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(57) **Abrégé/Abstract:**

The present invention relates to methods for treating a haematopoietic tumor by administering a drug inhibiting calcineurin and/or the calcineurin/NFAT pathway, alone or in combination with others cancer therapy, pharmaceutical compositions useful in such methods, and screening methods for identifying a compound useful for treating a haematopoietic tumor.



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(57) Abstract: The present invention relates to methods for treating a haematopoietic tumor by administering a drug inhibiting calcineurin and/or the calcineurin/NFAT pathway, alone or in combination with others cancer therapy, pharmaceutical compositions useful in such methods, and screening methods for identifying a compound useful for treating a haematopoietic tumor.

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NEW METHOD FOR TREATING CANCER BASED ON THE MODULATION OF CALCINEURIN

FIELD OF THE INVENTION

5 The present invention relates to methods for treating a haematopoietic tumor, pharmaceutical compositions useful in such methods, and screening methods for identifying a compound useful for treating a haematopoietic tumor.

BACKGROUND OF THE INVENTION

10

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children < 10 years-old whereas its occurrence in adults steadily increases with age. Non Hodgkin lymphoma (NHL) is the most common hematopoietic malignancy and is currently the 5th most common cancer in the western world. It includes a number of
15 clinical entities, as defined in the REAL or WHO classification, with a significant clinical overlap between precursor T- and B-cell lymphoblastic lymphoma and ALL. Remission in these clinical entities is induced by intensive combination chemotherapy (e.g. CHOP in disseminated NHL). Relapse is rare in childhood ALL but frequent in adult ALL. In NHL, depending on the entity, only 40 to 70 % of patients achieve long
20 term remission using CHOP or CHOP-based primary chemotherapy. Improvement of existing treatment regimens is therefore required. Approaches along these lines include the search for novel clinical, histological and molecular prognostic factors, the use of high dose chemotherapy followed by hematopoietic stem cell transplantation in relapsed cases, the search and integration of novel therapies into existing treatment strategies. In
25 addition, the repeated multidrug treatment of ALL and NHL is associated with severe immediate toxicity and poor quality of life and long term sequelae, including other cancers. Lymphoma/leukemia patients would therefore benefit greatly from novel therapeutic approaches, in particular those that directly target the molecular mechanisms responsible for tumor cell survival and proliferation, or those involved in the essential
30 interactions between tumor cells and their micro-environment.

Calcineurin (PP2B) is an ubiquitously expressed serine/threonine protein phosphatase that is involved in many biological processes and which is essential for life.

Calcineurin is a heterodimer composed of a catalytic subunit (CnA ; three isoforms) and a regulatory subunit (CnB ; two isoforms). Besides its catalytic domain, CnA includes a CnB-binding helical domain, a calmodulin binding region and an auto-inhibitory domain (AID) (1). Engagement of cell surface receptors coupled to phospholipase C
5 activation results in the generation of inositol(1,4,5)trisphosphate (InsP3) and diacylglycerol (DAG). While DAG is involved in PKC activation, InsP3 mediates the release of calcium from internal stores. In turn, store depletion induces the opening of specific store-operated channels that result in the influx of extracellular calcium. This increase in calcium ions concentration induces the binding of calmodulin to calcineurin,
10 the release of calcineurin from AID inhibition and activation of its phosphatase activity. Mutation by homologous recombination of the ubiquitously expressed CnB1 gene results in the suppression of calcineurin activity in all somatic tissues and in embryonic lethality at day 11.5 of mouse development (2). Interestingly, the CnB1 mutant phenotype phenocopies that of a double knockout of NFAT3+4, indicating that NFAT
15 proteins are major downstream substrates of calcineurin in mouse development (2) .

The NFAT family of transcriptional regulators includes NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5. Except for NFAT5, the other NFAT proteins are activated by cell surface receptors coupled to phospholipase C activation and to store-operated Ca^{2+} entry, typically the pre-TCR and the T cell antigen receptor in T
20 lymphoid cells (for review, see (3)). NFAT1-4 share a similar modular structure, including N-terminal and C-terminal activation domains; a central Rel-homology domain that mediates DNA binding; a regulatory domain that includes multiple serine phosphorylation sites (4) and a calcineurin docking domain. The major docking site of calcineurin is localized in the N-terminal region of the regulatory domain and is
25 centered over a critical PxIxIT motif. In resting cells, NFAT1-4 are fully phosphorylated in their regulatory domain, are cytosolic and in a conformation inhibiting their DNA binding activity. Ca^{2+} /calmodulin-induced activation of calcineurin induces the concerted dephosphorylation of NFATs, their nuclear accumulation and the activation of their DNA binding activity. Several constitutive and
30 signal-induced export protein kinases have been implicated in the maintenance of NFAT hyperphosphorylation in resting cells and in their nuclear re-phosphorylation after signal-evoked dephosphorylation, including casein kinase 1, glycogen synthase kinase 3, DYRK1A, DYRK2, Jun kinase 1 (JNK1) and the related p38. NFAT1-4 bind

DNA as monomer to their cognate A/TGGAA binding site, as dimers at NF κ B-like response elements and as cooperative complexes (e.g. NFAT/AP1 ; NFAT/STAT4 ; NFAT/MAF/GATA3) on composite DNA response elements in specific cell lineages and/or in response to the activation of specific receptors.

