

Methods for the identification of ZAP-70 interacting molecules and for the purification of ZAP-70

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The present invention relates to methods for the identification of ZAP-70 interacting molecules and for the purification of ZAP-70 using aminopyrido-pyrimidine ligand 24 as a ligand for ZAP-70. Furthermore, the present invention relates to pharmaceutical compositions comprising said interacting molecules e.g. for the treatment of T-cell mediated diseases such as autoimmune disease, inflammation and transplant rejection.

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Protein kinases participate in the signaling events which control the activation, growth and differentiation of cells in response to extracellular mediators or stimuli such as growth factors, cytokines or chemokines. In general, these kinases are classified in two groups, those that preferentially phosphorylate tyrosine residues and those that preferentially phosphorylate serine and/or threonine residues. The tyrosine kinases include membrane-spanning growth factor receptors such as the epidermal growth factor receptor (EGFR) and cytosolic non-receptor kinases such as Src, Syk or ZAP-70.

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Inappropriately high protein kinase activity is involved in many diseases including cancer and inflammatory disorders. This can be caused either directly or indirectly by the failure of control mechanisms due to mutation, overexpression or inappropriate activation of the enzyme. In all of these instances, selective inhibition of the kinase is expected to have a beneficial effect.

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Protein tyrosine kinases - both receptor tyrosine kinases and non-receptor kinases - are essential for the activation and proliferation of cells of the immune system. Among the earliest detectable events upon the immunoreceptor activation in mast cells, T cells and B cells is the stimulation of non-receptor tyrosine kinases. Immune receptors such as the high-affinity IgE receptor (FcεRI), T cell antigen receptor (TCR) and B cell receptor, consist of antigen-binding subunits and signal transducing subunits. The signal transducing chain contains one or more copies of immunoreceptor tyrosine-based activation motifs (ITAMs). For TCR activation, ITAMs located in the CD3 molecule are phosphorylated by Lck and Fyn, two Src family tyrosine kinases, followed by recruitment and activation of ZAP-70, a member of the Syk family of tyrosine kinases. These activated tyrosine kinases then phosphorylate downstream adaptor molecules such as LAT (linker for activation of T cells) and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa). This step leads to

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the activation of multiple downstream signaling molecules such as inducible T cell kinase (ITK), PLC γ 1 and PI3 kinase (Wong, 2005, Current Opinion in Pharmacology 5, 1-8).

5 ZAP-70 (zeta-associated protein of 70 kDa) belongs to the Syk family of tyrosine kinases and is associated with the zeta subunit of the T cell receptor (Chan et al., 1991, Proc. Natl. Acad. Sci. USA 88, 9166-9170; Weiss, 1993, Cell 73, 209-212). ZAP-70 is primarily expressed in T cells and Natural Killer (NK) cells and plays an essential role in signaling through the TCR. The TCR-mediated activation of T cells is crucial for the immune response. Failure to adequately regulate T cell activation can lead to allergic and
10 autoimmune diseases. Therefore ZAP-70 is considered as an attractive target for the development of immunosuppressive agents for T cell mediated diseases.

Several approaches for the identification of selective ZAP-70 inhibitors have been reported. Vu suggested the structure-based design and synthesis of antagonists of the
15 tandem Src-homology 2 (SH2) domains of ZAP-70 (Vu, 2000, Curr. Med. Chem. 7(10), 1081-1100). Nishikawa screened a peptide library for the ability to bind to ZAP-70 and identified a peptide that inhibited ZAP-kinase activity by competing with protein substrates (Nishikawa et al., 2000, Molecular Cell 6, 969-974). Moffat used a ZAP-70 kinase assay with the non-physiological substrate polyGluTyr to identify ZAP-70 inhibitors (Moffat et
20 al., 1999, Bioorg. Med. Chem. Letters 9, 3351-3356). In addition, the three-dimensional structure of the ZAP-70 kinase domain in complex with Staurosporine was reported and suggested as basis for the structure-based design of inhibitors (Jin et al., 2004, J. Biol. Chem. 279(41), 42818-42825).

25 Although ZAP-70 and its role in TCR signaling was already discovered in 1991 (Chan et al., 1991, Proc. Natl. Acad. Sci. USA 88, 9166-9170) and soon afterwards recognized as a logical drug target for T cell mediated diseases, no ZAP-70 inhibitors have been approved as drugs yet. One reason for the failure to identify and develop selective ZAP-70 inhibitors is the lack of effective assay methods to identify compounds that interact with the
30 physiological form of ZAP-70 as it occurs in activated T-cells.

In view of the above, there is a need for providing effective methods for the identification of ZAP-70 interacting compounds as well as for methods for the purification of ZAP-70.

35 To comply with this need, the invention provides in a first aspect a method for the identification of a ZAP-70 interacting compound, comprising the steps of

- a) providing a protein preparation containing phosphorylated ZAP-70,
- b) contacting the protein preparation with aminopyrido-pyrimidine ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex,
- c) incubating the aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex with a given compound, and
- d) determining whether the compound is able to separate phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidine ligand 24.

In a second aspect, the present invention relates to a method for the identification of a ZAP-70 interacting compound, comprising the steps of

- a) providing a protein preparation containing phosphorylated ZAP-70,
- b) contacting the protein preparation with aminopyrido-pyrimidine ligand 24 immobilized on a solid support and with a given compound under conditions allowing the formation of an aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex, and
- c) detecting the aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex formed in step b).

In a third aspect, the invention provides a method for the identification of a ZAP-70 interacting compound, comprising the steps of:

- a) providing two aliquots of a protein preparation containing phosphorylated ZAP-70,
- b) contacting one aliquot with aminopyrido-pyrimidine ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex,
- c) contacting the other aliquot with aminopyrido-pyrimidine ligand 24 immobilized on a solid support and with a given compound under conditions

allowing the formation of an aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex, and

- d) determining the amount of aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex formed in steps b) and c).

In a fourth aspect, the invention relates to a method for the identification of a ZAP-70 interacting compound, comprising the steps of:

- a) providing two aliquots comprising each at least one cell containing phosphorylated ZAP-70,
- b) incubating one aliquot with a given compound,
- c) harvesting the cells of each aliquot,
- d) lysing the cells in order to obtain protein preparations,
- e) contacting the protein preparations with aminopyrido-pyrimidine ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex, and
- f) determining the amount of aminopyrido-pyrimidine ligand 24 – phosphorylated ZAP-70 complex formed in each aliquot in step e).

In the context of the present invention, it has been surprisingly found that aminopyrido-pyrimidine ligand 24 is a ZAP-70 ligand which recognizes preferably phosphorylated ZAP-70 (see Figure 2). This enables the use of aminopyrido-pyrimidine ligand 24 in screening assays, e.g. in competitive screening assays as well as in methods for the purification of phosphorylated ZAP-70.

The structure of aminopyrido-pyrimidine ligand 24 is given in Fig. 1. This compound (7-(4-Aminomethyl-phenylamino)-3-(2,6-dichloro-phenyl)-1-methyl-1H-[1,6]naphthyridin-2-one) is a substituted aminopyrido-pyrimidine compound. The aminopyrido-pyrimidine ligand 24 can be covalently coupled to a suitable solid support material via the primary amino group and be used for the isolation of binding proteins. The synthesis of aminopyrido-pyrimidine ligand 24 is described in Example 1. According to the invention,

the expression "aminopyrido-pyrimidine ligand 24" also includes compounds comprising the core, e.g. the identical Pyrido[2,3]pyrimidine core structure as described by Klutchko and colleagues (Klutchko et al., 1998, J. Med. Chem. 41(17) :3276-3292) but which have another linker, preferably coupled to the amino group not being part of the cyclic structures, for linkage to the solid support. Typically linkers have backbone of 8, 9 or 10 atoms. The linkers may contain either a carboxy-, hydroxy or amino-active group.

Therefore, preferably the expression "aminopyrido-pyrimidine ligand 24" also includes compounds having the same (7-phenylamino)-3-(2,6-dichloro-phenyl)-1H-[1,6]naphthyridin-2-one) core which may be optionally substituted at the 4-position of the 7-phenylamino, e.g. with an alkyl having e.g. 1-15 C-atoms, which may be substituted with an amino-, hydroxy- or carboxy-group, and which may be additionally substituted at the 1H position of the N-atom of the 1H-[1,6]naphthyridin-2-one, e.g. with an alkyl having e.g. 1-15 C-atoms. More preferably, the expression "aminopyrido-pyrimidine ligand 24" also includes compounds with the same (7-phenylamino)-3-(2,6-dichloro-phenyl)-1H-[1,6]naphthyridin-2-one) core which are only substituted at the 4-position of the 7-phenylamino with an alkyl having e.g. 1-15 C-atoms, which may be substituted with an amino-, hydroxy- or carboxy-group and at the 1H-position of the 1H-[1,6]naphthyridin-2-one with an alkyl having e.g. 1-15 C-atoms.

Most preferably, compounds designated as "aminopyrido-pyrimidine ligand 24" are selected from the group consisting of (7-(4-Aminomethyl-phenylamino)-3-(2,6-dichloro-phenyl)-1-methyl-1H-[1,6]naphthyridin-2-one) and of compounds with the same (7-phenylamino)-3-(2,6-dichloro-phenyl)-1H-[1,6]naphthyridin-2-one) core which are only substituted at the 4-position of the 7-phenylamino with an alkyl having e.g. 1-15 C-atoms, which may be substituted with an amino-, hydroxy- or carboxy-group and at the 1H-position of the 1H-[1,6]naphthyridin-2-one with an alkyl having e.g. 1-15 C-atoms.

Pyrido[2,3]pyrimidine derivatives were initially described as ATP-competitive inhibitors of receptor tyrosine kinases (platelet-derived growth factor receptor, PDGFR; fibroblast growth factor receptor, FGFR; epidermal growth factor receptor, EGFR) and the non-receptor tyrosine kinase c-Src (Klutchko et al., 1998, J. Med. Chem. 41(17): 3276-3292). Subsequently, it was reported that the PD173955 compound potently inhibited the Bcr-Abl fusion protein and the c-Kit receptor tyrosine kinase (Wisniewski et al., 2002, Cancer Research 62, 4244-4255) and also the anaplastic lymphoma kinase (ALK) (Gunby et al., 2006, J. Med. Chem. 49(19):5759-5768).

In a chemical proteomics study it was found that immobilized pyrido[2,3-d]pyrimidine ligands interact with more than 30 human protein kinases and potently inhibited both the p38 and RICK kinases (Wissing et al., 2004, Mol. Cell. Proteomics 3(12):1181-1193). In independent proteomic studies it was observed that the immobilized PD173955 compound
5 interacts with several ephrin receptor tyrosine kinases (WO2006/056467A1). Yet another publication demonstrated that this compound potently inhibited the kinase activity of ephrin receptors (Caligiuri et al., 2006, Chemistry & Biology 13, 711-722).

According to the present invention, the expression "ZAP-70" does not only mean the
10 human protein as shown in Figure 4 but also a functionally active derivative thereof, or a functionally active fragment thereof, or a homologue thereof, or a variant encoded by a nucleic acid that hybridizes to the nucleic acid encoding said protein under low stringency conditions. Preferably, these low stringency conditions include hybridization in a buffer comprising 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02%
15 PVP, 0.02% BSA, 100 ug/ml denatured salmon sperm DNA, and 10% (wt/vol) dextran sulfate for 18-20 hours at 40°C, washing in a buffer consisting of 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS for 1-5 hours at 55°C, and washing in a buffer consisting of 2X SSC, 25 mM Tris-HCl (pH 7.4) 5 mM EDTA, and 0.1% SDS for 1.5 hours at 60°C.

