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**Effects of the small-molecule inhibitor of integrin α4, TBC3486, on pre-B-ALL cells**

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The treatment of patients with chemotherapy-resistant leukemia remains a challenge. A role of the microenvironment for drug resistance of leukemia cells has been proposed. We have identified the adhesion molecule integrin α4 as a central mediator of drug resistance of pre-B-cell acute lymphoblastic leukemia (ALL). We thus demonstrated that chemotherapy-resistant pre-B-ALL cells can be eradicated in a xenograft model by concurrent blockade of α4 using natalizumab, a humanized anti-α4 antibody in clinical use against multiple sclerosis, and Crohn’s Disease. Here, we extended our studies to an alternative α4 inhibitor, the non-peptidic small molecule TBC3486. Previous in vitro assays and molecular modeling studies indicated that TBC3486 behaves as a ligand mimetic, competing with VCAM-1 for the MIDAS site of integrin α4. As such, the compound has shown efficacy in integrin α4-dependent models of inflammatory and autoimmune disease and has shown efficacy in mice with autoimmune encephalomyelitis, a model for multiple sclerosis. As opposed to natalizumab, which will inhibit both members of the α4 integrin family, α4β1 and α4β7, TBC3486 is 200-fold more potent in inhibiting α4β1 than α4β7. In addition, it is completely inactive against all other integrins tested, including members of the β2, β3 as well as other members of the β1 family of integrins. The potential usefulness of this novel inhibitor for pre-B-ALL treatment was tested in our established in vitro and in vivo assays. We evaluated the effect of TBC3486 on de-adhesion of patient-derived ALL cells (LAX7R) using established adhesion assays. As a control for our studies, a close structural analog was used that lacks activity toward α4β1 integrin (THI0012). After activating LAX7R cells with 1 mM Mn²⁺, leukemia cells were co-cultured with the murine stromal cell line O9P9. Subsequently, LAX7R cells were treated with different doses of TBC3486 (5, 10 and 25 μM) and its control, THI0012 (5, 10 and 25 μM), for 4 days. TBC3486 dose-dependently inhibited adhesion of ALL cells (Figure 1a), albeit the adhesion was not completely blocked. The dose of 25 μM was selected for subsequent studies. The concentrations of compound required for inhibition in these assays are higher than previously reported. This is due to the fact that TBC3486 is highly protein bound in the presence of 20% serum (used in these assays), which significantly reduces the amount of free compound available to bind to the integrin receptor. Next, we determined whether TBC3486 decreases binding of three xenograft cells derived from primary pre-B-ALL cases (LAX7R, ICN3 and SFO3) to the counter-receptor of α4 integrin, human VCAM-1. Adhesion assays were performed as previously described by culturing ALL cells with TBC3486 (25 μM) or THI0012 (25 μM) on hVCAM-1-coated plates for 2 days. Compared with the control group, TBC3486-treated ALL cells showed significantly less adhesion to hVCAM-1 (Figures 1c and g; however, the adhesion was not completely blocked. CD49d (MFI) is expressed with higher intensity in LAX7R compared with the other two samples (ICN3 and SFO3) (data not shown), which may explain why TBC3486 blocked a larger percentage of LAX7R adhesion to VCAM-1. In addition to blocking cell adhesion, TBC3486 treatment also specifically targeted the expression of integrin α4, but not integrin α5 and α6 (Figure 1b). The treatment with TBC3486 did not affect cell viability in all three cases (Figures 1d, f and h) compared with the THI0012 control. Taken together, TBC3486 leads to the partial de-adhesion of pre-B-ALL cells from its counter-receptor VCAM-1 under the conditions described.

We previously showed that antibody-mediated integrin α4 blockade can sensitize leukemia cells to chemotherapy. Therefore, we evaluated whether TBC3486 can not only de-adhere cells from their counter-receptors but also sensitize them to chemotherapy. To study the drug sensitivity of human leukemia, ALL (LAX7R) cells were co-cultured with murine calvaria-derived OP9 stromal cells, which allow in vitro studies beyond 2 days. Then this co-culture was exposed to chemotherapy as previously described. ALL cells were treated with chemotherapy (vincristine, dexamethasone, 1-asparaginase; VDL) or saline as control for 4 days, with TBC3486 (25 μM) or control THI0012. TBC3486 with VDL treatment reduced the cell viability of human leukemia LAX7R (51 ± 1% vs 31 ± 1%; P < 0.05) (Figure 2a), ICN3 (60 ± 3% vs 49 ± 1%; P < 0.05) (Figure 2b) and SFO3 (78 ± 1% vs 65 ± 1%; P < 0.05) (Figure 2c), indicating that this combination therapy sensitized leukemia cells to chemotherapy in vitro. Nevertheless, all chemoprotection afforded by OP9 cells was removed by TBC3486 in spite of only partial de-adhesion. This observation at least suggests that effects over and above adhesion and physical chemoprotection provided by stromal cells are mediated through α4 integrin.
To determine whether TBC3486 can prolong the survival of xenotolerant mice bearing human leukemia cells in vivo, LAX7R cells were injected into NOD/SCID hosts. Three days after leukemia cell transfer, mice received either TBC3486 or THI0012 (control) (10 mg/kg/d) daily for 2 weeks with two daily intraperitoneal injections to ensure stable plasma levels, with or without VDL chemotherapy. TBC3486-treatment \((n = 7)\) resulted in prolonged survival time compared with THI0012 control mice \((n = 3)\) (median survival time (MST) = 41 vs 33 days, \(P = 0.001)\). In combination with chemotherapy, the Kaplan–Meier survival curve indicated that TBC3486 + VDL-treatment \((n = 6)\) resulted in a significantly prolonged survival time (compared with the VDL + THI0012 control \((n = 7)\) (MST = 82 vs 58 days, \(P = 0.0003)\; Figure 2d).