5 NFAT1-4 play critical roles in many developmental processes and in the immune response. The best characterized function of the calcineurin/NFAT pathway is its essential role in T cell activation following co-engagement of the TCR and co-activator receptors like CD28 by antigen-presenting cells. In this response, NFAT1 and NFAT2 play a redundant role and activate the expression of a number of activation-specific genes through their binding, together with c-JUN/C-FOS to composite NFAT/AP1 response elements in the promoter region of these genes ((5) and references
10 therein). Remarkably, NFAT1 plays a prominent role in the inhibition of TCR signaling in T cells subjected to an anergizing stimuli e.g. Ca²⁺ signaling without concomitant PKC/MAPkinase activation. In that situation, NFAT1 regulates the transcription of a different set of genes either through its ability to bind specific response elements as
15 homodimer, or in synergy with transcriptional partners different from AP1. The calcineurin/NFAT pathway, plays a major role in T cell development, in particular in positive selection during the transition of immature CD4CD8 double positive (DP) thymocytes to mature CD4 and CD8 SP T cells (6) and in the functional differentiation
20 of T cells, most notably in both Th1 and Th2 differentiation from naive T helper cells through cooperation with specific STATs and lineage-specific transcription factors (for review, see (7))

 Since calcineurin and its downstream NFAT substrates have a central role in T cell activation, this pathway is a critical target for therapeutic control of pathological
25 immune responses (for review, see (8)). Two inhibitors of calcineurin, cyclosporinA and FK506 (Prograf) act by binding to specific intracellular receptors, cyclophilin and FKBP12, respectively. The respective drug/receptor complexes binds calcineurin and inhibit its activity, resulting in the full rephosphorylation of NFATs and their accumulation in the cytoplasm. Both CsA and FK506 are extensively used as
30 immunosuppressive agents in human medicine to facilitate allograft survival and autoimmune diseases. More specific inhibitors of NFAT activation have been generated, in particular a high affinity version of the PXIXIT domain, known as the VIVIT peptide; when expressed in cells as a GFP fusion, this peptide selectively blocks

NFAT dephosphorylation and NFAT-dependent transcription (9). Recently, several pharmacological compounds have been identified that block the NFAT-calcineurin interaction, but are at present of limited interest in vivo due to cell toxicity (10).

Although critically important in many aspects of T cell survival, activation and proliferation, the calcineurin/NFAT pathway has so far not been involved in T cell lymphoma/leukemia development.

More generally, a role of this pathway in tumorigenesis is suspected but not clear and not proven. Indeed, in vitro studies have shown that (i) expression of a constitutively nuclear mutant of NFAT2 interferes with the differentiation of the 3T3-L1 fibroblastic cell line into adipocytes and induces morphological transformation of these cells and their growth as tumors in immunosuppressed mice (11); (ii) both NFAT1 and NFAT5 expression is induced in response to integrin signaling in a breast carcinoma-derived cell line and participate in the activation of cell migration and invasion of matrigel, but this response is not dependent on calcineurin activity (12); (iii) NFAT2 is nuclear in a subset of human leukemia, including diffuse large B-cell lymphoma (LBCL) and is involved in cell growth of LBCL cell lines in vitro (13).

The patent application US2005100897 describes that NFAT may be involved in promoting carcinoma invasion based on in vitro observations. NFAT1 and NFAT5 are expressed at high levels and are constitutively active in cell lines derived from human breast and colon carcinomas. They showed that an increase in matrigel invasion can be blocked in vitro with a dominant negative NFAT mutant, but not cyclosporin A or FK506.

WO 03/099362 discloses a method for treating lung metastasis with compositions comprising a cyclosporin A-liposomal complex and paclitaxel-liposomal complex for aerosol delivery. Cyclosporin A increases the bioavailability of paclitaxel by antagonizing plasma membrane glycoprotein (P-glycoprotein). No direct effect of cyclosporin A on metastatic cells is disclosed. Ross et al (1997, Clinical Cancer Research, 3, 57-62) discloses that cyclosporin A has been successfully used to reverse the resistance of neoplastic cells to paclitaxel against leukemia and respiratory epithelial cancers. It indicates that CsA alone has little or no anti-proliferative activity. No survival increase has been observed with CsA alone.

WO 02/24957 discloses a method for inhibiting angiogenesis by administering inhibitors of the calcineurin/NFAT pathway. This method can be used for treating

vascularized tumors.

WO 2004/004644 discloses a method for treating a cancer, including hematopoietic tumors, comprising the administration of an inhibitor of mTOR in combination to a tyrosine kinase inhibitor. Rapamycin (Sirolimus) is an example of
5 mTOR inhibitor. However, rapamycin is not a calcineurin inhibitor as demonstrated in several articles (e.g., 19, 20).

US 2004/0039010 discloses a method for treating an acute lymphoblastic leukemia comprising the administration of rapamycin, optionally in combination with an IL-7 inhibitor or an anti-tumoral agent. As indicated above, rapamycin is not a
10 calcineurin inhibitor.

Smart et al, (1988, *Transplantation Proceedings*, No 3, Suppl. 3, 900-912) discusses the use of cyclosporin A (CsA) in a spontaneous acute T cell leukemia in the rat. However, it concludes negatively because of a modest effect in blood of animals carrying established tumors, the inability of CsA to significantly affect lymphoid tissue
15 and non lymphoid organs infiltration, a synergistic nephrotoxicity with the tumor and no increase of host survival. In addition, to show an effect, CsA has to be co-injected with the transplanted tumor and used in a long term treatment.

Cesano et al (1995, *Cancer Immunology and immunotherapy*, 40, 139-151) discloses a comparison between normal LAK cells and a cytotoxic leukemic T cell clone to aim treating cancer by immunotherapy, and particularly concerns their capacity
20 to maintain cytotoxic activity after a treatment by irradiation and CsA (immunosuppressive treatment).

The abstract of Cabrelle et al (2002, *Blood*, 100) discloses in vitro the apoptotic effect of CsA in B-chronic leukemic cells and a modest effect on an uncharacterized cell
25 population in CLL patients. However, no data is provided concerning the dose and the regimen. Moreover, these data have not been confirmed by any subsequent scientific article.

Despite considerable research efforts in this area, there is still a strong need for novel, targeted and more efficient treatment for hematopoietic tumors. In addition, the
30 medicine is looking for the most appropriate treatment for each case. Indeed, antitumoral treatments have a lot of side effects and a treatment is preferably used if it is possible to predict his efficiency.