20 In some aspects of the invention, first a protein preparation containing phosphorylated ZAP-70 is provided. The methods of the present invention can be performed with any protein preparation as a starting material, as long as the phosphorylated ZAP-70 is solubilized in the preparation. Examples include a liquid mixture of several proteins, a cell
25 lysate, a partial cell lysate which contains not all proteins present in the original cell or a combination of several cell lysates.

The presence of phosphorylated ZAP-70 protein species in a protein preparation of interest can be detected on Western blots probed with antibodies that are specifically directed
30 against ZAP-70 phosphorylation sites, for example phosphorylated Tyrosine residues at position 126, 292, 319, 492 or 493 (Watts et al., 1994, The Journal of Biological Chemistry 269(4), 29520-29529). Antibodies directed against phosphorylated Serine or threonine can also be used. Such phospho-specific anti-ZAP-70 antibodies can be obtained from commercial suppliers (e.g. Upstate or Cell Signaling). The use of such anti-phospho
35 antibodies in conjunction with Western blot analysis to detect phosphorylated ZAP-70 has been described (Houtman et al., 2005, The Journal of Immunology 175(4), 2449-2458).

Cell lysates or partial cell lysates can be obtained by isolating cell organelles (e.g. nucleus, mitochondria, ribosomes, golgi etc.) first and then preparing protein preparations derived from these organelles. Methods for the isolation of cell organelles are known in the art (Chapter 4.2 Purification of Organelles from Mammalian Cells in "Current Protocols in Protein Science", Editors: John.E. Coligan, Ben M. Dunn, Hidde L. Ploegh, David W. Speicher, Paul T. Wingfield; Wiley, ISBN: 0-471-14098-8).

In addition, protein preparations can be prepared by fractionation of cell extracts thereby enriching specific types of proteins such as cytoplasmic or membrane proteins (Chapter 4.3 Subcellular Fractionation of Tissue Culture Cells in "Current Protocols in Protein Science", Editors: John.E. Coligan, Ben M. Dunn, Hidde L. Ploegh, David W. Speicher, Paul T. Wingfield; Wiley, ISBN: 0-471-14098-8).

Furthermore protein preparations from body fluids can be used (e.g. blood, cerebrospinal fluid, peritoneal fluid and urine).

For example whole embryo lysates derived from defined development stages or adult stages of model organisms such as *C. elegans* can be used. In addition, whole organs such as heart dissected from mice can be the source of protein preparations. These organs can also be perfused in vitro in order to obtain a protein preparation.

Furthermore, the protein preparation may be a preparation containing phosphorylated ZAP-70 which has been recombinantly produced. Methods for the production of recombinant proteins in prokaryotic and eukaryotic cells are widely established (Chapter 5 Production of Recombinant Proteins in "Current Protocols in Protein Science", Editors: John. E. Coligan, Ben M. Dunn, Hidde L. Ploegh, David W. Speicher, Paul T. Wingfield; Wiley, 1995, ISBN: 0-471-14098-8).

In a preferred embodiment of the methods of the invention, the provision of a protein preparation includes the steps of harvesting at least one cell containing phosphorylated ZAP-70 and lysing the cell.

The ZAP-70 protein is preferentially expressed in T lymphocytes and Natural Killer (NK) cells. Therefore cells isolated from peripheral blood represent a suitable biological material. Procedures for the preparation and culture of human lymphocytes and lymphocyte subpopulations obtained from peripheral blood (PBLs) are widely known (W.E Biddison, Chapter 2.2 "Preparation and culture of human lymphocytes" in Current

Protocols in Cell Biology, 1998, John Wiley & Sons, Inc.). For example, density gradient centrifugation is a method for the separation of lymphocytes from other blood cell populations (e.g. erythrocytes and granulocytes). Human lymphocyte subpopulations can be isolated via their specific cell surface receptors which can be recognized by monoclonal antibodies. The physical separation method involves coupling of these antibody reagents to magnetic beads which allow the enrichment of cells that are bound by these antibodies (positive selection). The isolated lymphocyte cells can be further cultured and stimulated by adding antibodies directed against the T-cell receptor or co-receptors such as CD-3 to initiate T-cell receptor signaling and subsequently phosphorylation of ZAP-70 (Houtman et al., 2005, The Journal of Immunology 175(4), 2449-2458).

As an alternative to primary human cells cultured cell lines (e.g. Jurkat cells) can be used and stimulated in analogous fashion with anti-CD3 antibodies to obtain phosphorylated ZAP-70 (Kim and White, 2006, The Journal of Immunology 176(5):2833-2843).

Alternatively, it is also possible to incubate said lymphocytes or cell lines with phosphatase inhibitors in order to keep ZAP-70 in its phosphorylated state. Such methods are known in the art (D.C. Weiser and S. Shenolikar, Chapter 18.10 in *Current Protocols in Molecular Biology*, 2003, John Wiley & Sons, Inc.).

In a preferred embodiment, the cell is part of a cell culture system and methods for the harvest of a cell out of a cell culture system are known in the art (literature supra).

The choice of the cell will mainly depend on the expression of ZAP-70, since it has to be ensured that the protein is principally present in the cell of choice. In order to determine whether a given cell is a suitable starting system for the methods of the invention, methods like Westernblot, PCR-based nucleic acids detection methods, Northernblots and DNA-microarray methods ("DNA chips") might be suitable in order to determine whether a given protein of interest is present in the cell.

The choice of the cell may also be influenced by the purpose of the study. If the in vivo efficacy for a given drug needs to be analyzed then cells or tissues may be selected in which the desired therapeutic effect occurs (e.g. T cells, NK cells or ZAP-70 positive CLL cells). By contrast, for the elucidation of protein targets mediating unwanted side effects the cell or tissue may be analysed in which the side effect is observed (e.g. bone marrow, thymus).

Furthermore, it is envisaged within the present invention that the cell containing phosphorylated ZAP-70 may be obtained from an organism, e.g. by biopsy. Corresponding methods are known in the art. For example, a biopsy is a diagnostic procedure used to obtain a small amount of tissue, which can then be examined microscopically or with
5 biochemical methods. Biopsies are important to diagnose, classify and stage a disease, but also to evaluate and monitor drug treatment.

It is encompassed within the present invention that by the harvest of the at least one cell, the lysis is performed simultaneously. However, it is equally preferred that the cell is first
10 harvested and then separately lysed.

Methods for the lysis of cells are known in the art (Karwa and Mitra: Sample preparation for the extraction, isolation, and purification of Nuclei Acids; chapter 8 in "Sample Preparation Techniques in Analytical Chemistry", Wiley 2003, Editor: Somenath Mitra, print ISBN: 0471328456; online ISBN: 0471457817). Lysis of different cell types and
15 tissues can be achieved by homogenizers (e.g. Potter-homogenizer), ultrasonic desintegrators, enzymatic lysis, detergents (e.g. NP-40, Triton X-100, CHAPS, SDS), osmotic shock, repeated freezing and thawing, or a combination of these methods.

20 According to the methods of the invention, the protein preparation containing phosphorylated ZAP-70 is contacted with the aminopyrido-pyrimidine ligand 24 immobilized on a solid support under conditions allowing the formation of a aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex.

25 In the present invention, the term "an aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex" denotes a complex where aminopyrido-pyrimidine ligand 24 interacts with ZAP-70, e.g. by covalent or, most preferred, by non-covalent binding.

The skilled person will know which conditions can be applied in order to enable the
30 formation of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex.

In the context of the present invention, the term "under conditions allowing the formation of the complex" includes all conditions under which such formation, preferably such binding is possible. This includes the possibility of having the solid support on an
35 immobilized phase and pouring the lysate onto it. In another preferred embodiment, it is also included that the solid support is in a particulate form and mixed with the cell lysate.

In the context of non-covalent binding, the binding between aminopyrido-pyrimidine ligand 24 and phosphorylated ZAP-70 is, e.g., via salt bridges, hydrogen bonds, hydrophobic interactions or a combination thereof.

5 In a preferred embodiment, the steps of the formation of the aminopyrido-pyrimidine ligand 24 - phosphorylated ZAP-70 complex are performed under essentially physiological conditions. The physical state of proteins within cells is described in Petty, 1998 (Howard R. Petty¹, Chapter 1, Unit 1.5 in: Juan S. Bonifacino, Mary Dasso, Joe B. Harford, Jennifer Lippincott-Schwartz, and Kenneth M. Yamada (eds.) *Current Protocols in Cell*
10 *Biology* Copyright © 2003 John Wiley & Sons, Inc. All rights reserved. DOI: 10.1002/0471143030.cb0101s00 Online Posting Date: May, 2001 Print Publication Date: October, 1998).

The contacting under essentially physiological conditions has the advantage that the
15 interactions between the ligand, the cell preparation (i. e. the enzyme to be characterized) and optionally the compound reflect as much as possible the natural conditions. “Essentially physiological conditions” are *inter alia* those conditions which are present in the original, unprocessed sample material. They include the physiological protein concentration, pH, salt concentration, buffer capacity and post-translational modifications
20 of the proteins involved. The term “essentially physiological conditions” does not require conditions identical to those in the original living organism, wherefrom the sample is derived, but essentially cell-like conditions or conditions close to cellular conditions. The person skilled in the art will, of course, realize that certain constraints may arise due to the experimental set-up which will eventually lead to less cell-like conditions. For example,
25 the eventually necessary disruption of cell walls or cell membranes when taking and processing a sample from a living organism may require conditions which are not identical to the physiological conditions found in the organism. Suitable variations of physiological conditions for practicing the methods of the invention will be apparent to those skilled in the art and are encompassed by the term “essentially physiological conditions” as used
30 herein. In summary, it is to be understood that the term “essentially physiological conditions” relates to conditions close to physiological conditions, as e. g. found in natural cells, but does not necessarily require that these conditions are identical.

For example, “essentially physiological conditions” may comprise 50-200 mM NaCl or
35 KCl, pH 6.5-8.5, 20-45°C, and 0.001-10 mM divalent cation (e.g. Mg⁺⁺, Ca⁺⁺,); more preferably about 150 mM NaCl or KCl, pH 7.2 to 7.6, 5 mM divalent cation and often include 0.01-1.0 percent non-specific protein (e.g. BSA). A non-ionic detergent (Tween, NP-40,

Triton-X100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (volume/volume). For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents.

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Preferably, "essentially physiological conditions" mean a pH of from 6.5 to 7.5, preferably from 7.0 to 7.5, and / or a buffer concentration of from 10 to 50 mM, preferably from 25 to 50 mM, and / or a concentration of monovalent salts (e.g. Na or K) of from 120 to 170 mM, preferably 150 mM. Divalent salts (e.g. Mg or Ca) may further be present at a concentration of from 1 to 5 mM, preferably 1 to 2mM, wherein more preferably the buffer is selected from the group consisting of Tris-HCl or HEPES.

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In the context of the present invention, aminopyrido-pyrimidine ligand 24 is immobilized on a solid support. Throughout the invention, the term "solid support" relates to every undissolved support being able to immobilize a small molecule ligand on its surface.

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According to a further preferred embodiment, the solid support is selected from the group consisting of agarose, modified agarose, sepharose beads (e.g. NHS-activated sepharose), latex, cellulose, and ferro- or ferromagnetic particles.

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Aminopyrido-pyrimidine ligand 24 may be coupled to the solid support either covalently or non-covalently. Non-covalent binding includes binding via biotin affinity ligands binding to streptavidin matrices.

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Preferably, the aminopyrido-pyrimidine ligand 24 is covalently coupled to the solid support.

