Pre T-cell receptor alpha (pTα) expression patterns and functional analysis in human T-cell lymphoblastic leukemia

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Abstract. Background: The pTα/preTCR regulates the β-selection, a crucial T-cell developmental checkpoint, providing a most potent survival advantage to thymocytes mediated by the src-kinase p56Lck.

Methods: To define the relevance of pTα in human T-cell lymphoblastic leukemia (T-ALL), we analyzed in T-ALL cell lines (n = 14) pTα and p56Lck mRNA and protein expression as also the tyrosine-phosphorylation. The p56Lck specific src-protein-tyrosine kinase inhibitor (PTK-I) PP1 was used in growth inhibition assays. IC50 value determination, cell cycle- and apoptosis analyses were performed in T-ALL-, non-T-ALL- and murine transgenic cell lines.

Results: pTα expression patterns were markedly different in T-ALL cell lines as compared to those reported for normal lymphoid counterparts. PP1 induced in 6/11 T-ALL cell lines a survival disadvantage resulting from a cell cycle arrest in the G1/S phase in thymic lymphoblastic cells and apoptosis induction in the immature cell line HSB-2, respectively. PP1 sensitive cell lines expressed the target protein p56Lck and showed a corresponding P-Tyr signal.

Conclusion: Sensitivity of thymic T-ALLs to PP1 clearly underlines the impact of pTα mediated proliferation in this leukemic sub-type. In addition, p56Lck represents also independently of pTα a promising therapeutical target for the src-kinase inhibitors in neoplastic lymphoid diseases.

Keywords: T-ALL, pTα, PP1, molecular targeted therapy, tyrosine kinase inhibitor

Abbreviations

IC50  Cytotoxic half maximal inhibitory concentration;  
PP1  4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine;  
PTK(-I)  protein-tyrosine kinase (inhibitor).

1. Background

Emergence of T-ALL is thought to result from specific interference of T-cell oncogenes with develop-
While the relevance of pTα/preTCR expression in leukemogenesis is currently a disputed topic, leukemogenesis in Notch3-ICN transgene mice showed a striking dependence on pTα expression [1,3,7,20,27]. Additionally, the interpretation of the functional relevance of pTα/preTCR in human T-ALL cells is controversial due to the lack of antibodies, low surface expression and novel insights that implicate pTα itself, independently of other components of the preTCR as the mediator of this early survival signal [26].

Here, we analyzed pTα gene- and protein-expression patterns and employed the src-PTK-1 PP1 to study the biological relevance of the pTα/preTCR in human T-ALL cell lines.

2. Materials and methods

Cell lines (n = 18) were obtained from the DSMZ (Braunschweig, Germany, http://www.dsmz.de). T-ALL cell lines (n = 14) were classified according to the EGIL-criteria [4], while pre- and pro-T-ALL due to the low number of available cell lines were categorized as immature T-ALL. Murine SCB.29 and SCBγ/δ.28 were provided by H. von Boehmer (Dana-Faber Cancer Institute, Boston, MA, USA) [17].

pTαa (wild-type) expression was quantified by realtime RT-qPCR and normalized to β-actin. RT-PCR was performed for: pTαb (truncated isoform), β-actin and p56Lck PCR conditions and primer sequences can be obtained from the corresponding author.

Western blot analysis of the pTα-chain (murine monoclonal antibody 2F5, Hybridoma J2.3, obtained by H. von Boehmer, Boston, MA, USA) and actin (murine monoclonal antibody, C2, Santa Cruz Biotechnology, CA, USA) was accomplished according to standard protocols, using equal protein lysate amounts (3 × 105 cells; testing the effect of PP1: 1.5 × 105 cells) by Western blotting according to standard protocols (n = 15; analyzed at least twice) [12,16]. β-actin was used as a loading control (monoclonal mouse antibody, AC-47, Sigma, Germany). The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (Santa Cruz Biotechnology, CA, USA and Cell Signaling, USA). Summarizing heat maps were compiled for quantitative pTαa-, semi-quantitative pTαb- and p56Lck-mRNA and pTα protein expression with Genesis (V. 1.7.2, A. Sturn, Graz University of Technology, Austria) [19].