SUMMARY OF THE INVENTION

The inventors have found that calcineurin is activated in lymphoid malignancies. The activation of calcineurin in these cancer cells was difficult to observe. Indeed, the
5 activation of calcineurin can be assessed through the activation of NFAT by dephosphorylation and the activation of NFAT disappears as soon as the cells are maintained in culture.

The inventors have shown that calcineurin is a target of therapeutic interest in lymphoid malignancies. Surprisingly, inhibitors of calcineurin are shown to be of
10 therapeutic interest to control the evolution of lymphoid malignancies, by affecting either the tumor cell itself and/or its stromal micro-environment.

Therefore, the present invention concerns the use of a drug inhibiting calcineurin for the preparation of a medicament for treating a haematopoietic tumor. In a preferred embodiment, said haematopoietic tumor has a sustained calcineurin activity. In a
15 preferred embodiment, the drug inhibiting calcineurin can be cyclosporin A and FK506. In a most preferred embodiment, the drug inhibiting calcineurin is FK506. In a preferred embodiment, the haematopoietic tumor is a lymphoma and/or a leukemia. In a preferred embodiment, the drug inhibiting calcineurin is used in combination with a cancer therapy.

20 The present invention further concerns a product containing a drug inhibiting calcineurin, preferably FK506, and an anticancer drug as a combined preparation for simultaneous, separate or sequential use in a cancer therapy. The present invention also concerns a pharmaceutical composition comprising a drug inhibiting calcineurin, preferably FK506, and an anticancer drug.

25 The present invention further concerns a method for staging or characterizing a haematopoietic tumor in a subject, comprising determining the activity of calcineurin in cells of the haematopoietic tumor isolated from said subject. In a particular embodiment, a tumor cell having a sustained or increased activity of calcineurin is related to an invasive capacity, a metastatic potential, and/or a relapse probability.

30 The present invention also concerns a method for selecting a subject having a haematopoietic tumor to be treated by a calcineurin inhibitor comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject, and selecting the subject having tumoral cells with a sustained calcineurin activity.

In addition, the present invention concerns a method of assessing the responsiveness of a subject having a haematopoietic tumor to a treatment with a calcineurin inhibitor, comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject, a sustained calcineurin activity of said
 5 cells being indicative of a positive responsiveness to said treatment. In addition, the present invention concerns a method for screening, identifying or selecting a drug for treating a haematopoietic tumor, comprising contacting in vitro or in vivo a test compound with a calcineurin substrate under conditions in which calcineurin is able to dephosphorylate said substrate and determining whether said test compound affects the
 10 phosphorylation state of the substrate. In a preferred embodiment, the calcineurin substrate is NFAT.

LEGEND TO THE FIGURES

15 Figure 1. NFAT1 expression and activation in TEL-JAK2 leukemia.

Fig. 1A : Whole cell extracts of control thymocytes (WT) and Tg TEL-JAK2 leukemic cells (TJ2) (14) isolated from an invaded thymus were analyzed by SDS/PAGE and western blot using a NFAT1-specific antibody (upper panel). Samples were normalized using either an anti-STAT5 (middle) or an anti-ERK2 antibody
 20 lower panel). **Fig. 1B :** same as in Fig. 1A, except that cells used in lanes 3 and 4 were maintained in tissue culture in the presence of cyclosporin A (CsA), or ionomycin (Iono), as indicated.

Figure 2. TgTEL-JAK2 leukemic cells express activated NFATs : analysis by electrophoretic mobility shift assays (EMSA)

25 **Fig. 2A :** Nuclear extracts obtained from control thymocytes (WT) and TgTEL-JAK2 leukemic cells were analyzed for NFAT DNA binding activity by EMSA, using as DNA probe a double stranded oligonucleotide corresponding to the mouse IL2 promoter -45 NFAT-response element (top panel). Migration of the probe in the absence of any extract is shown in lane 1. The bottom panel displays the binding
 30 activity of the nuclear extracts used to a probe specific of the ubiquitously-expressed Sp1. Note that equal binding to the Sp1 probe is observed in the WT and TgTEL-JAK2 nuclear extracts. **Fig. 2B :** as in Fig. 2A, except that the DNA binding reaction mixture included 1 µl of the indicated NFAT antibodies (anti-NFAT1 ; anti-NFAT4) or a pan-

NFAT antibody, specific of NFAT1-4. The negative control used is a c-Rel-specific antibody.

Figure 3. Activated Notch-induced T cell leukemia activate NFAT

Fig. 3A : tumor cells from a series of independent ICN1-induced T cell leukemia
 5 obtained directly from diseased mice (lanes 1-7), or maintained in culture for 60
 minutes in either the presence of CsA (lane 8) or ionomycin (lane 9) were analyzed by
 western blot for expression and activation of NFAT1 (top panel) and NFAT2 (bottom
 panel), using antibodies specific for NFAT1 or NFAT2, respectively. Phosphorylated
 and de-phosphorylated isoforms are indicated by coloured arrows. **Fig. 3B** : Schematic
 10 representation of the different NFAT2 splicing isoforms and their relative migration in
 their fully phosphorylated (ionomycin) or dephosphorylated states (CsA).

Figure 4. NFAT activation is not under the sole control of TEL-JAK2
 oncoprotein in TgTEL-JAK2 leukemic cells but requires a proper tumor micro-
 environment.