Before the coupling, the matrixes can contain active groups such as NHS, Carbodimide etc. to enable the coupling reaction with the aminopyrido-pyrimidine ligand 24. The aminopyrido-pyrimidine ligand 24 can be coupled to the solid support by direct coupling (e.g. using functional groups such as amino-, sulfhydryl-, carboxyl-, hydroxyl-, aldehyde-, and ketone groups) and by indirect coupling (e.g. via biotin, biotin being covalently attached to aminopyrido-pyrimidine ligand 24 and non-covalent binding of biotin to streptavidin which is bound to solid support directly).

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The linkage to the solid support material may involve cleavable and non-cleavable linkers. The cleavage may be achieved by enzymatic cleavage or treatment with suitable chemical methods.

5 Preferred binding interfaces for binding aminopyrido-pyrimidine ligand 24 to solid support material are linkers with a C-atom backbone. Typically linkers have backbone of 8, 9 or 10 atoms. The linkers contain either a carboxy- or amino-active group.

10 The skilled person will appreciate that between the individual steps of the methods of the invention, washing steps may be necessary. Such washing is part of the knowledge of the person skilled in the art. The washing serves to remove non-bound components of the cell lysate from the solid support. Nonspecific (e.g. simple ionic) binding interactions can be minimized by adding low levels of detergent or by moderate adjustments to salt concentrations in the wash buffer.

15 According to the identification methods of the invention, the read-out system is either the detection or determination of phosphorylated ZAP-70 (first aspect of the invention), the detection of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex (second aspect of the invention), or the determination of the amount of the aminopyrido-
20 pyrimidine ligand 24-phosphorylated ZAP-70 complex (second, third and forth aspect of the invention).

In the method according to the first aspect of the invention, the detection or determination of separated phosphorylated ZAP-70 is preferably indicative for the fact that the compound
25 is able to separate phosphorylated ZAP-70 from the immobilized aminopyrimidin ligand 24. This capacity indicates that the respective compound interacts, preferably binds to ZAP-70, which is indicative for its therapeutic potential.

30 In one embodiment of the method according to the second aspect of the invention, the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex formed during the method of the invention is detected. The fact that such complex is formed preferably indicates that the compound does not completely inhibit the formation of the complex. On the other hand, if no complex is formed, the compound is presumably a strong interactor with ZAP-70, which is indicative for its therapeutic potential.

35 According to the methods of the second, third and forth aspect of the invention the amount of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex formed during

the method is determined. In general, the less complex in the presence of the respective compound is formed, the stronger the respective compound interacts with phosphorylated ZAP-70, which is indicative for its therapeutic potential.

5 The detection of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex according to the second aspect of the invention can be performed by using labeled antibodies directed against phosphorylated ZAP-70 and a suitable readout system.

10 According to a preferred embodiment of the second aspect of the invention, the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex is detected by determining its amount.

In the course of the second, third and forth aspect of the invention, it is preferred that phosphorylated ZAP-70 is separated from the immobilized aminopyrido-pyrimidine ligand
15 24 in order to determine the amount of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex.

According to invention, separating means every action which destroys the interactions between aminopyrido-pyrimidine ligand 24 and phosphorylated ZAP-70. This includes in a
20 preferred embodiment the elution of phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidine ligand 24.

The elution can be achieved by using non-specific reagents as described in detail below (ionic strength, pH value, detergents). In addition, it can be tested whether a compound of
25 interest can specifically elute the phosphorylated ZAP-70 from aminopyrido-pyrimidine ligand 24. Such ZAP-70 interacting compounds are described further in the following sections.

Such non-specific methods for destroying the interaction are principally known in the art and depend on the nature of the ligand enzyme interaction. Principally, change of ionic
30 strength, the pH value, the temperature or incubation with detergents are suitable methods to dissociate the target enzymes from the immobilized ligand. The application of an elution buffer can dissociate binding partners by extremes of pH value (high or low pH; e.g. lowering pH by using 0.1 M citrate, pH2-3), change of ionic strength (e.g. high salt
35 concentration using NaI, KI, MgCl₂, or KCl), polarity reducing agents which disrupt hydrophobic interactions (e.g. dioxane or ethylene glycol), or denaturing agents

(chaotropic salts or detergents such as Sodium-dodecyl-sulfate, SDS; Review: Subramanian A., 2002, Immunoaffinity chromatography).

In some cases, the solid support has preferably to be separated from the released material.

- 5 The individual methods for this depend on the nature of the solid support and are known in the art. If the support material is contained within a column the released material can be collected as column flowthrough. In case the support material is mixed with the lysate components (so called batch procedure) an additional separation step such as gentle centrifugation may be necessary and the released material is collected as supernatant.
- 10 Alternatively magnetic beads can be used as solid support so that the beads can be eliminated from the sample by using a magnetic device.

- In step d) of the method according to the first aspect of the invention, it is determined if phosphorylated ZAP-70 has been separated from the immobilized aminopyrido-pyrimidine
- 15 ligand 24. This may include the detection of phosphorylated ZAP-70 or the determination of the amount of phosphorylated ZAP-70.

- Consequently, at least in preferred embodiments of all identification methods of the invention, methods for the detection of separated phosphorylated ZAP-70 or for the
- 20 determination of its amount are used. Such methods are known in the art and include physico-chemical methods such as protein sequencing (e.g. Edmann degradation), analysis by mass spectrometry methods or immunodetection methods employing antibodies directed against phosphorylated ZAP-70.

- 25 Preferably, phosphorylated ZAP-70 is detected or the amount of phosphorylated ZAP-70 is determined by mass spectrometry or immunodetection methods.

- The identification of proteins with mass spectrometric analysis (mass spectrometry) is known in the art (Shevchenko et al., 1996, Analytical Chemistry 68: 850-858, (Mann et al.,
- 30 2001, Analysis of proteins and proteomes by mass spectrometry, Annual Review of Biochemistry 70, 437-473) and is further illustrated in the example section.

- Preferably, the mass spectrometry analysis is performed in a quantitative manner, for example by using iTRAQ technology (isobaric tags for relative and absolute quantification)
- 35 or cICAT (cleavable isotope-coded affinity tags) (Wu et al., 2006. J. Proteome Res. 5, 651-658).

According to a further preferred embodiment of the present invention, the characterization by mass spectrometry (MS) is performed by the identification of proteotypic peptides of phosphorylated ZAP-70. The idea is that phosphorylated ZAP-70 is digested with proteases and the resulting peptides are determined by MS. As a result, peptide frequencies for peptides from the same source protein differ by a great degree, the most frequently observed peptides that “typically” contribute to the identification of this protein being termed “proteotypic peptide”. Therefore, a proteotypic peptide as used in the present invention is an experimentally well observable peptide that uniquely identifies a specific protein or protein isoform.

According to a preferred embodiment, the characterization is performed by comparing the proteotypic peptides obtained in the course of practicing the methods of the invention with known proteotypic peptides. Since, when using fragments prepared by protease digestion for the identification of a protein in MS, usually the same proteotypic peptides are observed for a given enzyme, it is possible to compare the proteotypic peptides obtained for a given sample with the proteotypic peptides already known for enzymes of a given class of enzymes and thereby identifying the enzyme being present in the sample.

As an alternative to mass spectrometry analysis, the eluted phosphorylated ZAP-70 (including coeluted binding partners or scaffold proteins), can be detected or its amount can be determined by using a specific antibody directed against phosphorylated ZAP-70, preferably with an antibody recognizing a Tyrosine phosphorylation at position 493 of phosphorylated ZAP-70. Such antibody is known in the art (Cell Signaling Technologies, #2704).

Since aminopyrido-pyrimidine ligand 24 preferentially recognizes phosphorylated ZAP-70, It is also possible to use anti-ZAP-70 antibodies that recognize non-phosphorylated epitopes on ZAP-70.

Furthermore, in another preferred embodiment, once the identity of the coeluted binding partner has been established by mass spectrometry analysis, each binding partner can be detected with specific antibodies directed against this protein.

Suitable antibody-based assays include but are not limited to Western blots, ELISA assays, sandwich ELISA assays and antibody arrays or a combination thereof. The establishment of such assays is known in the art (Chapter 11, Immunology, pages 11-1 to 11-30 in: Short

Protocols in Molecular Biology. Fourth Edition, Edited by F.M. Ausubel et al., Wiley, New York, 1999).

These assays can not only be configured in a way to detect and quantify a ZAP-70
5 interacting protein of interest (e.g. another kinase such as LAT which is a substrate of ZAP-70), but also to analyse posttranslational modification patterns such as ubiquitin modification.

Furthermore, the identification methods of the invention involve the use of compounds
10 which are tested for their ability to be an ZAP-70 interacting compound.

Principally, according to the present invention, such a compound can be every molecule
which is able to interact with ZAP-70, eg. by inhibiting its binding to aminopyrido-
pyrimidine ligand 24. Preferably, the compound has an effect on ZAP-70, e.g. a
15 stimulatory or inhibitory effect.

Preferably, said compound is selected from the group consisting of synthetic or naturally
occurring chemical compounds or organic synthetic drugs, more preferably small
molecules, organic drugs or natural small molecule compounds. Preferably, said compound
20 is identified starting from a library containing such compounds. Then, in the course of the present invention, such a library is screened.

Such small molecules are preferably not proteins or nucleic acids. Preferably, small
molecules exhibit a molecular weight of less than 1000 Da, more preferred less than 750
25 Da, most preferred less than 500 Da.

A "library" according to the present invention relates to a (mostly large) collection of
(numerous) different chemical entities that are provided in a sorted manner that enables
both a fast functional analysis (screening) of the different individual entities, and at the
30 same time provide for a rapid identification of the individual entities that form the library.
Examples are collections of tubes or wells or spots on surfaces that contain chemical
compounds that can be added into reactions with one or more defined potentially
interacting partners in a high-throughput fashion. After the identification of a desired
"positive" interaction of both partners, the respective compound can be rapidly identified
35 due to the library construction. Libraries of synthetic and natural origins can either be
purchased or designed by the skilled artisan.

Examples of the construction of libraries are provided in, for example, Breinbauer R, Manger M, Scheck M, Waldmann H. Natural product guided compound library development. *Curr Med Chem*. 2002 Dec;9(23):2129-45, wherein natural products are described that are biologically validated starting points for the design of combinatorial libraries, as they have a proven record of biological relevance. This special role of natural products in medicinal chemistry and chemical biology can be interpreted in the light of new insights about the domain architecture of proteins gained by structural biology and bioinformatics. In order to fulfill the specific requirements of the individual binding pocket within a domain family it may be necessary to optimise the natural product structure by chemical variation. Solid-phase chemistry is said to become an efficient tool for this optimisation process, and recent advances in this field are highlighted in this review article. Other related references include Edwards PJ, Morrell AI. Solid-phase compound library synthesis in drug design and development. *Curr Opin Drug Discov Devel*. 2002 Jul;5(4):594-605.; Merlot C, Domine D, Church DJ. Fragment analysis in small molecule discovery. *Curr Opin Drug Discov Devel*. 2002 May;5(3):391-9. Review; Goodnow RA Jr. Current practices in generation of small molecule new leads. *J Cell Biochem Suppl*. 2001;Suppl 37:13-21; which describes that the current drug discovery processes in many pharmaceutical companies require large and growing collections of high quality lead structures for use in high throughput screening assays. Collections of small molecules with diverse structures and "drug-like" properties have, in the past, been acquired by several means: by archive of previous internal lead optimisation efforts, by purchase from compound vendors, and by union of separate collections following company mergers. Although high throughput/combinatorial chemistry is described as being an important component in the process of new lead generation, the selection of library designs for synthesis and the subsequent design of library members has evolved to a new level of challenge and importance. The potential benefits of screening multiple small molecule compound library designs against multiple biological targets offers substantial opportunity to discover new lead structures.