IC50 values were determined by MTT assays (n = 16; average was obtained from five independent experiments, each performed in triplicates; 5 mg/ml, 6 h incubation; Sigma, Germany) following PP1 incubation (96 h; tested PP1 concentrations: 0.1, 1, 10 and 20 µM; Biolum, USA). Cell cycle analyses were performed by acridine-orange (http://www.healthcare.uiowa.edu) based DNA quantification (n = 10 cell lines, 3 independent experiments, each as duplicate) (Sigma, Germany). Cells (8 × 105 cells) were cultured 12 h in serum-deprived medium (RPMI-1640 medium containing no FCS) and then subsequently incubated (48 h) in accordant RPMI medium prior to cell cycle staining (PP1 treated sample: 8 µM PP1 + 10% FCS; starvation control: 0.02% DMSO + 1% FCS; growth control: 0.02% DMSO + 10% FCS). Controls enabled clarification of SubG0-phase- and G2M-phase populations. Estimated S-phase- and G2M-populations were summarized as the proliferative fraction of gated cells using doublet discrimination mode. From the same samples apoptotic bodies were visualized with Hoechst33258 (Sigma, Germany), and the relative numbers of vital cells out of 100 counted cells were calculated. Apoptosis was further validated and quantified by flow cytometry using an annexin-V/propidium iodide staining kit, following the manufacturer’s instructions (two separate experiments; Roche Applied Science, Germany).

3. Results and conclusion

Analysis of peripheral blood mononuclear cells (PBMCs) from T-ALL patients at disease onset and in remission identified combined expression of pTαa, its isoform pTαb and Notch3 of particular impor-
tance for sustaining T-cell leukemogenesis, potentially representing a pathognomonic molecular signature of human T-ALL [3]. In contrast to this finding but in agreement with Asnafi et al. [1], we detected mRNA expression of pTα isoforms in patients with T-ALL, B-ALL, AML and also in healthy controls (data not shown). Expression analysis of pTα in PBMCs from T-ALL patients might be misleading since pTα expression in these specimens is most likely not originating from the leukemic population, but rather from CD4\(^+\)CD3\(^-\) extrathymic T-cell progenitors [6], whose characteristics in T-ALL are unknown. To avoid this bias, our analyses were performed in homogenous T-ALL cell lines.

Among all analyzed T-ALL subtypes, immature T-ALLs showed the highest average pTα\(^a\) mRNA expression, and pTα\(^b\) mRNA was found in two out of three immature T-ALLs (Fig. 1A, B). pTα protein was detected in three out of three immature T-ALLs (Fig. 1D). Intermediate pTα\(^a\) mRNA expression was found in thymic T-ALLs, and pTα\(^b\) was detected in most of these cells (Fig. 1A, B). pTα protein was detected in six out of six thymic T-ALLs (Fig. 1D). Mature T-ALLs expressed the lowest level of pTα\(^a\) mRNA, and only two out of four mature T-ALLs expressed pTα\(^b\) (Fig. 1A, B), whereas all mature T-ALLs expressed pTα protein (Fig. 1D).

Physiological pTα isoform expression is differentially regulated during T-cell maturation [2,15]. Normally, pTα\(^a\) expression increases until β-selection onset, while the transcriptional onset of pTα\(^b\) is delayed, peaking after the β-selection and potentially regulating a rapid loss of pTα\(^a\) after β-selection, thus terminating the cellular and molecular effects of pTα [2,15]. During the thymic window, where the β-selection is supposed to occur, pTα\(^b\) is believed to strengthen the pTα\(^a\) signal [2,15]. Quantitative and qualitative differences were observed by comparing pTα mRNA expression patterns in T-ALLs with expression patterns known from thymic T-cell development. In contrast to thymocytes, maximum pTα\(^b\) expression was found prior to the β-selection in immature T-ALLs [2,15]. Furthermore, pTα\(^a\) was still expressed in mature T-ALLs, while pTα\(^b\) was rarely detected. The variation of pTα\(^a\) gene expression level throughout the immunophenotypical compartments is impressively higher among T-ALL subtypes (approximately 10,000-fold) than during intrathymic differentiation (approximately 80-fold) [15].