Fig. 4A : Western blot analysis of NFAT1 activation in total extracts from
 15 thymocytes (lane1) and TgTEL-JAK2 leukemic cells (lanes 2 and 3). Analysis of
 extracts prepared from leukemic cells directly obtained from diseased animals (lane 2)
 shows that, in contrast to normal thymocytes, NFAT1 is mainly present in a
 dephosphorylated, active state in TgTEL-JAK2 animals (compare lanes 1 and 2).
 20 However, after 15min in culture as isolated cells (lane 3), TgTEL-JAK2 leukemic cells
 exhibit rephosphorylation of the almost complete intracellular pool of NFAT1 proteins
 (compare lanes 2 and 3). **Fig. 4B** : Top : western blot analysis of NFAT1 activation in
 total extracts from TgTEL-JAK2 leukemic cells directly obtained from diseased animals
 (lane 1), or after 2 hours in culture (lane 2). Middle : western blot analysis of STAT5
 25 activation TgTEL-JAK2 leukemic cells directly obtained from diseased animals (lane1),
 or after 2 hours in culture (lane 2), as analyzed using a STAT5 phosphotyrosine
 antibody. Bottom : analysis of STAT5 expression, using a STAT5-specific antibody.
 Note that under these conditions, TEL-JAK2 tyrosine kinase activity is maintained as
 shown by the phosphorylation of STAT5 (bottom panel).

Figure 5. Proper tumor micro-environment is required for NFAT activation in
 30 ICN1-induced leukemia and EBV-induced human lymphoma.

Fig. 5A : Western blot analysis of NFAT1 expression and activation in total
 extracts directly prepared from the leukemic cells obtained from ICN1-induced

leukemia (lane 1), or the same cells maintained in culture for 1 hour (lane 2). Note that NFAT1 is in its phosphorylated (activated) form in ICN-1 leukemic cells and that activation is rapidly lost when leukemic cells are removed from their normal micro-environment and maintained in culture as isolated cells. **Fig. 5B** : Western blot analysis of NFAT1 expression and activation in total extracts directly prepared from the leukemic cells obtained from an EBV-induced human B cell lymphoma (lane 1), or the same cells maintained in culture as isolated cells for 1 hour without further treatment (lane 2), or in the presence of ionomycin (lane 3) or CsA (lane 4). Note that NFAT1 is activated in leukemic cells in situ, but that activation is lost when cells are removed from their normal tumoral micro-environment.

Figure 6. Calcineurin inhibitors cyclosporinA (CsA) and FK506 (Prograf) inhibit progression of TgTEL-JAK2 leukemia.

Fig. 6A : TgTEL-JAK2 leukemic cells were grafted into syngenic recipient mice. Under these conditions, leukemic cells engraft and proliferate in peripheral lymphoid organs and metastasize to non hematopoietic organs such as liver. (A) Three cohorts of mice were compared. Control (untreated, NT) ; mice treated with CsA ; mice treated with Prograf. Note that spleen invasion is inhibited by CsA and Prograf treatment (left), as analyzed by measuring spleen weight. The weight of age-matched control mice is shown for comparison (Normal). **Fig. 6B** : Pictures of representative spleens, as indicated in the legend. Normal spleen ; Leukemic spleen (untreated) ; Leukemic spleen from CsA- and Prograf-treated mice.

Figure 7. CsA and Prograf treatment inhibits NFAT factors activation.

Fig. 7A : Western blot analysis of NFAT1 (top) and NFAT4 (middle) expression and phosphorylation in non treated (NT) and CsA-treated TgTEL-JAK2 leukemia. **Fig. 7B** : Western blot analysis of NFAT1 (top) and NFAT4 (middle) expression and phosphorylation in non treated (NT) and Prograf-treated TgTEL-JAK2 leukemia. Note the fast migrating (activated) forms of NFAT1 and NFAT4 in the untreated leukemia and the fully phosphorylated, inactive species in CsA- and Prograf-treated leukemias. Western blot analysis of ERK expression (bottom) is shown as loading control.

Figure 8. CsA and Prograf treatment strongly interferes with leukemia progression.

Imprints of: normal bone marrow (**Fig. 8A**), leukemic bone marrow from untreated TgTEL-JAK2 leukemic mouse (**Fig. 8B**) and bone marrow from CsA-treated

(**Fig. 8C**) and Prograf-treated TgTEL-JAK2 leukemic mice (**Fig. 8D**) were stained with May-Grunwald-Giemsa. Note that the majority of cells in normal bone marrow (**Fig. 8A**) are of myeloid origin (granulocytic morphology); in contrast, the leukemic bone marrow obtained from an untreated animal is composed of an homogeneous population of T lymphoblastic cells that replaces the normal cells (**Fig. 8B**). Treatment with CsA (**Fig. 8C**) and Prograf (**Fig. 8D**) results in severe diminution in leukemic cells numbers and in the recovery of a cell composition and morphology close from that of normal bone marrow.

Figure 9. CsA and Prograf treatment inhibits invasion of leukemic cells in non-hematopoietic organs.

HES (Hematoxylin Eosin Safran) staining of parafin- included sections of liver from either normal mouse (**Fig. 9A**), a leukemic, untreated mouse (**Fig. 9B**), a leukemic CsA-treated mouse (**Fig. 9C**) and a leukemic, Prograf-treated mouse (**Fig. 9D**). **Fig. 9A** shows the normal structure of the liver parenchyma. The untreated TgTEL-JAK2 leukemic mouse shows massive infiltration of leukemic cells (stained in blue) in the liver parenchyma through the portal areas and sinusoids (**Fig. 9B**). Treatment with CsA (**Fig. 9C**) or Prograf (**Fig. 9D**) shows severe reduction of liver invasion by leukemic cells.