In a preferred embodiment of the second aspect of the invention, phosphorylated ZAP-70 is first incubated with the compound and then with the immobilized aminopyridopyrimidine ligand 24.

Preferably, the phosphorylated ZAP-70 is first incubated with the compound for 10 to 60 minutes, more preferred 30 to 45 minutes at a temperature of 4°C to 37°C, more preferred 4°C to 25°C, most preferred 4°C. Preferably compounds are used at concentrations ranging

from 1 μ M to 1 mM, preferably from 10 to 100 μ M. The second step, contacting with the immobilized ligand, is preferably performed for 10 to 60 minutes at 4°C.

Furthermore, steps a) to c) of the second aspect of the invention may be performed with several protein preparations in order to test different compounds. This embodiment is especially interesting in the context of medium or high throughput screenings (see below).

In a preferred embodiment of the method of the invention according to the third or forth aspect, the amount of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex formed in step c) is compared to the amount formed in step b)

In a preferred embodiment of the method of the invention according to the third or forth aspect, a reduced amount of the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex formed in step c) in comparison to step b) indicates that phosphorylated ZAP-70 is a target of the compound. This results from the fact that in step c) of this method of the invention, the compound competes with the ligand for the binding of phosphorylated ZAP-70. If less phosphorylated ZAP-70 is present in the aliquot incubated with the compound, this means preferably that the compound has competed with the inhibitor for the interaction with the enzyme and is, therefore, a direct target of the enzyme and vice versa.

Preferably, the identification methods of the invention are performed as a medium or high throughput screening.

The interaction compound identified according to the present invention may be further characterized by determining whether it has an effect on ZAP-70 activity, for example on its kinase activity (Isakov et al., The Journal of Biological Chemistry 271(26), 15753-15761).

The interaction compound identified according to the present invention may also be characterized by measuring whether it has an effect on T cell receptor (TCR) signaling in a cell based assay using a T cell line or primary T cells. Cellular activation that is initiated by TCR signaling occurs as a result of a series of molecular events that include tyrosine phosphorylation of the CD3 zeta (CD3 ζ) chain, recruitment of ZAP-70, phosphorylation of phospholipase gamma (PLC γ), inositol 1,4,5-triphosphate production and release of calcium stores from the endoplasmic reticulum to the cytoplasm. Methods for measuring intracellular calcium release using fluorescent indicators for cytosolic calcium after TCR stimulation have been described (Meinl et al., 2000, J. Immunol. 165(7):3578-3583).

According to the classical TCR signaling model the CD3 ζ chain and ZAP-70 are substrates of the Lck kinase, while SLP76, LAT and PLCgamma are downstream of ZAP-70 (Schwartzberg et al. 2005, Nat. Rev. Immunology 5, 284-295). The activity of ZAP-70 can be assessed by detecting specific phosphorylation events on downstream targets with specific anti-phospho-Tyrosine antibodies. For example, ZAP-70 phosphorylates the “linker for activation of T cells” (LAT) protein on the tyrosine residue at position 191 which can be detected by an anti-pTyr 191 antibody (Houtman et al., 2005, J. Immunol. 175(4): 2449-2458). This assay can be used as a readout for ZAP-70 activity or measuring the effect of ZAP-70 interacting compounds on the kinase ZAP-70 activity.

The compounds identified according to the present invention may further be optimized (lead optimisation). This subsequent optimisation of such compounds is often accelerated because of the structure-activity relationship (SAR) information encoded in these lead generation libraries. Lead optimisation is often facilitated due to the ready applicability of high-throughput chemistry (HTC) methods for follow-up synthesis.

One example of such a library and lead optimization is described in Wakeling AE, Barker AJ, Davies DH, Brown DS, Green LR, Cartlidge SA, Woodburn JR. Specific inhibition of epidermal growth factor receptor tyrosine kinase by 4-anilinoquinazolines. Breast Cancer Res Treat. 1996;38(1):67-73.

The invention further relates to a method for the preparation of a pharmaceutical composition comprising the steps of

- a) identifying a ZAP-70 interacting compound as described above, and
- b) formulating the interacting compound to a pharmaceutical composition.

Therefore, the invention provides a method for the preparation of pharmaceutical compositions, which may be administered to a subject in an effective amount. In a preferred aspect, the therapeutic is substantially purified. The subject to be treated is preferably an animal including, but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

The compounds identified according to the invention are useful for the prevention or treatment of diseases where ZAP-70 plays a role, e.g. immune or autoimmune diseases or disorders mediated by T lymphocytes. Such diseases or disorders comprise asthma, rheumatoid arthritis, psoriasis, inflammatory bowel disease (e.g. Crohn's disease or ulcerative colitis), multiple sclerosis, and acute or chronic rejection of organ or tissue allo-
or xenografts.

In addition, the compounds of the invention can be used to treat or ZAP-70 positive B-cell chronic lymphocytic leukaemia (B-CLL).

In general, the pharmaceutical compositions of the present invention comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, including but not limited to peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered orally. Saline and aqueous dextrose are preferred carriers when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions are preferably employed as liquid carriers for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated, in accordance with routine procedures, as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in
5 sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of
10 active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

15 The therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free carboxyl groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., those formed with free amine groups such as those derived from isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc., and those derived from sodium, potassium,
20 ammonium, calcium, and ferric hydroxides, etc..

The amount of the therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, in vitro assays may
25 optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per
30 kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. In general, suppositories may contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

35 Various delivery systems are known and can be used to administer a therapeutic of the invention, e.g., encapsulation in liposomes, microparticles, and microcapsules: use of

recombinant cells capable of expressing the therapeutic, use of receptor-mediated endocytosis (e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432); construction of a therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion, by bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral, rectal and intestinal mucosa, etc.), and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the therapeutic can be delivered in a vesicle, in particular a liposome (Langer, 1990, Science 249:1527-1533).

In yet another embodiment, the therapeutic can be delivered via a controlled release system. In one embodiment, a pump may be used (Langer, supra). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose

The invention further relates to a method for the purification of phosphorylated ZAP-70, comprising the steps of

- a) providing a protein preparation containing phosphorylated ZAP-70,

- b) contacting the protein preparation with aminopyrido-pyrimidine ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex, and

5

- c) separating phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidine ligand 24.

10 As mentioned above, it has been surprisingly found that aminopyrido-pyrimidine ligand 24 is a ligand which recognizes phosphorylated ZAP-70. This enables efficient purification methods for phosphorylated ZAP-70.

15 With respect to phosphorylated ZAP-70, the protein preparation containing phosphorylated ZAP-70, the conditions for contacting with aminopyrido-pyrimidine ligand 24, immobilized aminopyrido-pyrimidine ligand 24, the aminopyrido-pyrimidine ligand 24-phosphorylated ZAP-70 complex, the separation of phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidine ligand 24, and the detection of phosphorylated ZAP-70 or the determination of its amount, the embodiments as defined above for the identification methods of the invention also apply to the purification method of the
20 invention.

In a preferred embodiment, the purification method of the invention further comprises after step c) the identification of proteins being capable of binding to phosphorylated ZAP-70. This is especially interesting when the formation of the complex is performed under
25 essentially physiological conditions, because it is then possible to preserve the natural condition of the enzyme which includes the existence of binding partners, enzyme subunits or post-translational modifications, which can then be identified with the help of mass spectrometry (MS).

30 Consequently, in a preferred embodiment, the purification method of the invention further comprises after step c) the determination whether the phosphorylated ZAP-70 is further posttranslationally modified, e.g. by ubiquitine modification.

35 The invention further relates to the use of aminopyrido-pyrimidine ligand 24 for the identification of ZAP-70 interacting compounds and for the purification of phosphorylated ZAP-70. The embodiments as defined above also apply to the uses of the invention.

It has been described that ZAP-70 is a prognostic marker for chronic lymphocytic leukaemia (CLL) which represents a B-cell malignancy (Hamblin and Hamblin, 2005, Expert Opinion in Therapeutic Targets 9(6):1165-1178). Originally CLL subtypes were distinguished by the mutational status of the immunoglobulin variable region genes, but it is now established that a CLL subtype can be identified by the expression of ZAP-70. The ZAP-70 kinase is normally expressed in T cells rather than B cells, but it is anomalously expressed in the more aggressive subtype of CLL. Consequently, the aminopyrido-pyrimidine ligand 24 is an important tool for the diagnosis of subtypes of CLL or for the prognosis of disease progression by determining the probability that a CLL patient will suffer from advanced stage disease.

Therefore, in a further aspect, the invention also relates to the use of the aminopyrido-pyrimidine ligand 24 for the preparation of a diagnosticum for chronic lymphocytic leukaemia (CLL).

The invention further relates to a method for the diagnosis/prognosis of CLL, comprising the step of determining the presence/amount of phosphorylated ZAP-70 in a sample in relation to the total amount of ZAP-70, wherein an increased amount of phosphorylated ZAP-70 in comparison to the total amount of ZAP-70 (non-phosphorylated plus phosphorylated ZAP-70) in a sample from a CLL patient is indicative for having a higher probability for an aggressive form of CLL.

In this context, a sample is a protein preparation derived from the subject. Consequently, all embodiments discussed above with respect to protein preparation derive from subjects also apply to this aspect of the invention.

In a preferred embodiment of the diagnosis method of the invention, the presence or amount of phosphorylated ZAP-70 and of total ZAP-70 in a sample is determined by the methods described above, e.g. by MS or with the help of a specific antibody. Then, the respective amounts are compared.

According to the invention, "subject" means any mammal, preferably a human being.

The amount of phosphorylated ZAP-70 can be determined as described above.

In the context of the present invention, "diagnosis" means both the diagnosis of an existing disease as well as the prognosis that a given subject has a probability of more than 50 % to develop the respective disease.

- 5 The invention is further illustrated by the following figures and examples, which are not considered as being limiting for the scope of protection conferred by the claims of the present application.

Short description of the figures

Figure 1: Structure of aminopyrido-pyrimidin ligand 24.

- 5 The free primary amino group can be used for covalent coupling to a solid support material.

Figure 2: Drug pulldown experiment with immobilized aminopyrido-pyrimidin ligand 24 and Western blot analysis.

- 10 As biological material cell lysates prepared from non-treated or pervanadate treated Jurkat cells were used. The drug pulldown experiment was performed as described in Example 2 with lysate samples containing 50 mg of protein. Input lysate, flowthrough (non-bound fraction) and SDS-eluates (bound fraction) were separated on a SDS-polyacrylamide gel and transferred to a membrane. The blot was probed first with a general anti-ZAP-70
15 antibody (2A) and then with an antiphospho-ZAP-70 antibody (2B). Secondary detection antibodies were labeled with fluorescent dyes for detection with the Odyssey infrared imaging system.

Lysates from non-treated Jurkat cells (lanes 1 to 3):

Lane 1: Lysate control, not subjected to drug pulldown experiment

- 20 Lane 2: Flow-through (non-bound fraction)

Lane 3: Eluate (SDS-eluted bound fraction)

Lysates from pervanadate-treated Jurkat cells (lanes 4 to 6):

Lane 4: Lysate control, not subjected to drug pulldown experiment

Lane 5: Flow-through (non-bound fraction)

- 25 Lane 6: Eluate (SDS-eluted bound fraction)

2A: The upper blot was probed with the general ZAP-70 antibody (L1E5, mouse monoclonal antibody directed against residues surrounding the aminoterminal part of human ZAP-70, Cell Signaling #2709).

- 30 2B: The lower blot was probed with the phospho-ZAP-70 antibody (Phospho-ZAP-70 Tyr493, Cell Signaling #2704).

