3,5q7d
  - Endpoint assessment (overall survival, time to progression):
    - WBC count 2x/week, IgM+CD19+CD5+CD3- clone size 2x/week, weight 1x/week
    - mRNA-extraction (peripheral blood) 1x/week, RT-PCR (qualitative, then quantitative) regarding Hh components/targets (SMO, PTCH1, BCL2, CCND1, GLI1-3)
2.3.3. Results:
- Phase 1: Time To Leukemia Onset (TTLO)
  - median TTLO for IPI-926 group: 36d
  - median TTLO for vehicle/control group: not reached
  - Log-Rank-Test (Cox-Mantel); p = 0.03

![Time to Leukemia Onset graph]

- no delaying effect regarding leukaemia onset through IPI-926 (in fact, there was even a significant difference in favour of the control group)
**Phase 1: Overall Survival (OS)**
- median OS for IPI-926 group: 65d
- median OS for vehicle/control group: 77d
- Log-Rank-Test (Cox-Mantel): p=0.12

Overall Survival

![Overall Survival Graph]

- no difference regarding Overall Survival between groups treated with IPI-926 and vehicle respectively

**Phase 2:**
- due to lack of any significant effect of IPI-926 in phase 1, phase 2 was not conducted (as prespecified)

2.3.4. Conclusions:
- There is no evidence of any effect of IPI-926 in this in-vivo model with NOD-SCID mice transplanted with TCL1-splenocytes – absence of any effect in delaying disease onset if given as prophylactic treatment, led us to abandon further testing, not to go on into phase 2 respectively.

2.4. In-vitro Testing of IPI-926 (in co-culture with bone marrow stromal cells) – selection of results shown

2.4.1. Aim:
- After controversial results in previous publications, we want to assess in-vitro efficacy of IPI-926 in lymphoid cell lines and in patient-derived CLL cells in a limited sample number (n=5-10)
- Additional testing of IPI-926’s efficacy in a co-culture with bone marrow stromal cells (HS-5)
2.4.2.1. Dose escalation of IPI-926 in different lymphoid cell lines

a) Methods
24h-, 48h-, and 72h-viability assay (Annexin V/7ADD) after IPI-926 exposure (concentrations: 0.1, 0.25, 0.5, 1, 2.5, 5 and 10μM – DMSO <1%) in different lymphoid cell lines (MEC1, Raji, NALM-6) and patient-derived CLL cells

b) Results (only 48h and 72h shown)

![Graphs showing cell viability](image)

Conclusion:
Any effect on viability of the cell lines examined was visible at higher dose levels of IPI-926 only; MEC1 – the cell line most akin to CLL – seems to be insensitive to IPI-926

2.4.2.2. Viability of patient-derived CLL cells after IPI-926 exposure in combination with different conventional cytotoxics

a) Methods
24h- and 48h-viability assay (Annexin V/7ADD) in patient-derived CLL-cells: Exposure to IPI-926 with/without co-administration of fludarabine or bendamustine

b) Results (only 48h shown)

![Graph showing cell viability](image)

Conclusion:
IPI-926 did affect CLL cell survival on its own and did not sensitize for effects of conventional cytotoxics
2.4.2.4. Viability of patient-derived CLL cells after IPI-926 exposure in combination with different conventional cytotoxics

a) Methods
24h-, 48h-, and 72h-viability assay (Annexin V/7ADD) in patient-derived CLL-cells after IPI-926 exposure (1uM) with/without fludarabine (4uM) with/without B-Cell stimuli (BCR, IL-4, co-culture with HS-5 and CD40L-expressing NIH cells)

b) Results

Viability of patient-derived CLL cells (48h - n=2)

![Viability graph]

c) Conclusion
Obvious pro-survival effects of BCR-, IL4- and CD40L-stimulation on patient-derived CLL cells were seen – as well as significant cytotoxic effects of fludarabine, inhibited by CD40L-stimulation. No effect of IPI-926 – neither alone nor in combination with fludarabine.

[HS-5 co-culture did not result in a clear pro-survival effect – probably due to a too small number of HS-5 cells used – HS-5 number was increased in the following tests (with visible pro-survival effects – see below)]

Cave - very small sample size; initially, a sample size of n=5-10 was planned, but further analyses were canceled due to negative results, lack of IPI-926 effects, in additional in vitro-analyses (see below).

2.4.2.6 Viability of patient-derived CLL cells after IPI-926 exposure in comparison to cyclosporine and in co-culture with HS-5 bone marrow stromal cells

a) Methods
48h-, and 72h-viability assay (Annexin V/7ADD) in patient-derived CLL-cells after IPI-926 exposure (1uM) or cyclosporine (5uM) with/without fludarabine (4uM) and with/without co-culture with sufficient amount of HS-5 bone marrow stromal cells
b) Results

IPI-926 in HS-5 co-cultured CLL cells (48h, n=5)

IPI-926 in HS-5 co-cultured CLL cells (72h, n=5)
c) Conclusion
Although cyclopamine did show a (significant) tendency to reduce viability in patient-derived CLL cells (p=0.004, native vs Cyclopamine 5μM), this effect was abrogated in the co-culture setting with HS-5 bone marrow stromal cells—which is, in fact, quite contrary to the suggestion, that smoothend inhibition through cyclopamine would alter stromal/CLL cell interaction. IPI-926 did not at all alter CLL-cell survival in vitro.

Discussion/Conclusion

3. Discussion
On the basis of early promising in vitro-data on Hh-inhibition in CLL (13), we have investigated the potent SMO-inhibitor IPI-926 in an in vivo model, in a TCL1-mouse model. However, the disease TCL1-mice develop does not only express a quite similar immunophenotype and is not only reportedly sensitive to conventional cytotoxics used in human CLL (15), but also shows the typically diverse disease manifestations (leukemic vs lymphomatous) and a variable disease course (indolent vs rather aggressive) as its human counterpart – this diversity is an obvious finding when breeding TCL1-mice, although the biologic characteristics of murine TCL1 leukemia match more to the ones of an aggressive form of human CLL (17).