Figure 10 : Sustained calcineurin activation in leukemic cells from intracellular NOTCH1- and TEL-JAK2-induced T-ALL. (**Fig. 10a**) Primary thymocytes from wild-type mice (WT) and TEL-JAK2 (TJ2) and intracellular NOTCH1 (ICN1) leukemic cells were analyzed by Western blot for the phosphorylation of NFATc2 (upper panels) and NFATc1 (lower panels) either in freshly isolated cells (*in vivo*, lanes 1, 5, 8), or after *ex-vivo* culture for 60 minutes in the presence of 1µg/ml ionomycin (Ion , lanes 2, 6, 9), 1µg/ml cyclosporine A (CsA ; 3, 7 ,10) or left untreated (Unt, lane 4). The fully phosphorylated and dephosphorylated forms of NFATc2 and NFATc1 are indicated as filled and open arrowheads, respectively. In line with published data, NFATc1 migrates as three isoforms generated by alternative splicing. (**Fig. 10b**) Western blot analysis of NFATc2 phosphorylation in leukemic cells obtained from TJ2/Rag2^{-/-} and TJ2/CD3ε^{-/-} compound mice (lanes 2, 3, 5 and 6) and their control littermates TJ2/Rag2^{+/-} and TJ2/CD3ε^{+/-} (lanes 1 and 4). (**Fig. 10c**) TJ2 or ICN1 leukemic cells were analyzed by Western blot for the phosphorylation of NFATc2 (upper panels) either in freshly resected cells or after one hour *ex vivo* culture in RPMI +10%FCS. (**Fig. 10d**) TJ2

samples of panel (Fig. 10c) were analyzed by Western blot for STAT5 tyrosine phosphorylation (upper panel) and expression (lower panel). (Fig. 10e) Sustained calcineurin activation in tumor cells from mouse models of human lymphoma/leukemia. Cells obtained from a tumor induced in nude mice by subcutaneous injection of a cell line derived from an $Ik^{L/L}$ leukemia (T64) and the tumor cells obtained from a xenograft model of a human Burkitt-like lymphoma were analyzed by Western blot for the phosphorylation of NFATc2 (upper panels) and NFATc1 (lower panel) either directly (*in vivo* ; lanes 1, 4), or following *ex-vivo* culture for 60 minutes in the presence of 1 μ g/ml ionomycin (Ion ; lane 2) or 1 μ g/ml cyclosporinA (CsA ; lanes 3, 5).

10 **Figure 11** : CsA and Prograf induce T-ALL regression and prolong mouse survival. (**Fig. 11a**) Bone marrow cytopins were prepared from wild-type mice (WT) and ICN1 leukemic mice that were treated for 5 days with either the solvent carrier alone (ICN1 Unt), Prograf (ICN1 Prog) or with CsA (ICN1 CsA) and analyzed after May-Grünwald Giemsa staining. Original magnification: X 800. (**Fig. 11b**) Spleen weights of CsA (λ), Prograf (z) or solvent carrier (σ)-treated leukemic mice are shown by scatter plot. The weights of spleen from normal individuals are shown for comparison ($\diamond$). A substantial reduction in TEL-JAK2 (TJ2) and ICN1 tumor load was evident after 10 days and 5 days of treatment, respectively. WT, n=2; TJ2 Unt, n=5; TJ2 CsA, n=4; TJ2 Prog, n=4; ICN1 Unt, n=3; ICN1 CsA, n=3; ICN1 Prog, n=2. These data are representative of at least 3 independent experiments. *P*-values for the differences in median spleen weights are indicated as: $p<0.05$ (*), $p<0.01$ (**) and $p<0.0001$ (***). (**Fig. 11c**) Liver sections were prepared from wild-type mice (WT unt) and ICN1 or TJ2 leukemic mice that were treated with either the solvent carrier alone (ICN1 unt; TJ2 unt) or Prograf (ICN1 Prog; TJ2 Prog) or CsA (ICN1 CsA; TJ2 CsA) and analyzed after Hematoxylin-Eosin-Safran (HES) staining. Low original magnification: X40; High original magnification: X 800. (**Fig. 11d**) Kaplan-Meier survival curves of syngeneic mice transplanted with $5 \cdot 10^6$ ICN1 leukemic cells and then treated (open line) or not (filled line) with Prograf (3mg/kg/day) for 14 days. The number of mice in each group and the mean survival are indicated between parentheses. The *P*-value was calculated using the log-rank test. (**Fig. 11e**) Therapeutic treatment of TEL-JAK2-diseased mice with CsA or Prograf induces leukemia regression in bone marrow cells. May-Grünwald Giemsa staining of bone marrow cytopins from TJ2 leukemic mice that were treated for 10 days with either the solvent carrier alone (TJ2 Unt) or with Prograf (TJ2 Prog) or

with CsA (TJ2 CsA). Original magnification: X 800.

Figure 12 : *In vivo* calcineurin inhibition leads to reduced proliferation and induces apoptosis of leukemic cells in mouse models of human leukemia. (**Fig. 12a**) NFATc2 phosphorylation was assessed by Western blot in leukemic cells obtained from the spleens of either solvent carrier-(lanes 1-4 and 7-10) or Prograf-(lanes 5 and 6), or CsA-treated (lanes 11 and 12) TEL-JAK2 (TJ2) mice (described in Fig. 11). Fully phosphorylated and dephosphorylated forms of NFATc2 are indicated with filled and open arrowheads, respectively. (**Fig. 12b**) Semi-thin liver sections stained by toluidine blue obtained from either solvent carrier- or Prograf-treated TJ2 leukemic mice. Hepatocytes and leukemic cells are indicated with filled and open arrowheads, respectively. Note that only the nuclei of leukemic cells but not of hepatocytes are pycnotic in the Prograf-treated sample. Original magnification: X 1500. (**Fig. 12c**) Electron microscopic examination of ultra-thin liver section obtained from a representative Prograf-treated TJ2 mouse. Note the typical chromatin condensation characteristics of apoptotic cells in the TJ2 leukemic cells. (**Fig. 12d**) Representative field of histological analysis and TUNEL staining to evaluate the proportion of apoptotic leukemic cells in livers obtained from solvent carrier- or Prograf-treated TJ2 leukemic mice. Similar observations were made after CsA treatment (data not shown). Original magnification: X 800. (**Fig. 12e**) Left panel: analyses of the proportion of AnnexinV-positive (apoptotic) and BrdU-positive (proliferating) leukemic cells in the liver of ICN1 leukemic mice treated for 5 days either with the solvent carrier or with CsA or Prograf, as indicated. The percentage of BrdU-positive and AnnexinV-positive cells is indicated on the right of each graph. Right panel: the same experiment was performed for TJ2 leukemic mice that were treated either with the solvent carrier alone or with CsA or Prograf for 2 days. The percentage of AnnexinV-positive cells is indicated on the right of the graph. (**Fig. 12f**) *In vivo* antiproliferative effect of Prograf and CsA on TJ2 leukemic cells. TJ2 leukemic cells were subcutaneously injected to nu/nu mice. Under these conditions TJ2 cells formed a tumor at the site of injection after 10 to 15 days, but also invaded lymphoid (spleen, lymph nodes) and non-lymphoid organs (kidney, liver). Two weeks later, mice were randomized to receive Prograf (3mg/kg/day) or PBS control by intratumoral injection. Cell cycle distribution of leukemic cells was assessed using BrdU-FITC and 7-AAD double staining. Results are representative of 2 independent experiments. The percentage of cells in the G0/G1 (R6),