Figure 3: Drug pulldown experiment with immobilized aminopyrido-pyrimidin ligand 24 for mass spectrometry analysis of proteins and phosphopeptides.

Proteins bound to immobilized aminopyrido-pyrimidin ligand 24 were eluted with SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A protein gel after staining with Coomassie blue is shown. The indicated gel areas were cut out as gel slices and proteins were subjected to analysis by mass spectrometry. The position of ZAP-70 is in gel slice 10 and 10a.

Figure 4: Peptides identified of ZAP-70.

The peptides that were identified by mass spectrometry analysis of the human ZAP-70 sequence (IPI00329789.5, 628 amino acids) are shown in bold type and underlined.

Figure 5: Assay for the identification of ZAP-70 interacting compounds.

The experiment was performed as described in example 3. ZAP-70 protein was captured by immobilized aminopyrido-pyrimidin ligand 24 from Jurkat cell lysates (pervanadate treated Jurkat cells) and eluted by the compounds as indicated. Eluates were transferred to a nitrocellulose membrane and ZAP-70 was detected with the Odyssey Infrared Imaging system. First antibody: anti-phosphoZAP-70 (Tyr493). Second antibody: goat anti-rabbit Irdye800CW. Relative Odyssey units are shown. Compounds used for elution: SDS, positive control for maximal elution; DMSO, solvent control; ligand 24, aminopyrido-pyrimidin ligand 24; Staurosporine; JNK inhibitor SP600125; p38 inhibitor SB 202190.

Figure 6: Compound profiling by adding compounds to cell lysates (example 4).

A test compound was added to Jurkat cell lysates at various concentrations followed by incubation with the affinity matrix and the analysis of captured proteins. A: ZAP-70 and Lck proteins were detected and quantified using specific antibodies and the Odyssey imaging system. 25 µg of cell lysate were used as a control. B: Dose response curve for test compound CZC15497.

Figure 7: Compound profiling by incubating compounds with living cells (example 4).

Jurkat cells were incubated with the test compound at various concentrations for 30 minutes, then the cells were treated for 30 minutes with pervanadate. A cell lysate was prepared, mixed with the affinity matrix and the captured proteins were analyzed. A: ZAP-70 and Lck proteins were detected and quantified using specific antibodies and the Odyssey imaging system. 25 µg of cell were used as a control. B: Dose response curve for test compound CZC15497.

Figure 8: Test of compound CZC15497 in a cell-based calcium release assay.

Calcium release was measured in a real-time by flow cytometry assay using Jurkat cells (density of 1.5×10^6 cells/ml) that were activated with an anti-CD3 antibody. Inhibition of
5 calcium release is plotted against compound concentration. An IC_{50} value of $0.24 \mu M$ was obtained. The experiment was performed as described in example 5.

Example 1: Preparation of affinity matrix

This example illustrates the preparation of an affinity matrix for affinity capture of kinases from cell lysates. A capturing ligand was covalently immobilized on a solid support through covalent linkage using an amino functional group. This affinity matrix was used in example 2 and example 3.

Synthesis of aminopyrido-pyrimidin ligand 24: 7-(4-Aminomethyl-phenylamino)-3-(2,6-dichloro-phenyl)-1-methyl-1H-[1,6]naphthyridin-2-one

The first seven steps of the synthesis were performed as described in Klutchko, S.R. et al., 1998, Journal of Medicinal Chemistry 41, 3276-3292. The remaining steps were performed as described below.

Steps 1-7: 6-(2,6-Dichlorophenyl)-2-methanesulfonyl-8-methyl-8H-pyrido[2,3-d]pyrimidin-7-one was synthesized from 4-chloro-2-methylsulfonyl-5-pyrimidinecarboxylate ethyl ester following the procedure in *J. Med. Chem.* 1998, 41, 3276-3292.

Step 8: {4-[3-(2,6-Dichloro-phenyl)-1-methyl-2-oxo-1,2-dihydro-[1,6]naphthyridin-7-ylamino] benzyl}-carbamic acid tert-butyl ester

6-(2,6-Dichlorophenyl)-2-methanesulfonyl-8-methyl-8H-pyrido[2,3-d]pyrimidin-7-one (0.100g, 0.2mmol) and 3-(N-Boc-methylamino)aniline (0.421g, 2.0mmol) were mixed as solids and heated to 140°C for 30 mins. The crude reaction mixture was dissolved in dichloromethane and washed with 2N HCl (aq) x 2. The organic layer was dried with anhydrous magnesium sulfate, filtered and concentrated. The crude product was recrystallised from hot ethyl acetate to afford {4-[3-(2,6-Dichloro-phenyl)-1-methyl-2-oxo-1,2-dihydro-[1,6]naphthyridin-7-ylamino] benzyl}-carbamic acid *tert*-butyl ester as a yellow solid (0.031g- 25%). ¹H NMR (DMSO-*d*₆) δ 10.18 (s, 1H); 8.83 (s, 1H); 7.76 (d, 2H); 7.58 (d, 2H); 7.46 (dd, 1H); 7.32 (brt, 1H); 7.23 (d, 2H); 4.10 (d, 2H); 3.66 (s, 3H); 1.40 (s, 9H). LCMS: method A, RT=5.60min.

Step 9: 7-(4-Aminomethyl-phenylamino)-3-(2,6-dichloro-phenyl)-1-methyl-1H-[1,6]naphthyridin-2-one

{4-[3-(2,6-Dichloro-phenyl)-1-methyl-2-oxo-1,2-dihydro-[1,6]naphthyridin-7-ylamino]benzyl}-carbamic acid *tert*-butyl ester (0.026g, 0.05mmol) was dissolved in methanol (3ml) and hydrochloric acid (4N in dioxane, 1.2ml) was added. The reaction was stirred at room temperature for 1.5 hours when HPLC showed no remaining starting material. The solvent was removed *in vacuo*. The residue was dissolved in water and the solution basified with sodium carbonate (sat., aq.). The resulting precipitate was collected and dried to afford 7-(4-Aminomethyl-phenylamino)-3-(2,6-dichloro-phenyl)-1-methyl-1H-[1,6]naphthyridin-2-one (0.021g -100%) as a yellow solid. ¹H NMR (DMSO-*d*₆) δ 10.20 (brd, 1H); 8.83 (d, 1H); 7.90 (d, 1H); 7.76 (d, 1H); 7.72 (d, 1H); 7.60 (dd, 2H); 7.47 (ddd, 1H); 7.33 (d, 1H); 7.24 (d, 1H); 4.07 (d, 2H); 3.66 (s, 3H). LCMS: method A, RT=4.44min, [MH⁺=426].

All reactions were carried out under inert atmosphere. NMR spectra were obtained on a Bruker dpx400. LCMS was carried out on an Agilent 1100 using a zorbax SBC-18, 4.6mmx150mm-5μ column. Column flow was 1ml/min and solvents used were water and acetonitrile (0.1%TFA) with an injection volume of 10ul. Wavelengths were 254 and 210nm. Methods are described below.

Table 1: Analytical methods

Method	Easy Access Method Name	ChemStation Method Name	Flow Rate	Solvent	Run Time
A	Analytical positive 7 mn	ANL_POS7.M	1ml/min	0-2.5 min 5-95% MeCN 2.5-6 min 95% MeCN	7 min
B	Analytical positive Ion	ANAL_POS.M	1ml/min	0-11 min 5-95% MeCN 11-13 min 95% MeCN	15 min

Table 2: Abbreviations used in chemistry protocols

aq	aqueous
d	doublet
DMSO	dimethyl sulfoxide
g	gram
HCl	Hydrochloric acid
HPLC	high pressure liquid chromatography
LCMS	liquid chromatography – mass spectrometry
m	multiplet
mins	minute
mmol	millimole
N	Normal
NMR	nuclear magnetic resonance
q	quartet
RT	retention time
s	singlet
sat	saturated
t	triplet

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Immobilization of ligand containing amine group

NHS-activated Sepharose 4 Fast Flow (Amersham Biosciences, 17-0906-01) was equilibrated with anhydrous DMSO (Dimethylsulfoxid, Fluka, 41648, H₂O ≤ 0.005%). 1 ml of settled beads was placed in a 15 ml Falcon tube, compound stock solution (usually 100 mM in DMF or DMSO) was added (final concentration 0.2-2 μmol/ml beads) as well as 15 μl of triethylamine (Sigma, T-0886, 99% pure). Beads were incubated at room temperature in darkness on an end-over-end shaker (Roto Shake Genie, Scientific Industries Inc.) for 16 - 20 hours. Coupling efficiency is determined by HPLC. Non-reacted NHS-groups were blocked by incubation with aminoethanol at room temperature

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on the end-over-end shaker over night. Beads were washed with 10 ml of DMSO and were stored in isopropanol at -20°C. These beads were used as the affinity matrix in example 2 and 3. Control beads (no ligand immobilized) were generated by blocking the NHS-groups by incubation with aminoethanol as described above.

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Example 2: Drug pulldown of a phosphorylated form of ZAP-70 using immobilized aminopyrido-pyrimidin ligand 24

10 This example demonstrates the use of the immobilized aminopyrido-pyrimidin ligand 24 for the identification of ZAP-70 from cell lysates of a human T cell line (Jurkat cells). To this end lysate of non-treated and treated Jurkat cells was contacted with the affinity matrix described in example 1. Proteins binding to the aminopyrido-pyrimidin ligand 24 were identified by Western blot and mass spectrometry (MS) analysis.

15 For Western blot analysis bound proteins were eluted from the affinity matrix and subsequently separated by SDS-Polyacrylamide gel electrophoresis. ZAP-70 was detected with a general monoclonal antibody binding to the non-phosphorylated aminoterminal part of the protein and in addition with a phospho-specific antibody (Figure 2).

20 The results of the Western blot analysis show that immobilized aminopyrido-pyrimidin ligand 24 preferentially pulls down a phosphorylated form of ZAP-70. The phosphorylated ZAP-70 was only significantly detected from treated Jurkat cell lysates compared to non-treated lysates.

25 For the identification of proteins and phosphopeptides by mass spectrometry analysis proteins captured by the affinity matrix were eluted and subsequently separated by SDS-Polyacrylamide gel electrophoresis. Suitable gel bands were cut out and subjected to in-gel proteolytic digestion with trypsin. Phosphorylated peptides were then enriched via immobilized metal affinity chromatography (IMAC). Retained phospho-peptides and non-bound peptides were separately analyzed by LC-MS/MS mass spectrometry.

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The peptide sequence coverage of ZAP-70 is shown in Figure 4. In total 9 ZAP-70 phosphopeptides were identified by mass spectrometry (Table 4).

1. Cell culture

Jurkat cells (clone E6-1 from ATCC, number TIB-152) were grown in 1 litre Spinner flasks (Integra Biosciences, #182101) in suspension in RPMI 1640 medium (Invitrogen, #21875-034) supplemented with 10% Fetal Bovine Serum (Invitrogen) at a density
5 between 0.15×10^6 and 1.2×10^6 cells/ml. For stimulation experiments cells were treated with $30 \mu\text{M}$ pervanadate (final concentration) at a density of about 1.0×10^6 cells/ml for one hour and subsequently harvested by centrifugation. After intensive washing with 1 x PBS buffer (Invitrogen, #14190-094) cell pellets were frozen in liquid nitrogen and subsequently stored at -80°C .

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For stimulation experiments cells were treated with H_2O_2 treated sodium orthovanadate in order to inhibit protein tyrosine phosphatases (D.C. Weiser and S. Shenolikar, Chapter 18.10 in *Current Protocols in Molecular Biology*, 2003, John Wiley & Sons, Inc.). For cell
15 treatment 30 ml of mix 3 was added to 10 liters of culture medium resulting in a final concentration of $30 \mu\text{M}$ pervanadate. Jurkat cells were kept at a density between 0.8 to 1.2×10^6 cells/ml for one hour and then harvested.