TBC3486 preferentially inhibits the high-affinity form of the integrin \(\alpha_4\). It may thus interfere with the function of lymphocytes entertaining the inflammatory response in multiple sclerosis, but may not interfere with that of lymphocytes causing inflammatory bowel disease. Which \(\beta\)-integrin is the more relevant partner for \(\alpha_4\) in the context of drug resistance in ALL is unclear. In addition, TBC3486 mimics VCAM-1 binding, inducing the high-affinity conformation of integrin \(\alpha_4\); by contrast, natalizumab recognizes and blocks the ligand-binding moiety of \(\alpha_4\) in any conformation and without affecting activation status. Whether, therefore, TBC3486-binding of integrin \(\alpha_4\) might elicit outside-in signaling would have to be addressed in further studies. Although unknown off-target effects of the compound cannot be excluded, it should be emphasized that the control compound, THI0012, is structurally identical to TBC3486 except it is the opposite enantiomer. The fact that this control compound has no

![Figure 1. Effects of TBC3486 treatment on adhesion and integrin expression of ALL cells. (a) Percentage of adhesion of LAX7R cells treated with different doses of TBC3486 and THI0012 on OP9 for 4 days. * \(P < 0.05\), mean ± s.d., performed in triplicates. (b) Expression of \(\alpha_4\), \(\alpha_5\) and \(\alpha_6\) integrins presented as histograms after 4 days of TBC3486 (25 \(\mu M\)) and THI0012 (25 \(\mu M\)) treatment. Three ALL cells \((c,d): LAX7R; \(e,f): ICN3; \(g,h): SF03\) were treated with THI0012 control (white bar) or TBC3486 (red bar) on plates coated with human VCAM-1 or 2% bovine serum albumin as control for 2 days. \((c, e, g): Adhesion of ALL cells (× 400 magnification) and % of adhering cells is shown. \((d, f, h): Viability of leukemia cells treated with TBC3486 or THI0012 control was determined by trypan blue exclusion.\]
activity in any of the in vitro or in vivo studies described here also argues that the efficacy of TBC3486 in these studies is directly related to its activity toward α4β1 integrin.

Like many small molecules, TBC3486 is endowed with a rather shorter half-life, requiring more frequent dosing than natalizumab. A shorter half-life should likely be an advantage in leukemia treatment, but challenges at establishing the optimal timing of anti-integrin treatments (how often, how much and so on) relative to the chemotherapy must not be underestimated. Natalizumab led to the complete eradication of pre-B-ALL in our xenotransplant model in two out of three pre-B-ALL cases. By contrast, adjuvant treatment with TBC3486 was associated only with prolonged survival, not with leukemia cell eradication. Although formal side-by-side comparisons were not performed, this may at least in part be due to the differences in treatment schedules (TBC3486: twice daily for 2 weeks, natalizumab: once weekly for 4 weeks), but additional differences of the two compounds may also contribute. As the half-life of natalizumab is much longer, therapeutic drug levels are likely to be continuously maintained for the entire duration of the 4-week VDL cycle.

Taken together, our data demonstrate that small-molecule inhibition of integrin α4 using TBC3486, currently in preclinical evaluation, is a promising approach for targeting chemotherapy-resistant leukemia. Further studies are warranted to understand and evaluate preclinically adjuvant small-molecule inhibition of integrins to overcome relapse of ALL.