S (R3) and G2/M (R5) phases of the cell cycle are indicated in each corresponding square. Similar results were obtained when tumors were analyzed after CsA treatment (data not shown).

Figure 13 : Ectopic expression of a constitutively active mutant of CnA (CnA*) in leukemic cells favors leukemia progression and invasion. **(Fig. 13a)** Leukemic cells from the spleens of four mice intravenously injected with either mock-transduced (TJ2) or CnA*-transduced (TJ2+ CnA*) TJ2 cells were isolated and analyzed for CnA* expression by immunoprecipitation using the anti-HA tag antibody followed by western blot using the anti-CnA antibody. **(Fig. 13b)** Spleen (upper panels) and liver weights (lower panels) from mice bearing ICN1 or ICN1+CnA* leukemia were compared (left panels; ICN1, n=7; ICN1+CnA*, n=8). The same comparisons were made for the TJ2 model (right panels; TJ2, n=5; TJ2+CnA*, n=7). The *P*-values were calculated using the log-rank test and are indicated as: $p<0.05$ (*), $p<0.01$ (**) and $p<0.0001$ (***). **(Fig. 13c)** Kidney sections from representative TJ2 and TJ2+CnA* leukemic mice stained with Hematoxylin-Eosin-Safran (HES). Original magnification: X 12,5. Note the massive infiltration of the renal mesenchyma in TJ2+CnA* leukemic mice. **(Fig. 13d)** Histological analyses of sternum sections from TJ2 and TJ2+CnA* leukemic mice. Note the higher cell density of leukemic cells in the bone marrow of TJ2+CnA* mice as compared to TJ2 mice (Upper panels). As observed at high magnification (X 80), TJ2+CnA* leukemic cells massively expand beyond the marrow compartment to invade adjacent muscles (Lower panels). **(Fig. 13e)** Increased tumor load in the kidney of mice transplanted with CnA*-transduced TJ2 cells as compared to mice engrafted with mock-transduced TJ2 cells. The kidney weights of mice transplanted with either mock-transduced TJ2 cells (TJ2, n=5) or CnA*-transduced TJ2 cells (TJ2+ CnA*, n=7) were determined and reported on the scatter plot. The *P*-value was calculated using the log-rank test ($p<0,0001$ [***]).

DETAILED DESCRIPTION OF THE INVENTION

The present data show that calcineurin is a target of therapeutic interest in lymphoid malignancies. They furthermore show that two inhibitors of calcineurin widely used in other indications in human medicine, namely CsA and FK506, could be of therapeutic interest to control the evolution of leukemia and lymphoma, by affecting

either the tumor cell itself and/or its stromal micro-environment.

The inventors demonstrate in the present invention that sustained calcineurin activation is observed in the mouse models of human T-cell malignancies tested. In particular, the inventors showed that the cancer cells display a persistent
5 dephosphorylation of NFAT. In intracellular NOTCH1(ICN1)- or TEL-JAK2-induced T-cell acute lymphoblastic leukemia (T-ALL), two mouse models relevant to human malignancies, *in vivo* inhibition of calcineurin activity by CsA or FK506 induced apoptosis of leukemic cells, rapid tumor clearance and significantly prolonged mouse survival. Conversely, ectopic expression of a constitutively activated mutant of
10 calcineurin favored leukemia progression. Thus, calcineurin activation is critical for the maintenance of the leukemic phenotype *in vivo*, identifying this pathway as a novel therapeutic target in T-cell malignancies. Indeed, CsA and FK506 treatment results in severe inhibition of tumor load in lymphoid organs, the near disappearance of leukemic cells from the bone marrow, accompanied by the restoration of normal hematopoiesis
15 and the essentially complete disappearance of leukemic cells from invaded liver. In addition, the inventors observed a specificity of the cytotoxicity of CsA and FK506 as the liver cells are not affected by CsA or FK506 treatment.

In addition, the inventors establish the conditions in which such a treatment can be beneficial for the patient. Indeed, the haematopoietic tumor has to show a sustained
20 activation of calcineurin in order to have an efficient treatment by calcineurin inhibitors.

Definition

Where "comprising" is used, this can preferably be replaced by "consisting essentially of", more preferably by "consisting of".

25 Whenever within this whole specification "treatment of a haematopoietic tumor" or the like is mentioned with reference to a drug inhibiting calcineurin, there is meant:

a) a method of treatment (=for treating) of a haematopoietic tumor, said method comprising the step of administering (for at least one treatment) a drug inhibiting calcineurin, (preferably in a pharmaceutically acceptable carrier material) to a subject,
30 especially a human, in need of such treatment, in a dose that allows for the treatment of said haematopoietic tumor (=a therapeutically effective amount);

b) the use of a drug inhibiting calcineurin and for the treatment of a haematopoietic tumor; or a drug inhibiting calcineurin, for use in said treatment

(especially in a human);

c) the use of a drug inhibiting calcineurin an for the manufacture of a pharmaceutical preparation for the treatment of a haematopoietic tumor;

d) a pharmaceutical preparation comprising a dose of a drug inhibiting
5 calcineurin that is appropriate for the treatment of a haematopoietic tumor; and/or

e) a product containing a drug inhibiting calcineurin and an anticancer drug as a combined preparation for simultaneous, separate or sequential use in the treatment of a hematopoietic tumor.

