An enzyme-linked immunosorbent assay to screen for inhibitors of the oncogenic anaplastic lymphoma kinase

Gunby, Rosalind Helen; Tartari, Carmen Julia; Porchia, Francesca; Donella-Deana, Arianna; Scapozza, Leonardo; Gambacorti-Passerini, Carlo

Correspondence: Emmanuel Raffoux, Département d'Hématologie, Hôpital Saint-Louis, 1, avenue Claude Vellefaux, 75010 Paris, France. Phone: international +33.1.42499643. Fax: international +33.1.42499345. E-mail: emmanuel.raffoux@sls.aphp.fr

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Malignant Lymphomas

An enzyme-linked immunosorbent assay to screen for inhibitors of the oncogenic anaplastic lymphoma kinase

The discovery of novel anti-cancer drugs targeting anaplastic lymphoma kinase (ALK), an oncogenic tyrosine kinase, raises the need for *in vitro* assays suitable for screening compounds for ALK inhibition. To this aim we have developed and optimized an ALK-specific enzyme-linked immunosorbent assay that employs a novel ALK peptide substrate and purified ALK kinase domain.

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The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase normally expressed in the developing nervous system.[1] However, chromosomal translocations involving the *ALK* gene (2p23) lead to the expression of constitutively activated ALK fusion proteins such as NPM/ALK, in tissues outside the nervous system.[2,3] ALK fusion proteins stimulate mitogenic and anti-apoptotic signaling pathways, leading to malignant transformation in cancers such as anaplastic large cell lymphoma (ALCL).[4-6] Therefore, ALK represents a valid target for pharmaceutical intervention. In the present study, we describe the development of an enzyme-linked immunosorbent assay (ELISA) that can be used to rapidly screen compounds for their ability to inhibit ALK *in vitro*.

His-tagged recombinant ALK (rALK) protein containing the predicted ALK kinase domain spanning amino acid residues 1116-1392 (NCBI Accession Code: Q9UM73), was expressed in Sf9 cells using a baculovirus expression system. rALK protein was purified to apparent homogeneity as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and was shown to maintain autophosphorylation activity throughout the purification procedure, indicating the correct folding of the purified protein (Figure 1A). Using purified rALK we identified a specific peptide substrate of ALK (peptide#1: ARDIYRASFFRKGGCAMLPVK) resembling the ALK activation loop (ARDIYRASYYRKGGCAMLPVK), which displayed a phosphorylation rate 3-fold higher than that of the general substrate, polyGlu4Tyr (Figure 1B). In the ALK-ELISA, peptide#1 was immobilized on an ELISA plate and phosphorylated by purified rALK in the presence of ATP and co-factors. The immobilized phosphorylated substrate was detected using anti-phosphotyrosine and horse-radish peroxidase (HRP)-conjugated antibodies. Addition of HRP substrate stimulated a colorimetric reaction which was detected by spectrophotometry. Assay conditions were optimized for measuring ALK inhibition. A saturating amount of peptide#1 (2.5 µg/well) and an amount of rALK within the linear range of the colorimetric response (15 – 150 ng protein/well) were used. Plotting phosphorylation of peptide#1 by rALK against time indicated that there was a linear increase in absorbance up to 16 minutes (Figure 1C). No increase in absorbance was observed in the absence of peptide or with a negative control peptide, indicating that background noise was negligible. A reaction time within the linear range (10 min) was chosen for maximum sensitivity in measuring ALK inhibition.