Preparation of pervanadate solutions

Dissolve sodium orthovanadate (Sigma, #6508) at 100 mM final concentration in sterile,
20 distilled water and adjust the pH value of the solution to pH10.0 with 0.1 M NaOH. Place the solution in a boiling water bath until the solution clarifies or becomes translucent. If necessary, readjust pH to 10.0 and store the stock solution at -20°C . Avoid repeated rounds of freezing and thawing of the stock solution.

Mix $500 \mu\text{l}$ of 30% H_2O_2 (Sigma, Cat. No. H1009) and $375 \mu\text{l}$ 1 x PBS (Mix 1).

25 Mix 3.5 ml 100 mM vanadate solution (pH 10.0) with 31.5 ml 1 x PBS (Mix 2).

Mix $650 \mu\text{l}$ Mix 1 and 32.5 ml Mix 2 (Mix 3). Incubate Mix 3 for 10 minutes at room temperature before applying to cell cultures.

2. Preparation of cell lysates

30 Jurkat cells were homogenized in a Potter S homogenizer in lysis buffer: 50 mM Tris-HCl, 0.8% NP40, 5% glycerol, 150 mM NaCl, 1.5 mM MgCl_2 , 25 mM NaF, 1 mM sodium vanadate, 1 mM DTT, pH 7.5. One complete EDTA-free tablet (protease inhibitor cocktail, Roche Diagnostics, 1 873 580) per 25 ml buffer was added. The material was dounced 10

times using a mechanized POTTER S, transferred to 50 ml falcon tubes, incubated for 30 minutes on ice and spun down for 10 min at 20,000 g at 4°C (10,000 rpm in Sorvall SLA600, precooled). The supernatant was transferred to an ultracentrifuge (UZ)-polycarbonate tube (Beckmann, 355654) and spun for 1 hour at 100.000 g at 4°C (33.500 rpm in Ti50.2, precooled). The supernatant was transferred again to a fresh 50 ml falcon tube, the protein concentration was determined by a Bradford assay (BioRad) and samples containing 50 mg of protein per aliquot were prepared. The samples were immediately used for experiments or frozen in liquid nitrogen and stored frozen at -80°C.

3. Compound pull-down experiment

Sepharose-beads with immobilized compound (100 µl beads per pull-down experiment) were equilibrated in lysis buffer and incubated with a cell lysate sample containing 50 mg of protein on an end-over-end shaker (Roto Shake Genie, Scientific Industries Inc.) for 2 hours at 4°C. Beads were collected, transferred to Mobicol-columns (MoBiTech 10055) and washed with 10 ml lysis buffer containing 0.5% NP40 detergent, followed by 5 ml lysis buffer with 0.25 % detergent. To elute the bound protein, 60 µl 2 x SDS sample buffer was added, the column was heated for 30 minutes at 50°C and the eluate was transferred to a microfuge tube by centrifugation. Proteins were then separated by SDS-Polyacrylamide electrophoresis (SDS-PAGE).

4. Protein detection by Western blot analysis

Western blots were performed according to standard procedures and the ZAP-70 protein was detected and quantified by using a specific anti-phospho ZAP-70 antibody (first antibody), a fluorescently labeled secondary antibody and the Odyssey Infrared Imaging system from LI-COR Biosciences (Lincoln, Nebraska, USA) according to instructions provided by the manufacturer (Schutz-Geschwendener et al., 2004. Quantitative, two-color Western blot detection with infrared fluorescence. Published May 2004 by LI-COR Biosciences, www.licor.com).

The phospho-ZAP-70 antibody (pTyr493, rabbit polyclonal, Cell Signaling #2704) detects ZAP-70 only when phosphorylated at tyrosine residue 493. The general ZAP-70 antibody (L1E5, mouse monoclonal antibody, Cell Signaling #2709) is directed against the amino-terminus of human ZAP-70.

5. Protein Identification by Mass Spectrometry

5.1 Protein digestion prior to mass spectrometric analysis

5 Gel-separated proteins were reduced, alkylated and digested in gel essentially following the procedure described by Shevchenko et al., 1996, Anal. Chem. 68:850-858. Briefly, gel-separated proteins were excised from the gel using a clean scalpel, reduced using 10 mM DTT (in 5mM ammonium bicarbonate, 54°C, 45 min) and subsequently alkylated with 55 mM iodoacetamid (in 5 mM ammonium bicarbonate) at room temperature in the dark (30
10 minutes). Reduced and alkylated proteins were digested in gel with porcine trypsin (Promega) at a protease concentration of 12.5 ng/μl in 5mM ammonium bicarbonate. Digestion was allowed to proceed for 4 hours at 37°C and the reaction was subsequently stopped using 5 μl 5% formic acid.

15 5.2 Sample preparation prior to analysis by mass spectrometry

Gel plugs were extracted twice with 20 μl 1% TFA and pooled with acidified digest supernatants. Samples were dried in a vacuum centrifuge and resuspended in 10 μl 0.1% formic acid. Dried samples were resuspended in 30 μl of a water containing 250 mM acetic acid, 30% acetonitrile and 4 μl PHOS-select Iron Affinity beads (Sigma, P9740; IMAC
20 material) and incubated for 2 hours at room temperature under gentle horizontal shaking. Subsequently, the slurry was loaded onto a gel loader tip containing a restriction to hold back the IMAC material. Beads were allowed to settle for a minute, then the solvent was pushed through the column using a P10 pipette and collected in an Eppendorf tube. The IMAC column was washed two times with 20 μl of an aqueous solution containing 250
25 mM acetic acid, 30% acetonitrile. The eluted fractions were combined and dried in a vacuum centrifuge. This fraction is referred to as the non-bound fraction (NBF). Phosphopeptides were eluted from the IMAC column using 20 μl of an aqueous solution containing 400 mM ammonia and 30% acetonitrile. The elution was repeated twice and the eluted fractions were combined and dried in a vacuum centrifuge (bound fraction, BF). Both
30 fractions (NBF and BF) were resuspended in 10 μl 0.1% formic acid prior to LC-MS/MS analysis (Pozuelo Rubio et al., 2005, Biochemical Journal 392(Part 1), 163-172).

5.3. Mass spectrometric data acquisition

Peptide samples were injected into a nano LC system (CapLC, Waters or Ultimate, Dionex) which was directly coupled either to a quadrupole TOF (QTOF2, QTOF Ultima, QTOF Micro, Micromass) or ion trap (LCQ Deca XP) mass spectrometer. Peptides were separated on the LC system using a gradient of aqueous and organic solvents (see below).

- 5 Solvent A was 5% acetonitrile in 0.5% formic acid and solvent B was 70% acetonitrile in 0.5% formic acid.

Table 3: Peptides eluting off the LC system were partially sequenced within the mass spectrometer.

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<u>Time (min)</u>	<u>% solvent A</u>	<u>% solvent B</u>
<u>0</u>	<u>95</u>	<u>5</u>
<u>5.33</u>	<u>92</u>	<u>8</u>
<u>35</u>	<u>50</u>	<u>50</u>
<u>36</u>	<u>20</u>	<u>80</u>
<u>40</u>	<u>20</u>	<u>80</u>
<u>41</u>	<u>95</u>	<u>5</u>
<u>50</u>	<u>95</u>	<u>5</u>

5.4. Protein identification

- The peptide mass and fragmentation data generated in the LC-MS/MS experiments were used to query fasta formatted protein and nucleotide sequence databases maintained and updated regularly at the NCBI (for the NCBIInr, dbEST and the human and mouse genomes) and European Bioinformatics Institute (EBI, for the human, mouse, D. melanogaster and C. elegans proteome databases). Proteins were identified by correlating the measured peptide mass and fragmentation data with the same data computed from the entries in the database using the software tool Mascot (Matrix Science; Perkins et al., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551-3567). Search criteria varied depending on which mass spectrometer was used for the analysis.

25 5.5 Phosphopeptide identification

The identification of phosphorylated peptides was achieved by allowing phosphorylation at Serine, Threonine and Tyrosine residues as variable modifications in Mascot search parameters followed by manual inspection of the spectrum to sequence assignments.

5 Table 4: Phospho-peptides of ZAP-70

The phospho-peptides that were identified by mass spectrometry analysis of the human ZAP protein sequence (IPI00329789.5, 628 amino acids) are indicated (Observed value: measured mass to charge ratio; Mr(exp.): 'observed value' x charge).

Peptide number	Start position	End position	Observed value	Mr(exp)	Mr(calc)	Delta	Sequence
1	176	186	624.297	1246.579	1246.559	0.02	KLpYSGAQTDGK
2	241	251	687.349	1372.684	1372.683	0.002	LKADGLIpYCLK
3	283	298	628.966	1883.876	1883.841	0.034	RIDTLNSDGpYTPEPAR
4	284	298	864.899	1727.783	1727.74	0.043	IDTLNSDGpYTPEPAR
5	299	306	497.244	992.474	992.469	0.005	ITpSPDKPR
6	485	496	691.795	1381.575	1381.555	0.02	ALGADDSpYYTAR
7	485	496	691.8	1381.585	1381.555	0.03	ALGADDSYpYTAR
8	485	496	731.792	1461.569	1461.521	0.048	ALGADDSpYpYTAR
9	604	612	571.749	1141.483	1141.451	0.032	ACYpYSLASK

Example 3: Assay for the identification of ZAP-70 interacting compounds

The preparation of the aminopyrido-pyrimidin ligand 24 affinity matrix was done as described in example 1. To screen maximally 80 compounds in a 96 well plate the elution experiment is performed as described below.

Elution assay

The affinity matrix (1600 µl of beads) was washed 2 x with 30 ml 1x DP-buffer. After each washing step the beads were collected by centrifugation for 4 minutes at 1300 rpm at 4°C in a Heraeus centrifuge. The supernatants were discarded. Finally, the beads were equilibrated in 50 ml binding buffer (1x DP buffer/0.4% NP40), and rotated for 30 to 45 minutes on a ROTO SHAKE GENIE (Scientific Industries, Inc.) at 4°C. After this incubation time the beads were harvested and mixed with 45 ml of Pervanadate activated cleared Jurkat cell lysate at a protein concentration of 5 mg/ml. The preparation of the lysate was done as described in example 2. Beads and the lysate were incubated for 2 hours at 4°C. After the incubation with the lysate beads were collected by centrifugation as described and washed three times with 25 ml binding buffer. During each washing step the beads were incubated for 8 minutes on a ROTO SHAKE GENIE (Scientific Industries, Inc.) at 4°C. After the third wash the beads were transferred to 2 ml columns (MoBiTec, #S10129) and washed with 20 ml 1x DP buffer/0.2% NP40. Once the washing buffer had run through the column completely the volume of beads left in the column was calculated (approximately 1000 µl). The beads were resuspended in 4 fold excess of 1x DP-buffer/0.2% NP40 (4 ml) to generate a 1:4 slurry. For compound elution tests 50 µl of this suspension was added to each well of a 96 well plate (Millipore MultiScreenHTS , MSBVN1210, with lid and 1.2µm hydrophilic low protein binding Durapore membrane). To remove residual buffer the 96 well plate was assembled with Assemble filter and collection plate and this sandwich assembly was spun down for 10 seconds at 800 rpm in a centrifuge. Then 40 µl of elution buffer (1x DP-buffer/0.2% NP40) supplemented with the test compound was added to the beads. Test compounds were prepared by diluting them in dilution buffer starting from 40 fold concentrated stock solution in DMSO. The plate was assembled on the collection plate, fixed on an Eppendorf incubator and incubated for 30 minutes at 4°C at 650 rpm shaking. To harvest the eluate the 96 well filter plate assembled on the 96 well collection plate was centrifuged for 1 minute at 800 rpm in a table top centrifuge at 4°C (Heraeus). The typical volume of the eluate is approximately 35 µl. Eluates were stored at -80°C. The eluates were checked for the presence of ZAP-70 by using a dot blot procedure.