10 The present invention concerns the use of a drug inhibiting calcineurin for the preparation of a medicament for treating a hematopoietic tumor. In a preferred embodiment, said haematopoietic tumor has a sustained or increased calcineurin activity. In a particular embodiment, the subject to be treated presents dephosphorylated NFAT in cells of the haematopoietic tumor isolated from said subject.

15 In the present invention, "a sustained or increased calcineurin activity" is intended to refer to a calcineurin activity which is at least 20, 30, 40, 50, 60, 70, 80, 90, or 100 % more than the activity observed for a healthy or normal lymphoid cell. In a "normal" calcineurin activity, a combination of phosphorylated and non-phosphorylated calcineurin substrate is observed. In a sustained or increased calcineurin activity, the
20 substrate is essentially in a non-phosphorylated state. By "being essentially" is intended that at least 70, 80, 90, 95, 99 % of the substrate is in a non-phosphorylated state. In a preferred embodiment, the assayed substrate is NFAT. Calcineurin activity can be determined by any means known in the art. The present invention further concerns a method for treating a hematopoietic tumor in a subject comprising administering a
25 therapeutic amount of a drug inhibiting calcineurin. Optionally, the method for treating a hematopoietic tumor in a subject comprises a previous step of determining the activity of calcineurin in cells of the haematopoietic tumor isolated from said subject. Indeed, presence of a sustained or increased activity of calcineurin is indicative of an efficiency of the drug inhibiting calcineurin for treating said haematopoietic tumor. In a particular
30 embodiment, the method for treating a hematopoietic tumor in a subject can comprise a previous step of determining the phosphorylation state of NFAT in cells of the haematopoietic tumor isolated from said subject, the presence of a dephosphorylated NFAT being indicative of an efficiency of the drug inhibiting calcineurin for treating

said haematopoietic tumor. Such a therapeutic amount is an amount sufficient to inhibit calcineurin activity in the target hematopoietic tumoral cells.

The inventors have shown that a drug which inhibits calcineurin induces apoptosis of cancer cells and inhibits the proliferation of cancer cells. Therefore, this
5 drug is of a great interest to block the progression of the cancer, in particular the spreading and the growth of cancer. This drug can also provides a cancer regression, a restoration of hematopoiesis and an increase survival.

The present invention also concerns the use of a drug inhibiting calcineurin for the preparation of a medicament for increasing the efficiency of a treatment of a
10 hematopoietic tumor. In a preferred embodiment, said haematopoietic tumor has a sustained or increased calcineurin activity. Preferably, the treatment of a hematopoietic tumor can be a cancer chemotherapy, an immunotherapy, a radiotherapy, a hormone or cytokine therapy, any other therapeutic method used for the treatment of a haematopoietic tumor or a combination thereof. More preferably, the treatment of a
15 hematopoietic tumor is a cancer chemotherapy. In particular, the invention relates to a method for increasing the survival time of a subject having a haematopoietic tumor comprising, administering to said subject an efficient amount of a drug inhibiting calcineurin; thereby increasing the survival time of said subject. Preferably, the method further comprises a previous step of determining calcineurin activity in cells of the
20 haematopoietic tumor isolated from said subject and administering the drug to the subject having tumoral cells with a sustained calcineurin activity.

Preferably, said subject is a mammal. More preferably, said subject is a human.

NFAT (Nuclear Factor of Activated T-cells) can be selected from the group consisting of NFAT1 (also called NFATP and NFATC2, Unigene Hs.356321), NFAT2
25 (also called NFATC1 and NFATC, Unigene Hs.534074), NFAT3 (also called NFATC4, Unigene Hs.77810), NFAT4 (also called NFATC3 and NFATX, Unigene Hs.341716) and any combination thereof. In a preferred embodiment, said NFAT is selected from the group consisting of NFAT1, NFAT2, and NFAT4. The disclosures of the Unigene files corresponding to the aforementioned accession numbers are incorporated herein by
30 reference.

More specifically, the present invention can be utilized for the treatment of a hematopoietic tumor. Preferably, said haematopoietic tumor is selected in the group consisting of B lymphoma, T lymphoma, B lymphoblastic leukemia and T

lymphoblastic leukemia. In a more preferred embodiment, said haematopoietic tumor is a T-cell leukemia and/or T cell lymphoma. For example, the hematopoietic tumor can be selected from the group consisting of a hematopoietic tumor of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell
5 lymphoma, T-cell lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma, hairy cell lymphoma and Burkitt lymphoma and a hematopoietic tumor of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukaemia. In a particular embodiment, the hematopoietic tumor is an aggressive leukemia or lymphoma. The cancer can be a primary tumor or a metastasis. The cancer to treat can
10 also be a relapse.

According to the present invention, a drug inhibiting calcineurin leads to an inactivation of NFAT, e.g. a phosphorylation of NFAT. Indeed, the inventors have observed an activation of NFAT in the primary cancer cells isolated from a human subject. NFAT activation includes protein-protein interaction between calcineurin and
15 NFAT, dephosphorylation of NFAT by calcineurin, and translocation of NFAT to the nucleus.

Calcineurin inhibitors are already used in therapy as an immunosuppressant to prevent rejection following organ transplantation. In immunosuppressive therapy, calcineurin inhibitors are used in high doses for long term treatment. Such drugs
20 include, but are not limited thereto cyclosporin A (Novartis International AG, Switzerland), FK506 (Fujisawa Healthcare, Inc., Deerfield, IL, USA), FK520 (Merck & Co, Rathway, NJ, USA), L685,818 and L732,731 (Merck & Co), ISATX247, (Hoffman-La Roche Ltd), FK523, and 15-0-DcMe-FK-520 (Liu, Biochemistry, 31:3896-3902 (1992)). WO2005087798 describes cyclosporine derivative inhibiting
25 calcineurin. WO2006078724 describes FK506 and FK520 analogs inhibiting calcineurin. This list is not intended to be limitative.