The reliability of the ALK-ELISA in measuring ALK inhibition was assessed using the general kinase inhibitor, staurosporine. In a conventional in vitro radioactive kinase assay, performed in the presence of 30 µM ATP, staurosporine inhibited phosphorylation of peptide#1 by rALK with an IC_{50} of 123 nM (Figure 2A). In the ALK-ELISA performed under the same conditions, the IC_{50} value for staurosporine was identical to that obtained in the radioactive assay (150 nM) (Figure 2B). In the presence of 300 µM ATP an IC_{50} value of approximately 700 nM was obtained in the ALK-ELISA. This increase in IC_{50} at higher concentrations of ATP reflects the competition between staurosporine and ATP for binding the ALK kinase domain. As the majority of kinase inhibitors developed so far are ATP competitors,[7] and considering the high concentrations of ATP observed in cells (>1 mM), it is desirable that in vitro screening assays for ALK inhibitors are performed in the presence of high concentrations of ATP. Unlike the radioactive assay, which is restricted by specific activity considerations, the ALK-ELISA can be performed at ATP concentrations approaching physiological concentrations, therefore should generate results closer to those observed in cells. Indeed, the IC_{50} value obtained in the presence of 300 µM ATP (700 nM) in the ALK-ELISA is closer to the

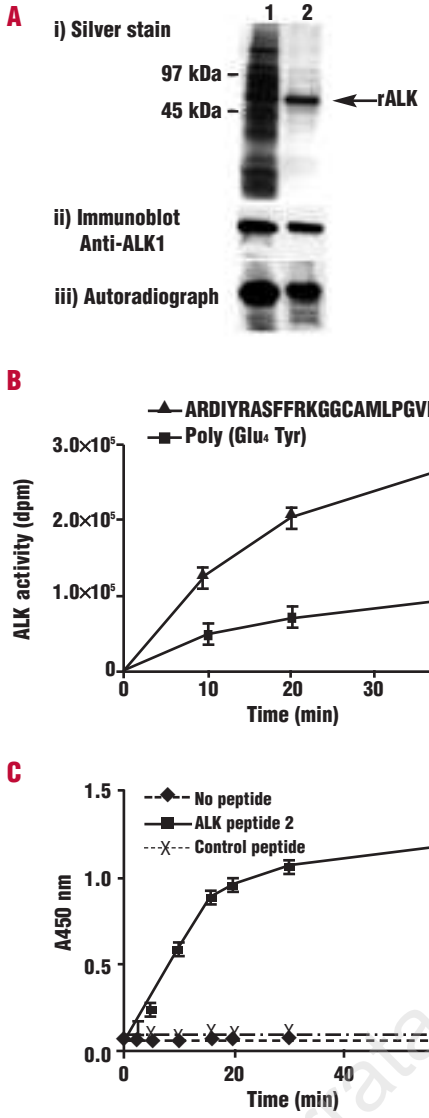

Figure 1. A. Production and purification of recombinant (6x)His-tagged ALK (rALK). rALK containing amino acids 1073–1459 (NCBI Accession Code: Q9UM73) was expressed in Sf9 cells using the MaxBac®2.0 baculovirus expression system (Invitrogen). rALK was purified on a Q-sepharose Fast Flow column followed by a HiTrap™-nickel column (Amersham-Pharmacia Biotech). Q-sepharose (lane 1) and HiTrap fractions (lane 2) were assessed for: (i) purity by SDS-PAGE and silver staining; (ii) the presence of ALK by anti-ALK1 immunoblotting; (iii) autophosphorylation activity by a radioactive kinase assay. B. Identification of a peptide substrate of rALK. Phosphorylation of the ARDIYRASFFRKGGCAMLPVK peptide#1 (200 μM) and the random polymer polyGlu4Tyr (0.1 mg/mL) by rALK was measured by an *in vitro* radioactive kinase assay. Reactions were performed in the presence of 50 mM Tris/HCl pH7.5, 5 mM $MnCl_2$, 10 μM Na-vanadate, 30 μM [γ^{32}P]ATP (specific activity 1000 cpm/pmol) and 10 units of rALK [1 unit = amount of enzyme transferring 1 pmol phosphate/min to polyGlu4Tyr] for 10 min at 30°C. Reactions were spotted onto P81 phosphocellulose paper, washed and labeled peptide measured by scintillation. Results are expressed as mean dpm ± s.dev. (n=3). C. Time course of the phosphorylation of peptide#1 in the ALK-ELISA compared with negative control peptide (GILGFVFTL) or no peptide. Peptide (2.5 μg/well) was immobilized on NuncImmuno plates and phosphorylated by rALK (50 ng/well) in the presence of 300 μM ATP, 50 mM Tris pH7.5, 5 mM $MnCl_2$, and 5 mM $MnCl_2$ at 30°C for 10 min. Phosphorylated peptide was detected using the monoclonal 4G10 anti-phosphotyrosine antibody (UpstateBiotech Ltd.), an anti-mouse IgG-HRP linked antibody (Amersham Pharmacia Biotech) and HRP substrate (tetramethylbenzidine substrate solution, Endogen). Absorbance was read at 450 nm using an Ultrospec® 300 microtiter plate reader (Amersham-Pharmacia Biotech). Results are expressed as the mean ± s.dev. (n=3).