As test compounds, Aminopyrido-pyrimidin ligand 24 (see Example 1), Staurosporine (Sigma S4400), JNK inhibitor SP600125 (Calbiochem 420119) and p38 inhibitor SB 202190 (Tocris 1264) were used. Typically all compounds are dissolved in 100 % DMSO (Fluka, cat.no 41647) at a concentration of 100 mM. Alternatively, 100 % DMF (Fluka, cat.no 40228) can be used for those compounds which cannot be dissolved in DMSO. Compounds are stored at -20°C. Dilution of test compound for elution experiments: Prepare 50 mM stock by diluting the 100 mM stock 1:1 with 100% DMSO. For elution experiments further dilute the compound with elution buffer (1x DP-buffer, 0.2% NP40).

10 Preparation of buffers

Table 5: Preparation of 5x-DP buffer

Substance:	Stock solution	Final conc. in 1 x lysis buffer	Add for 1l 5 x lysis buffer
Tris/HCl pH 7.5	1 M	50 mM	250 ml
Glycerol	87 %	5 %	288 ml
MgCl ₂	1 M	1.5 mM	7.5 ml
NaCl	5 M	150 mM	150 ml
Na ₃ VO ₄	100 mM	1 mM	50 ml

15 The 5x-DP buffer was filtered through 0.22 µm filter and stored in 40 ml-aliquots at -80°C. These solutions were obtained from the following suppliers: 1.0 M Tris/HCl pH 7.5 (Sigma, T-2663), 87% Glycerol (Merck, catalogue number 04091.2500); 1.0 M MgCl₂ (Sigma, M-1028); 5.0 M NaCl (Sigma, S-5150).

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Detection of eluted ZAP-70

The eluted ZAP-70 protein was detected and quantified by a dot blot procedure using a specific anti-phospho ZAP-70 antibody (first antibody), a fluorescently labeled secondary antibody and the Odyssey Infrared Imaging system from LI-COR Biosciences (Lincoln, Nebraska, USA) according to instructions provided by the manufacturer (Schutz-

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Geschwendener et al., 2004. Quantitative, two-color Western blot detection with infrared fluorescence. Publishe May 2004 by LI-COR Biosciences, www.licor.com).

Nitrocellulose membranes were treated with 20% ethanol for 5 seconds and subsequently washed with 1 x PBS buffer. Eluates (as described above) were combined with 10 µl of 4 x SDS loading buffer (200 mM Tris-HCl pH6.8, 8% SDS, 40% glycerol, 0.04% Bromphenol blue) and applied to the Nitrocellulose membrane with a BioRad dot blot appartus (BioRad, #170-6545) and washed once with 1 x PBS buffer.

For detection of ZAP-70 protein the membranes were first blocked by incubation with Odyssey blocking buffer for 1 hour. Blocked membranes were incubated for 16 hours at 4°C with the first antibody, a rabbit anti-phopspho ZAP-70 antibody recognizing phosphorylated Tyrosine residue 493 (pTyr493, rabbit polyclonal, Cell Signaling #2704) diluted 1:1000 in Odyssey blocking buffer supplemented with 0.1% Tween 20 and 0.02% SDS.

After washing the membrane four times for 5 minutes with 1 x PBS buffer containing 0.01% Tween 20 the membrane was incubated for 1 hour with the detection antibody (Goat anti-rabbit IRDye 800CW antibody from LI-COR, diluted 1:10 000 in Odyssey Blocking Buffer supplemented with 0.1% Tween 20 and 0.02% SDS). Afterwards the membrane was washed four times for 5 minutes with 1 x PBS buffer/0.1% Tween 20 and once for 5 minutes with 1 x PBS buffer. Afterwards the membrane was scanned with the Odyssey reader and data were analysed.

Example 4: Compound profiling of ZAP-70 interacting compounds by adding compounds to cell lysates or living cells

This example demonstrates a competitive binding assay in which test compounds are added directly into a cell lysate or incubated with living cells. For the cell lysate competitive binding assay various concentrations of test compounds were added to lysate samples and allowed to bind to the proteins contained in the lysate sample. Then the affinity matrix containing the immobilized aminopyrido-pyrimidin ligand 24 was added in order to capture proteins not bound to the test compound. After the incubation time the beads with captured proteins were separated from the lysate by centrifugation. Bound

proteins were then eluted and the presence of ZAP-70 was detected and quantified using a specific antibody and the Odyssey infrared detection system (Figure 6A). A dose response curve for compound CZC15497 was generated with an IC₅₀ value of 1.72 μ M (Figure 6B).

Alternatively, aliquots of life Jurkat cells were first incubated with various compound concentrations for 30 minutes and then treated with 30 μ M Pervanadate for another 30 minutes. The cells were harvested, cell lysates were prepared and the affinity matrix was added in order to capture proteins not bound to the test compound. After 90 minutes of incubation of the cell lysate with the affinity matrix the beads with the captured proteins were separated from the lysate by centrifugation. Bound proteins were then eluted and the presence of ZAP-70 was detected and quantified as described above using a specific antibody and the Odyssey infrared detection system (Figure 7A). A dose response curve for compound CZC15497 was generated with an IC₅₀ value of 0.65 μ M (Figure 7B).

A comparison of the results of both approaches (Figure 6 and 7; compound added to lysate versus compound incubated with cells) yield similar IC₅₀ values for the CZC15497 compound and the ZAP-70 kinase. Moreover, this compound specifically interacted with ZAP-70 kinase but not with Lck.

Washing of affinity matrix

The affinity matrix as described in example 1 (0.3 ml of dry volume) was washed two times with 15 ml of 1x DP buffer, then washed with 15 ml of 1x DP buffer containing 0.4% NP40 and finally resuspended in 0.3 ml of 1x DP buffer containing 0.4% NP40 (50% beads slurry).

Preparation of test compounds

For in lysate competition stock solutions of test compounds were prepared in DMSO corresponding to a 200fold higher concentration compared to the final desired test concentration (for example a 1 mM stock solution was prepared for a final test concentration of 5 μ M). This dilution scheme resulted in a final DMSO concentration of 0.5%. For control experiments (no test compound) a buffer containing 0.5% DMSO was used so that all test samples contained 0.5% DMSO.

Dilution of cell lysate

Cell lysates were prepared as described in example 2 from pervanadate treated Jurkat cells. For a typical experiment one lysate aliquot containing 50 mg of protein was thawed in a 37°C water bath and then kept at 4°C. To the lysate one volume of 1xDP containing

protease inhibitor buffer (1 tablet of protease inhibitor dissolved in 25 ml of 1x DP buffer or 25 ml of 1x DP buffer containing 0.4% NP40; EDTA-free tablet protease inhibitor cocktail from Roche Diagnostics, catalogue number 41647) was added so that a final NP40 concentration of 0.4% was achieved. The lysate was further dilute by adding 1x DP buffer
5 containing 0.4% NP40 and proteinase inhibitors so that a final protein concentration of 5 mg/ml was achieved.

Incubation of cell lysate with test compound and affinity matrix

To 1 ml of Jurkat lysate (containing 5 mg of protein) 5 µl of compound CZC15497 (diluted
10 in DMSO) were added and incubated for 45 minutes at 4°C. Then 20 µl of the affinity matrix with the immobilized aminopyrido-pyrimidin ligand 24 were added and incubated for one more hour at 4°C. After separation of the beads from the lysate by centrifugation bound proteins were eluted with 50 µl of 2x concentrated sample buffer (25 µl 4xNuPAGE LDS Sample Buffer, Invitrogen, NP0007; 2.5 µl 1 M Dithiothreitol (DTT); 22.5 µl H₂O).

Detection and quantification of ZAP-70

Western blots were performed according to standard procedures and the ZAP-70 protein was detected and quantified by using a specific anti-ZAP-70 antibody (primary antibody), a fluorescently labeled secondary antibody and the Odyssey Infrared Imaging system from
20 LI-COR Biosciences (Lincoln, Nebraska, USA) according to instructions provided by the manufacturer (Schutz-Geschwendener et al., 2004. Quantitative, two-color Western blot detection with infrared fluorescence. Published May 2004 by LI-COR Biosciences, www.licor.com). For the detection of Lck an anti-Lck antibody was used (Upstate 3A5).

Protein eluates were separated in SDS-Polyacrylamide gels and then transferred to a PVDF membrane. The membrane was incubated for one hour at room temperature with Odyssey buffer to block non-specific binding. After a wash the membrane was incubated for 16 hours at 4°C with the anti-ZAP-70 antibody (rabbit polyclonal antibody directed at phosphoTyr292 obtained from Abcam; dilution of 1:1000 in Odyssey buffer with 0.2% Tween 20). For the detection of Lck an anti-Lck antibody was used (Upstate 3A5). The
30 membrane was then washed four times five minutes with PBS buffer supplemented with 0.1% Tween 20. A secondary detection antibody labeled with a fluorescent dye was used in conjunction with the Odyssey infrared imaging system to visualize and quantitate protein bands (IRDye 800 nm anti-rabbit antibody (Licor) diluted 1:10000 in Odyssey
35 buffer with 0.2% Tween 20, 0.02% SDS, 1 hour incubation at room temperature). Again, the membrane was washed washed four times five minutes with PBS buffer supplemented with 0.1% Tween 20 and finally shortly washed with PBS buffer to remove the Tween 20.

Dose response curves were computed with the XL fit program (XLfit4 Excel Add-In Version 4.2.0 Build 13; IDBS, Guilford, UK).

Incubation of cells with test compound, preparation of cell lysate, incubation with the affinity matrix and detection of proteins

For cell treatments stock solutions of the test compound were prepared at a 5000 fold higher concentration compared to the final desired test concentration. The final DMSO concentration in the cell culture medium was 0.02% DMSO.

Jurkat cell cultures (1 litre volume, cell density 1.15×10^6 cells/ml) were treated with the compound CZC15497 at various concentrations (5.0 μ M, 1.0 μ M, 0.33 μ M, 0.11 μ M, 0.037 μ M and 0.012 μ M) for 30 minutes at 37°C and subsequently treated with 30 μ M Pervanadate for another 30 minutes (for the preparation of Pervanadate solutions see example 2). Then the cells were harvested, cell lysates were prepared as described above and the affinity matrix was added in order to capture proteins not bound to the test compound. After 90 minutes incubation of the cell lysate with the affinity matrix at 4°C the beads with the captured proteins were separated from the lysate by centrifugation. The bead bound proteins were eluted with 50 μ l of 2x concentrated sample buffer (25 μ l 4xNuPAGE LDS Sample Buffer, Invitrogen, NP0007; 2.5 μ l 1 M Dithiothreitol (DTT); 22.5 μ l H₂O). The presence of ZAP-70 in the eluate was detected and quantified using specific antibodies and the Odyssey infrared detection system (Figure 7A). For the detection of Lck an anti-Lck antibody was used (Upstate 3A5). A dose response curve for the interaction of compound CZC15497 with ZAP-70 was generated yielding an IC₅₀ value of 0.65 μ M (Figure 7B).

Example 5: Calcium release assay in Jurkat cells

Consistent with CZC15497 acting as a ZAP-70 inhibitor we found that the compound inhibits calcium release in anti-CD3 stimulated Jurkat T-cells with an IC₅₀ value of 0.24 μ M (Figure 8).