Even though pTα\(^a\) protein expression has been rarely reported in thymocyte populations corresponding to immunophenotypical immature T-ALL cells, we clearly demonstrated pTα protein translation in immature T-ALL cells (Fig. 1D). Our finding is supported by Gounari et al. [11] who observed human CD25 protein expression in murine lymphoid restricted progenitors, controlled by the pTα promoter. Since pTα\(^b\) expres-

![Heat map illustrating expression patterns in T-ALL cell lines](image-url)

**Fig. 1.** Expression patterns in T-ALL cell lines for pTα\(^a\)-, pTα\(^b\)-, p56Lck-mRNA and pTα protein. Heat map illustrations are shown with respect to the maturational stage of the particular leukemia cell line. (A) β-actin normalized pTα\(^b\) (wildtype) mRNA expression analyzed by RT-qPCR in human T-ALL cell lines (absolute quantification in copy numbers of pTα\(^a\)). (B) β-actin normalized pTα\(^b\) (truncated isoform) and (C) p56Lck mRNA expression by RT-PCR (relative quantification in arbitrary units; A.U.). (D) Actin normalized pTα protein expression analyzed by Western blotting (relative quantification in arbitrary units; A.U.). Cell lines with the highest expression are marked by white circles. White squares indicate negative control (AGS – gastric cancer cell line), or cell lines in which protein analysis was not performed (e.g., CCRF-CEM and MOLT-16). Arrows indicate the developmental stage with expected pTα function.
sion and function is physiologically down-regulated by pT\(\alpha\) in mature T-cells [2,15], it was surprising to observe pT\(\alpha\) protein expression in mature T-ALLs. This finding correlates with unexpectedly persistent pT\(\alpha\) mRNA expression concomitant with lower than expected pT\(\alpha\) expression in mature T-ALLs (Fig. 1A, B) [15], suggesting an aberrantly maintained pT\(\alpha\) signal due to a lack of physiological pT\(\alpha\) down-regulation in mature T-ALLs. Importantly, the regulation of pT\(\alpha\) mRNAs and pT\(\alpha\) protein expression is unknown. Here, we interestingly observed a tendency of mRNA/protein proportion from high/low in immature T-ALLs inverting during further maturated phenotypes to low/high in mature T-ALLs (Fig. 1A, D). Whereas the known function of T-cell receptors is in general linked to defined stages, cellular environment and pT\(\alpha\)/preTCR signal intensity, one might speculate that the mRNA/protein expression profile in the thymic window reflects the physiological differentiation; the expression profile in immature and mature T-ALL differs strikingly from this physiological situation, possibly presenting an abnormal and/or additional regulation of pT\(\alpha\) gene/protein expression.

To better define the functional relevance of pT\(\alpha\) in T-ALL cell lines, we used the src-PTK-I PP1 to block proximal pT\(\alpha\)/preTCR signaling, which is mainly mediated through p56\(^{\text{ck}}\) [22,23]. Initially, p56\(^{\text{ck}}\) mRNA (Fig. 1C) and protein expression as well as tyrosine-phosphorylation were analyzed (Fig. 2). The immature T-ALLs showed low levels of p56\(^{\text{ck}}\) mRNA (Fig. 1C). On the protein level, HSB-2 showed strong p56\(^{\text{ck}}\) and P-Tyr signals (Fig. 2C), whereas a second immature T-ALL cell line, RPMI-8402, exhibited lower p56\(^{\text{ck}}\) and total phosphorylated protein levels. All thymic T-ALLs

![Western blot analysis of p56\(^{\text{ck}}\) protein expression, total cellular tyrosine phosphorylated proteins (P-Tyr) and \(\beta\)-actin in human leukemic cell lines.](image)

(A) The human T-ALL cell line JURKAT (1 x 10^6 cells) was incubated either with the control (0.02% DMSO) or with PP1 at the respective concentrations (neg. – no protein control). After 48 h incubation equal cell lysate amounts (1.5 x 10^5 cells) were analyzed for p56\(^{\text{ck}}\) protein expression (56 kDa), P-Tyr status and \(\beta\)-actin (41 kDa) by Western blotting as described in Section 2. (B, C) Equal cell lysate amounts (3 x 10^5 cells) of human non-T-ALL cell lines (B) and human T-ALL cell lines (C) were analyzed for the expression of p56\(^{\text{ck}}\) (56 kDa), tyrosine phosphorylated proteins and \(\beta\)-actin (41 kDa). Abbreviations: sc = solvent control, RPMI = RPMI-8402, P12 = P12-ICHIKAWA.
showed p56Lck mRNA, protein expression and a corresponding P-Tyr signal. In the mature T-ALLs, strong p56Lck mRNA and protein expression were detected with a lower corresponding P-Tyr signal (Fig. 2C).