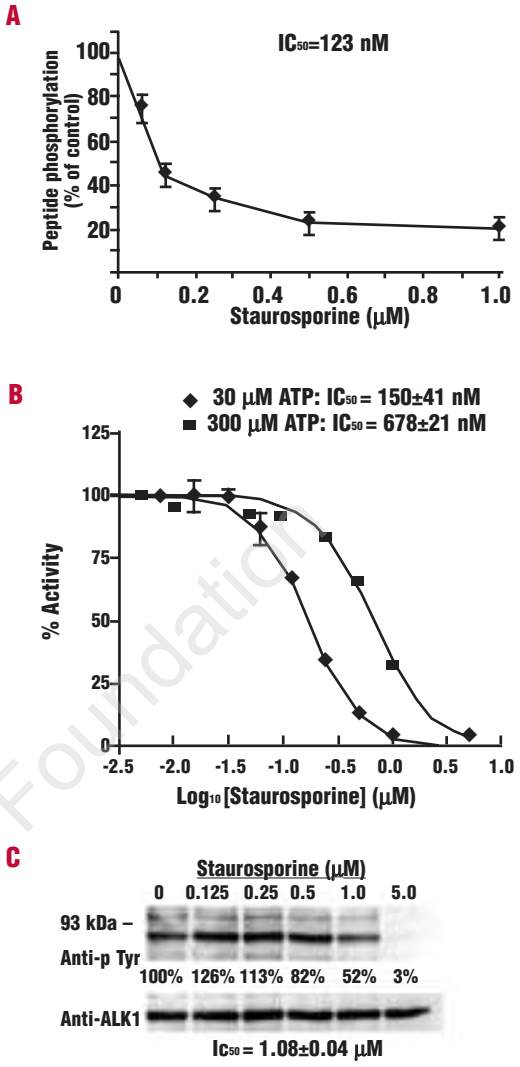

Figure 2. The effect of staurosporine on ALK activity. Staurosporine concentration-response curves showing inhibition of peptide#1 phosphorylation by purified rALK determined in the radioactive assay (A) and the ALK-ELISA (B). A. The kinase reaction was performed as described in Figure 1B in the presence of staurosporine, for 10 mins. B. The ALK-ELISA was performed as described in Figure 1C in the presence of 30 or 300 μM ATP and staurosporine. Results are normalized to vehicle control and expressed as the mean ± s.dev. (n=3). IC_{50} values were determined using GraphPad prism software fitting data using nonlinear regression. C. Inhibition of NPM/ALK autophosphorylation by staurosporine. BaF3 pro-B murine cells transfected with NPM/ALK were treated for 2h with staurosporine. NPM/ALK tyrosine phosphorylation was determined by anti-phosphotyrosine (anti-pTyr) immunoblotting and protein loading was controlled by anti-ALK1 immunoblotting. Band density was determined by densitometry and expressed as a percent of the control.

IC_{50} value (1 μM) observed for full length NPM/ALK expressed in BaF3 cells assessed by anti-phosphotyrosine immunoblotting and densitometry (Figure 2C).