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Figure 2. TBC3486 treatment sensitizes ALL cells to chemotherapy. (a–c) ALL cells (a) LAX7R; (b) ICN3; (c) SF03) were co-cultured with (right panel) or without OP9 cells (left panel) for 4 days and treated with TBC3486 (25 μM) or THI0012 (25 μM) in combination chemotherapy (VDL: V = 0.0005 μM, D = 0.005 μM, L = 0.0005 IU/ml) in vitro and viability of leukemia cells was determined by trypan blue exclusion *P<0.05; experiments were performed in triplicates. (d) ALL cells (LAX7R) were injected into NSG mice (5 × 10³ cells/mouse) and recipient mice were then treated with 10 mg/kg/day TBC3486 or THI0012 control, starting on day 3 post leukemia injection. The daily administrations were split into two intraperitoneal injections per day for 2 weeks. In addition, mice were treated with vincristine (0.5 mg/kg once a week), dexamethasone (8 mg/kg five times a week) and L-asparaginase (800 IU/kg five times a week) for 4 weeks. Kaplan–Meier survival curve was analyzed and MST was calculated for each group: THI0012 (n = 3, MST = 33 days) (black dashed); TBC3486 (n = 7, MST = 41 days) (red dashed); THI0012 + VDL (n = 6, MST = 58 days) (black solid); TBC3486 + VDL (n = 7, MST = 82 days) (red solid). *P<0.05, log-rank test.
MYD88 (L265P) mutation is an independent prognostic factor for outcome in patients with diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) encompasses an aggressive and heterogeneous group of malignancies. Gene expression profiling (GEP) unraveled two main subtypes based on their putative cell of origin named germinal center B-cell-like (GCB) and activated B-cell-like (ABC), with specific genetic alterations and different clinical outcome. Recent studies have reported several somatic mutations in the myeloid differentiation primary response gene 88 (MYD88) affecting B-cell lymphomas. Among them, the leucine to proline exchange at position 265 (L265P) is the most recurrent and biologically potent MYD88 variation, being found in about 30% of ABC-DLBCL but uncommon in GCB-DLBCL. Here, we have investigated the occurrence of MYD88 L265P mutation in adult patients with DLBCL and its relation to clinical and biological characteristics, including patients’ outcome.

A series of 175 retrospective patients diagnosed of DLBCL from July 2000 to July 2013 was selected from our lymphoma registry. To be included in this study, cases were required to have: newly diagnosed DLBCL that had not been previously treated with full clinical data available, treatment with remission intention, enough material for DNA extraction and absence of HIV infection. This clinical data available, treatment with remission intention, enough

Intermediate- or high IPI (HR = 3.0, 95% CI, 1.6–5.7, P = 0.001) and ABC phenotype assessed by Hans’ algorithm (P = 0.953). When adjusted in a multivariate Cox regression analysis, MYD88 L265P mutation remained as a significant risk factor for death (hazard ratio (HR) = 2.4, 95% confidence interval (CI), 1.2–4.7, P = 0.013) as well as intermediate- or high IPI (HR = 3.0, 95% CI, 1.6–5.7, P = 0.001). Regarding PFS, MYD88 mutation was a significant factor for inferior PFS (log rank, P = 0.049), but not ABC phenotype (P = 0.956). However, our results using the Hans’ algorithm for DLBCL subclassification should be taken with caution as algorithms based on immuno-histochemistry, even those recently proposed, have variable concordance with GEP.

Most patients were treated according to standard practice but some cases were enrolled into clinical trials or received specific treatments (for instance, primary brain lymphomas were treated

Reflected a particular referral pattern for several lymphoma types in our institution. MYD88 L265P mutation was found in 17 out of 175 cases (10%), a frequency slightly lower than that initially reported, although a recent study has shown an even lower incidence (6.5%), To the best of our knowledge, there are few studies analyzing the clinical features associated to MYD88 L265P mutation in DLBCL patients. Clinical characteristics of our series according to MYD88 mutational status are shown in Supplementary Table 2. MYD88 mutation occurred more frequently in males (P = 0.019), cases without B symptoms (P = 0.006) and those with primary extranodal disease (P = 0.02).

In agreement with recent observations, MYD88 mutation was infrequent in DLBCL arising in lymph nodes (3%), but more frequently found in extranodal sites such as central nervous system (50%), skin (33%) and testes (78%). Of note, and in contrast to other studies, we found no mutated cases among gastric, small intestine and large intestine DLBCL. Therefore, our results confirm the remarkable site-specific occurrence of MYD88 mutations at some immune-privileged locations. As somatic mutations in MYD88 are the most frequent alterations found in ABC-DLBCL, we further analyzed GCB or ABC phenotype by immunohistochemistry according to the criteria of Hans. As previously reported, MYD88 mutations were predominantly observed in the ABC type (14% vs 6% in GCB type, P = 0.214). For the whole cohort, the median follow-up time was 41 months (range, 2–171 months), with OS at 4 years of 69% and PFS at 4 years of 57%. Univariate analysis showed that intermediate- or high International Prognostic Index (IPI) (P = 0.001) and MYD88 L265P mutation (P = 0.001) (Figure 1a) were significantly associated with inferior OS, whereas no correlation was found with primary extranodal origin (P = 0.946) or ABC phenotype assessed by Hans’ algorithm (P = 0.953). When adjusted in a multivariate Cox regression analysis, MYD88 L265P mutation remained as a significant risk factor for death (hazard ratio (HR) = 2.4, 95% confidence interval (CI), 1.2–4.7, P = 0.013) as well as intermediate- or high IPI (HR = 3.0, 95% CI, 1.6–5.7, P = 0.001). Regarding PFS, MYD88 mutation was a significant factor for inferior PFS (log rank, P = 0.049), but not ABC phenotype (P = 0.956). However, our results using the Hans’ algorithm for DLBCL subclassification should be taken with caution as algorithms based on immuno-histochemistry, even those recently proposed, have variable concordance with GEP.

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