Our – admittedly small – TCL1 test cohort, did not indicate any effect of SMO-inhibition on prespecified disease parameters or on disease course. Instead of that, this test cohort did reveal the difficulty in the use of TCL1-mice as an in vivo-model. It is above all, the heterogeneity of disease manifestation and course, which hamper the reliable interpretation of in vivo-data in moderate sample sizes.

The use of a TCL1-SCID transplant mouse model is a pragmatic approach to this problem (18), resulting in a more homogenic test population on the one hand, and allowing to test a therapeutic intervention in a low-volume or even pre-symptomatic disease stage on the other hand. The latter probably provides a even more sensitive setting for efficacy testing.

However, unfortunately we did not detect any benefit of IPI-926 in our test cohort of 25 successfully transplanted NOD-SCID mice – neither regarding Time to Leukemia Onset (TLO) nor Overall Survival. Comparison of TLO even showed significance to the detriment of the mice treated with IPI-926.

Several restraints of interpretation of these observations have to be mentioned: The homogeneity of disease achieved by the transplantation of splenocytes from a small number of diseased TCL1-mice to a larger number of NOD-SCID mice, does include the possibility, that a subset of TCL1-mice – which were not chosen as a leukemia donor to NOD-SCID mice – still might respond to SMO-inhibition. In addition, the disease of the TCL1-mice used as leukemia donor usually was very advanced – potentially leading to the transplantation of highly malignant and resistant clones, masking any effect of SMO-inhibition, even in the low tumor burden setting.

Notwithstanding these limitations, we would have expected to detect any impact of IPI-926 on disease course in this sensitive setting; so our observation renders any relevant cytotoxic, pro-apoptotic or microenvironmental-depriving in vivo-effect unlikely.

Our in vitro-data on SMO-inhibition correspond to this absence of efficacy found in vivo-testing: Although Cyclopamine reduced viability of patient-derived CLL cells, this effect was abrogated in co-culture setting with bone marrow stromal cells – which is, in fact, quite contrary to the suggestion, that SMO-inhibition through cyclopamine would alter stromal/CLL cell interaction. Thus, an off-target effect seems likely – in addition, the more potent SMO-inhibitor IPI-926 did not alter CLL cell viability at all.

The recent report by Decker et al (19) offers a conclusive explanation for the somehow controversial in vitro-results regarding SMO-inhibition in CLL in different publications (13, 14): they found a highly diverse level of Hh pathway activity in CLL, where only ~30% of bone marrow samples in CLL express GLI1 in immunohistochemistry. Only the subgroup of CLL cells with trisomy 12 and high GLI1-expression did respond to SMO-inhibition. Moreover, in co-culture with bone marrow stromal cells, this pro-apoptotic effect was abrogated even in responding CLL cells. It was hypothesized, that enhanced Hh ligand secretion by the microenvironment (as a result of a negative feedback loop initiated by SMO-inhibition in the stromal cells) led to pronounced ligand-binding on PATCHED-receptor, what overruns downstream SMO-inhibition in the CLL cell by a non-canonical pathway (via ERK), bypassing inhibited SMO, leading to continuing activation of pro-survival target genes. This concept was confirmed conclusively by evidence of efficacy in the co-culture setting of a monoclonal antibody antagonizing PATCHED (leading to an inhibition more upstream of the signaling cascade on one side, and by proof of efficacy of a dual inhibition – SMO and ERK (leading to additional inhibition of the non-canonical pathway) – on the other side (19).
Our own data has much too small sample sizes to investigate specific subgroups of CLL – in fact, it did not include any CLL cells with known trisomy 12.

Besides a potential direct (cytotoxic or pro-apoptotic) or indirect (through reduction of microenvironmental support) effect of Hh inhibition, there are some considerations on relevance of the Hh pathway activity in (malignant) stem cell niche. Hh pathway activity has been found in bcr-arr positive human leukemic stem cells (20, 21), but not in notch1-mediated T-ALL (22), MLL-AF9-mediated AML (23) and not in physiologic hematopoiesis (22-24). Again, Lin et al (16) have investigated SMO-inhibition in a mouse model for B-ALL: after injection of human B-ALL cells into NOD-SCID mice, IPI-926 could not avert progression to leukemia, but – quite interesting – could prevent engraftment during secondary transplantation from these mice to further NOD-SCID mice (the latter were not treated with IPI-926). Thus, a detrimental effect of SMO-inhibition on the self-renewal subpopulation was postulated. This observation was our rationale to consider testing for any effect of IPI-925 in a minimal residual disease setting. However, the overall picture – including the relaying data and literature published by us and our own somewhat disappointing results with this compound in CLL – brought us to suspend further in vivo-testing. Additional in vitro-evidence of the existence of a malignant stem cell in CLL (and its dependence on Hh activity) need to precede further in vivo-investigation.

In summary, we did not find – neither in our in vitro- nor in our TCL1-SCID transplant mouse model – any hint on activity of SMO-inhibition in CLL. More recent preclinical data (19) suggests, that some subgroups of CLL (ie trisomy 12 and those with high GLI1-expression) might profit from Hh pathway inhibition – which should be achieved rather by a direct inhibition of the Hh ligand receptor PATCHED (or by dual SMO-JAER-inhibition) than by sole SMO-inhibition. Apart from the doubtlessly highly interesting pathogenetic insights gained recently, the future clinical relevance of Hh inhibition in CLL – even in the subgroup mentioned – is debatable.

References


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