Assay principle

The development of fluorescent probes makes it possible to measure the concentration of intracellular free Calcium ions in single living cells. Cells are first loaded with a cell-permeable Ca²⁺-sensitive dye, then the test compound is added. Finally, cells are activated through the T cell receptor with an anti-CD3 antibody shortly before data acquisition on

the flow cytometer. The release of Ca^{2+} is measured as a function of time after cell stimulation. This protocol describes the flow cytometry method using the Fluo-3 and Fluo-4 dyes (Minta et al., 1989, J. Biol. Chem. 264(14):8171-8178) to measure the intracellular Ca^{2+} concentration in Jurkat cells (see also June et al., 1997, Measurement of intracellular calcium ions by flow cytometry. In: Current Protocols in Cytometry (1997) Unit 9.8.1-9.8.19, John Wiley & Sons, Inc.).

Ca^{2+} release assay protocol

In general, cells should be handled in the shortest time possible to assure their stability.

Therefore the preparation of all materials in advance is highly recommended as well as using any incubation or centrifugation time to prepare the next steps (e.g. preparing compound dilution or starting the flow cytometer).

1) Prepare a work plan indicating the number of samples to be analyzed including controls.

2) Cell harvest: Centrifuge 50 to 100 ml of a Jurkat cell culture for 5 minutes at 1100 rpm. Discard the supernatant, pool the resuspended cell pellets in a single 50 ml Falcon tube, and fill up to 50 ml with PBS- CaCl_2 (without FCS). Centrifuge again sample again.

3) Resuspend cell pellet with PBS- CaCl_2 (without FCS) to achieve a concentration not lower than 10×10^6 cells/ml. Prepare an adequate dilution to evaluate the cell concentration and count the cells.

4) Separate the volume of cells needed to be loaded (for example 10^7 cells for 20 samples) in a 50 ml Falcon tube. Fill up with PBS- CaCl_2 to 900 μl for 10^7 cells.

5) Prepare in the dark a 1:1 mix of FLUO-4 (1 mM) + Pluronic F-127 (20%). 5 μl of Fluo-4 are needed per 10^7 cells.

6) Prepare a 1/10 dilution of this mix with PBS- CaCl_2 (in the dark; for example 5 μl Fluo-4 + 5 μl F-127 completed to 100 μl).

7) Add the dye solution to the cells. Do not load more than 30×10^6 cells per tube.

8) Incubate the sample for 30 minutes in a cell incubator (37°C , 5% CO_2). Mix from time to time.

9) During this time prepare the adequate test compound dilution in PBS- CaCl_2 (with 10% FCS). The solution should be 10 times more concentrated than the desired final concentration. Vortex each dilution. Prepare also the antibody dilutions: anti-CD3 (1/200) and GAM (1/25).

10) Label FACS tubes and distribute in the corresponding tube 30 μl of the compound dilution or only buffer for the control tubes.

11) After 30 minutes of incubation wash the cells twice in PBS-CaCl₂ (with 10% FCS). During the washing steps make sure that the flow cytometer is already "ON" in order to warm the laser.

12) Resuspend the cell pellet in PBS-CaCl₂ (with 10% FCS) at a concentration of 1.5×10^6 cells/ml.

13) Distribute 300 µl of cell suspension into the FACS tubes and mix gently. The compound incubation is starting. Keep samples at room temperature in the dark.

14) Open the settings of the cytometer. It is advised to have a template ready which is used for all measurements. Settings of the machine should also be the same from one experiment to the other which permits to evaluate the reproducibility of the cell preparation.

15) In Setup modus control your cell preparation on the cytometer. Cells should fit in the pre-defined gates.

16) During the compound incubation time, check also that cells are responding to the anti-CD3 activation as expected. Set a timer at 8 sec. Add 6 µl of anti-CD3 dilution (0.2 µg/ml final) and mix gently. Add 1 µl of GAM dilution (0.75 µg/ml final) and mix gently. Incubate the samples in water bath at 37°C while starting timer. After 8 seconds of incubation, acquire data at medium speed for 200 seconds. A clear increase of fluorescence should appear after 1 minute.

17) Make sure that the cells rest and equilibrate at room temperature 15 minutes before FACS data acquisition

18) Data acquisition: Samples to be compared should be measured consecutively.

19) For a better reproducibility, make shure that data for the loaded cells are acquired within 2 hours.

20) Analyze the data by using the FlowJo® software (Tree Star, Inc.).

Cell culture

The Jurkat cell line J77 was obtained from American Type Cell Collection (ATCC). Jurkat cells were maintained in RPMI 1640 medium (Gibco, ref. 21875-034) supplemented with heat-inactivated fetal calf serum (Gibco, ref. 10270-106. FCS is heat-inactivated by in water bath for 45 minutes at 56°C).

Reagents

Fluo-3, AM (Molecular Probes, F14218, supplied as 1 ml of ready made 1 mM solution in DMSO and stored in 5 µl or 7.5 µl aliquots at -20°C).

Fluo-4, AM (Molecular Probes, F14217, supplied as 1 ml of ready made 1 mM solution in DMSO and stored in 5 µl or 7.5 µl aliquots at -20°C).

Pluronic F-127 (Molecular Probes, P3000MP, supplied as 1 ml of ready made 20% solution in DMSO).

PBS-CaCl₂-MgCl₂ (Gibco, 14040-91).

Anti-CD3 antibody (Calbiochem, 217570, supplied at 1 mg/ml).

- 5 Goat anti-mouse IgG antibody (GAM; Sigma, M8890, supplied at 6 mg/ml).

Equipment

Flow cytometer (Becton-Dickinson, FACSCalibur) and FlowJo® software (Tree Star, Inc.).

Claims

- 5 1. A method for the identification of an ZAP-70 interacting compound, comprising the steps of
- a) providing a protein preparation containing phosphorylated ZAP-70,
- 10 b) contacting the protein preparation with aminopyrido-pyrimidin ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex,
- 15 c) incubating the aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex with a given compound, and
- d) determining whether the compound is able to separate phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidin ligand 24 .
- 20 2. The method of claim 1, wherein step d) includes the detection of separated phosphorylated ZAP-70 or the determination of the amount of separated phosphorylated ZAP-70.
- 25 3. The method of claim 2, wherein separated phosphorylated ZAP-70 is detected or the amount of separated phosphorylated ZAP-70 is determined by mass spectrometry or immunodetection methods, preferably with an antibody directed against phosphorylated ZAP-70, preferably with an antibody recognizing a phosphorylation at position 493 of phosphorylated ZAP-70.
- 30 4. A method for the identification of an ZAP-70 interacting compound, comprising the steps of
- a) providing a protein preparation containing phosphorylated ZAP-70,
- 35 b) contacting the protein preparation with aminopyrido-pyrimidin ligand 24 immobilized on a solid support and with a given compound under conditions

allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex, and

c) detecting the aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex formed in step b).

5. The method of claim 4, wherein in step c) said detecting is performed by determining the amount of the aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex.

6. The method of any of claims 4 or 5, wherein steps a) to c) are performed with several protein preparations in order to test different compounds.

7. A method for the identification of an ZAP-70 interacting compound, comprising the steps of:

a) providing two aliquots of a protein preparation containing phosphorylated ZAP-70,

b) contacting one aliquot with aminopyrido-pyrimidin ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex,

c) contacting the other aliquot with aminopyrido-pyrimidin ligand 24 immobilized on a solid support and with a given compound under conditions allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex, and

d) determining the amount of aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex formed in steps b) and c).

8. A method for the identification of an ZAP-70 interacting compound, comprising the steps of:

a) providing two aliquots comprising each at least one cell containing phosphorylated ZAP-70,

- b) incubating one aliquot with a given compound,
- c) harvesting the cells of each aliquot,
- 5 d) lysing the cells in order to obtain protein preparations,
- e) contacting the protein preparations with aminopyrido-pyrimidin ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex, and
- 10 f) determining the amount of aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex formed in each aliquot in step e).

9. The method of any of claims 7 or 8, wherein a reduced amount of aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex formed in the aliquot
15 incubated with the compound in comparison to the aliquot not incubated with the compound indicates that ZAP-70 is a target of the compound.

10. The method of any of claims 5 to 9, wherein the amount of the aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex is determined by separating
20 phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidin ligand 24 and subsequent detection of separated phosphorylated ZAP-70 or subsequent determination of the amount of separated phosphorylated ZAP-70.

25 11. The method of claim 10, wherein phosphorylated ZAP-70 is detected or the amount of ZAP-70 is determined by mass spectrometry or immunodetection methods, preferably with an antibody directed against phosphorylated ZAP-70, preferably with an antibody recognizing a phosphorylation at position 493 of phosphorylated ZAP-70.

30 12. The method of any of claims 1 to 11, performed as a medium or high throughput screening.

35 13. The method of any of claims 1 to 12, wherein said compound is selected from the group consisting of synthetic compounds, or organic synthetic drugs, more preferably small molecule organic drugs, and natural small molecule compounds.

14. The method of any of claims 1 to 13, wherein the ZAP-70 interacting compound is a ZAP-70 inhibitor.
15. The method of any of claims 1 to 14, wherein the solid support is selected from the group consisting of agarose, modified agarose, sepharose beads (e.g. NHS-activated sepharose), latex, cellulose, and ferro- or ferrimagnetic particles.
16. The method of any of claims 1 to 15, wherein the aminopyrido-pyrimidin ligand 24 is covalently coupled to the solid support.
17. A method for the preparation of a pharmaceutical composition comprising the steps of
- a) identifying a ZAP-70 interacting compound according to any of claims 1 to 16, and
 - b) formulating the interacting compound to a pharmaceutical composition.
18. A method for the purification of phosphorylated ZAP-70, comprising the steps of
- a) providing a protein preparation containing phosphorylated ZAP-70,
 - b) contacting the protein preparation with aminopyrido-pyrimidin ligand 24 immobilized on a solid support under conditions allowing the formation of an aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex, and
 - c) separating phosphorylated ZAP-70 from the immobilized aminopyrido-pyrimidin ligand 24 .
19. The method of claim 18, further comprising after step c) the identification of proteins being capable of binding to phosphorylated ZAP-70.
20. The method of claim 18, further comprising after step c) the determination of post-translational modifications phosphorylated ZAP-70.
21. The method of any of claims 18 to 20, wherein after step c) either the phosphorylated ZAP-70 and/or the proteins being capable of binding to

phosphorylated ZAP-70 are characterized by mass spectrometry or immunodetection methods.

22. The method of any of claims 1 to 21, wherein the provision of a protein preparation
5 includes the steps of harvesting at least one cell containing phosphorylated ZAP-70 and lysing the cell.

23. The method of any of claims 1 to 22, wherein the steps of the formation of the
10 aminopyrido-pyrimidin ligand 24 – phosphorylated ZAP-70 complex are performed under essentially physiological conditions.

24. Use of the aminopyrido-pyrimidin ligand 24 for the identification of ZAP-70 interacting compounds.

15 25. Use of the aminopyrido-pyrimidin ligand 24 for the purification of phosphorylated ZAP-70.

26. Use of the aminopyrido-pyrimidin ligand 24 for the preparation of a diagnosticum
20 for chronic lymphocytic leukaemia (CLL).

27. A method for the diagnosis/prognosis of CLL, comprising the step of determining
the presence/amount of phosphorylated ZAP-70 in a sample in relation to the total
amount of ZAP-70, wherein an increased amount of phosphorylated ZAP-70 in
comparison to the total amount of ZAP-70 (non-phosphorylated plus
25 phosphorylated ZAP-70) in a sample from a CLL patient is indicative for having a
higher probability for an aggressive form of CLL.