PP1 is a highly potent and specific inhibitor of the src-kinase family, mainly targeting p56Lck [12]. Cells depending essentially on the pTα/preTCR signal were expected to have survival disadvantages at lower PP1 concentrations compared to cells which are independent of pTα/preTCR signaling. As previously shown, PP1 reduced p56Lck tyrosine-phosphorylation in a dose-dependent manner (Fig. 2A) [12]. Furthermore, we tested PP1 activity in MOLT-4 and HL-60 cells (Fig. 3A): p56Lck-positive MOLT-4 cells were far more sensitive to PP1 (IC50: 5.99 µM) as compared to the p56Lck-negative AML cell line HL-60 (IC50: >20 µM) (Figs 1C, 2B, C, 3A). To investigate pTα/preTCR-dependent versus pTα/preTCR-independent p56Lck activation, two transgenic thymocyte cell lines were used. SCB.29 cells, in which p56Lck activation is known to be pTα-dependent, are twice as sensitive to PP1 in comparison to SCBγδ.28 cells, in which pTα/preTCR has no relevance (IC50: 6.9 µM vs. 13.99 µM, respectively) (Fig. 3A) [17].

Therefore, an IC50 <10 µM PP1 was considered as specific inhibition of pTα/preTCR proximal signaling. Using these criteria, we observed functional pTα/preTCR expression in six out of eleven tested T-ALL cell lines (Fig. 3A).

The highest PP1 responsiveness (IC50: 0.96 µM) was observed in HSB-2 (Fig. 3A), one of two tested immature T-ALLs; further analyses could demonstrate that PP1 exposure resulted in apoptosis induction of HSB-2 (Figs 3B, 4A, B), clearly shown by nearly 90% annexin-V positive cells. In four out of six thymic T-ALL cell lines PP1 induced a GI/G0 arrest and caused moderate growth inhibition in one out of three mature T-ALL cell lines, without any evidence of apoptosis (Figs 3B, 4). All PP1 sensitive cell lines expressed the target protein p56Lck and showed a corresponding P-Tyr signal (Fig. 2C). Two out of six thymic T-ALLs, which are early thymic T-ALLs according to the immunophenotype, did not show PP1 sensitivity, even though they expressed p56Lck with corresponding P-Tyr signals (Fig. 2C). One might speculate, that in these PP1 non-sensitive thymic T-ALL cell lines alternative oncogenic pathways independent of phosphory-

| subtype      | cell line | IC50 [µM] |
|--------------|-----------|-----------|
| immature T-ALL | HSB-2     | 0.96      |
| thymic T-ALL  | RPMI-8402 | 14.66     |
|               | P12-ICHIKAWA | 15.22     |
|               | PF-382    | 13        |
|               | KE-37     | 7.65      |
|               | JURKAT    | 7.76      |
| mature T-ALL  | MOLT-14   | 9.41      |
|               | PEER      | 20        |
| AML           | LOUCY     | >20       |
| B-ALL         | TANOUE    | 15.71     |
| murine        | SCB29     | 6.9       |
|               | SCBu8.28  | 13.99     |