Ten compounds derived from the tyrphostin, AG957, developed as a Bcr/Abl inhibitor,[8] were screened using the ALK-ELISA. These compounds were mostly inactive

on ALK with the most potent derivatives, adaphostin and NSC 689857, having IC_{50} values of 23 and 10 μM, respectively (*data not shown*). UCN-01, a 7-hydroxy staurosporine derivative, reported to have induced disease stability in a patient with ALK-positive ALCL in a phase I clinical trial,[9] was also tested. UCN-01 inhibited ALK in the presence of 30 μM ATP (IC_{50}=5 μM), however no inhibition was observed at 300 μM ATP (*data not shown*). The low potency of UCN-01 and its lack of specificity for NPM/ALK transformed cells in proliferation assays (*data not shown*) suggest that UCN-01 does not target ALK. Therefore, potent and specific ALK inhibitors still need to be developed. The ALK-ELISA is a robust and accurate method suitable for middle and high-throughput screening that can be applied to the discovery of ALK inhibitors.

Rosalind Helen Gunby, Carmen Julia Tartari,* Francesca Porchia,* Arianna Donella-Deana,° Leonardo Scapozza,# Carlo Gambacorti-Passerini*@*

**Department of Experimental Oncology, National Cancer Institute, Milan, Italy; °Department of Biological Chemistry and CRIBI, National Research Centre, Institute of Neuroscience, University of Padua, Italy; #Section des Sciences Pharmaceutiques, University of Geneva, Switzerland; @McGill University, Montreal, Canada; ^Department of Internal Medicine, University of Milan Bicocca, Milan, Italy*

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Correspondence: Rosalind Helen Gunby, Department of Experimental Oncology, National Cancer Institute, via Venezian 1, 20133 Milan, Italy. Phone: international +39.02.23902689. Fax: international +39.02.23903237. E-mail: rosalind.gunby@istitutotumori.mi.it

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Malignant Lymphomas

Highly active antiretroviral therapy and outcome of AIDS-related Burkitt's lymphoma or leukemia. Results of the PETHEMA-LAL3/97 study

Short, intensive cycles of chemotherapy have resulted in improved survival in Burkitt's lymphoma/leukemia (BL) in adults.[1] The prognosis of patients with immunodeficiency virus (HIV)-associated BL is considered to be poor, but these patients have seldom been treated with BL-specific protocols.[2] However, a study (PETHEMA-LAL3/97) in which patients with BL were treated regardless of their HIV status failed to find differences between HIV-infected and immunocompetent individuals.[3] Furthermore, patients who received highly active antiretroviral therapy (HAART) seemed to have a slightly better disease-free survival than those who did not (p=0.051). We extended the follow-up analysis to elucidate the role of HAART in the survival of HIV-infected patients included in the PETHEMA-LAL3/97 protocol.

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We present the longer-term results of the multicenter PETHEMA LAL3/97 study on Burkitt's lymphoma/leukemia carried out by the Spanish PETHEMA group. The diagnostic criteria, characteristics of treatment, response criteria and follow-up procedures were reported in the original analysis.[3] Briefly, patients over 15 years old with newly diagnosed advanced BL (leukemic disease, lymphoma in stages III-IV or in stage II with a bulky mass) were treated, irrespectively of HIV status, with eight cycles of chemotherapy including alternating combinations of cytarabine, methotrexate, cyclophosphamide, ifosfamide, doxorubicin, teniposide, vincristine and dexamethasone. Triple drug HAART,including at least one protease inhibitor and two nucleoside reverse transcriptase inhibitors,[4] was recommended from diagnosis for HIV-positive patients if they were not receiving it already and was continued thereafter. The present analysis includes an extended follow-up of the 14 original HIV-positive patients reported[3] and 5 additional patients included in the protocol but previously excluded from analysis because of insufficient follow-up at that time. Overall survival (OS) and disease-free survival (DFS) were censored in July 2004 or date of last contact. Virological response to HAART was defined as having