Fig. 3. Functional analysis in transgenic cell lines and T-ALL cell lines. (A) IC50 values in human cell lines (T-ALL and non-T-ALL) after PP1 exposure; mean IC50 value of 5 experiments is shown – each experiment was performed as triplicate. (B) Cell cycle analysis of PP1 sensitive (<10 µM PP1; dotted line) and non-sensitive cell lines (continuous line), previously defined by IC50 determination. Results for PP1 treated cell lines and controls (0.02% DMSO) are shown; average of 2 experiments is shown – each experiment was performed as triplicate. (*) p ≤ 0.01 for PP1 (8 µM) treated cells compared to control. (1) HSB-2, (2) RPMI-8402, (3) MOLT-4, (4) P12-ICHIKAWA, (5) PF-382, (6) MOLT-14, (7) LOUCY, (8) PEER, (9) TANOUE, (10) HL-60.
Fig. 4. Analysis of the growth inhibitory mechanism after PP1 treatment in human T-ALL cell lines. (A) Cell cycle analysis and apoptosis staining after PP1 exposure with Hoechst33258 performed with the cell lines HSB-2 (highly PP1 sensitive), MOLT-4 (PP1 sensitive) and LOUCY (PP1 insensitive). Cells cycle analyses were performed five times in triplicates as described in Section 2 (R1 – SubG10-fraction, R2 – G10-fraction; R3 – S-phase fraction, R4 – G2/M-phase fraction; red – starvation control; green – proliferative control, purple – PP1 treated cells; white arrow indicates apoptotic bodies). Summary of cell cycle analysis is shown in Fig. 3B. (B) Apoptosis was further determined by flow cytometry analysis of annexin-V/propidium iodid (PI) stained cells as described in Section 2. Representative FACS analyses are shown for the T-ALL cell lines HSB-2 (PP1 highly sensitive), MOLT-4 (PP1 sensitive), LOUCY (PP1 insensitive). Results from two independent experiments are given as range of the relative cell distribution in the dot-plots and histograms, respectively. Legend: Dot-plots: y-axis: PI, x-axis: annexin-V; lower left quadrant (LL) – vital cells, lower right quadrant (LR) – early apoptosis, upper right quadrant (UR) – late apoptosis, upper left quadrant (UL) – detritus; histograms: y-axis: event counts, x-axis: annexin-V, M1 – annexin-V negative cells, M2/A+ – annexin-V positive cells.
lated p56<sup>Lck</sup> deliver a more dominant survival advantage.

We were further interested in the high sensitivity of the immature T-ALL cell line HSB-2 to PP1, as pTα signaling in immature T-ALLs is very unlikely and suspected an oncogenic pathway other than pTα/preTCR. Interestingly, the chromosomal analysis of HSB-2 cells (data not shown) revealed the translocation t(1; 7)(q34; p34) [21,25], which causes independently of pTα a hyperactivation of p56<sup>Lck</sup> (Fig. 2C). Even though the incidence of the translocation t(1; 7)(q34; p34) is considerably low at the initial diagnosis of a T-ALL, it has been speculated whether or not this translocation, or that Lck in general, is involved in later genetic events causing a transformation to a progressively more aggressive leukemia [14,21,25]. Furthermore, alterations of the chromosome 1p are frequently observed in human neoplastic disease. Approximately 10% of non-Hodgkin Lymphomas had genetic alteration at the site 1p31-36, potentially affecting Lck expression [14] and thereby represent an interesting therapeutic target for src-PTK-I as PP1.

In the PP1-induced G<sub>1</sub>/0 arrest in thymic T-ALL cell lines supports the hypothesis that pTα/preTCR sustains proliferation in the leukemic thymic compartment, in which pTα activation is physiologically relevant for G<sub>1</sub>/0 to S-phase transition and the arrested leukemogenic clone might be supported from this potent survival mediator [5,9,23]. Interestingly, not all thymic T-ALLs respond in the defined PP1 concentration range (<10 µM) arguing for a very tightly defined time window of malignant arrest in which pTα is relevant: PP1 unresponsive cell lines are immunophenotypically early thymic T-ALLs, whereas the responsive cell lines are considered to be in the intermediate and late thymic developmental time window. With respect to T-cell leukemogenesis further dissection of the Notch/pTα/CyclinD3 signaling axis is needed, as Notch1 or CyclinD3 inhibition, upstream or downstream of the pTα/preTCR, also cause G<sub>1</sub>/0 arrest predominantly in thymic T-ALLs [8,18,24].

In summary, our data point at the crucial role of pTα in defined subtypes of T-ALL mediating survival advantages for the malignant cell-type. Additionally, using the src-PTK-I PP1, we demonstrated that p56<sup>Lck</sup> also independently of pTα represents a promising therapeutic target in neoplastic T-cell diseases.

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