Calcineurin is a serine/threonine protein phosphatase, which is a heterodimer composed of a catalytic subunit (Calcineurin A) and a regulator subunit (Calcineurin B). Then, the activity of calcineurin can also be inhibited by blocking its expression, in
30 particular the expression of one of its subunit. In a preferred embodiment, the activity of calcineurin can also be inhibited by blocking the expression of the regulator subunit B. The expression can be blocked by any mean known by one skilled in the art, e.g., by chemically synthesized oligonucleotides such as antisense oligonucleotides, ribozymes,

short interfering RNA (siRNA) and short hairpin RNA (shRNA). Antisense oligonucleotides are short single-strand molecules that are complementary to the target mRNA and typically have 10-50 mers in length, preferably 15-30 mers in length, more preferably 18-20 mers in length. Antisense oligonucleotides are preferably designed to
5 target the initiator codons, the transcriptional start site of the targeted gene or the intron-exon junctions (for review, 16). Ribozymes are single stranded RNA molecules retaining catalytic activities. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The ribozyme is engineered to interact with the target
10 RNA of interest comprising a cleavage NUH triplet, preferentially GUC (for review, 17). siRNA are usually 21 or 23 nucleotides long, with a 19 or 21 nucleotides duplex sequence and 2 nucleotides-long 3' overhangs. shRNA are designed with the same rules than for a sequence encoding a siRNA excepting several additional nucleotides forming a loop between the two strands of the siRNA. (For review 18)

15 Alternatively, the activity of calcineurin can be inhibited by a compound that inhibits the interaction between calcineurin subunits, in particular the interaction between subunits A and B. The activity of calcineurin can be inhibited by a compound that inhibits the interaction between calcineurin and calmodulin. Calcineurin inhibition can also be obtained by activation of endogenous inhibitors of calcineurin, including
20 cabin1, calcipressins and AKAP79.

The drug inhibiting calcineurin can be a compound that inhibits the interaction between calcineurin and its substrates, e.g. NFAT. Such a compound has been described in the US patent 6,686,450 which describes a polypeptide called Cabin 1 and fragment thereof that inhibit the interaction between calcineurin and NFAT, thereby inhibiting the
25 dephosphorylation of NFAT by calcineurin. The patent application WO2004/069200 disclosed peptides derived from NFAT capable of specifically inhibiting the interaction between calcineurin and NFAT and other substrates containing a PxIxIT binding interface, thereby inhibiting the dephosphorylation of these substrates by calcineurin. One example of such a peptide is a peptide comprising or consisting of the amino acid
30 sequence MAGPHPVIVITGPHEE. This patent application also describes small compounds, for example INCA-1, INCA-2 and INCA-6 capable of inhibiting the dephosphorylation of substrates by calcineurin.

Other drugs inhibiting calcineurin can be identified by screening methods

already disclosed in the art. As illustration, the US patents 6,875,581 and 6,338,946 describes screening methods useful for identifying modulators of calcineurin activity.

In a preferred embodiment, the drug inhibiting calcineurin is a drug that inhibits NFAT dephosphorylation.

5 The drug inhibiting calcineurin may be of various origin, nature and composition. It may be any organic or inorganic substance, such as a lipid, peptide, polypeptide, nucleic acid, small molecule, etc., in isolated or in mixture with other substances. In a preferred embodiment, the drug is a small molecule. In an other preferred embodiment, the drug is a peptide or a polypeptide. In an additional
10 embodiment, the drug is a nucleic acid, e.g., an antisense, a siRNA, a ribozyme.

 The drug inhibiting calcineurin can be used in association with a targeting moiety, the targeting moiety allowing to preferentially reach cancer cells rather than normal cell. Preferably, the targeting moiety allows the selective treatment of cancer cells. For example, B-subunit of Shiga toxin can be used as a cancer cell vectorization
15 means (for more details, see WO2004016148).

 According to the present invention, the drug inhibiting calcineurin can be used alone or in combination with usual cancer therapy. The cancer therapy can be selected from the group consisting of a cancer chemotherapy, an immunotherapy, a radiotherapy, a hormone or cytokine therapy, any other therapeutic method used for the treatment of a
20 haematopoietic tumor and a combination thereof. Preferably, the cancer therapy is a cancer chemotherapy. In a preferred embodiment, the drug inhibiting calcineurin is used in combination with a cancer chemotherapy. The drug inhibiting calcineurin can be administered before, at the same time or after the cancer therapy. In a first embodiment, the drug inhibiting calcineurin and the anticancer drug can be administered by the same
25 route. In an alternative embodiment, they are administered by different routes of administration.

 The present invention concerns a method of treating a hematopoietic tumor in a subject comprising administering a therapeutic amount of a drug inhibiting calcineurin and and a therapeutic amount of an anticancer drug. Preferably, the method further
30 comprises a previous step of determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject. More particularly, the method for treating a hematopoietic tumor in a subject comprises a previous step of determining the phosphorylation state of NFAT in cells of the haematopoietic tumor isolated from said

subject. Indeed, presence of a dephosphorylated NFAT is indicative of an efficiency of the drug inhibiting calcineurin for treating said haematopoietic tumor.

The present invention concerns a product containing a drug inhibiting calcineurin and an anticancer drug as a combined preparation for simultaneous,
5 separate or sequential use in the treatment of a hematopoietic tumor. In particular, the hematopoietic tumor has a sustained or increased calcineurin activity. In a preferred embodiment, said drug inhibiting calcineurin is FK506.

The present invention concerns a pharmaceutical composition comprising a drug inhibiting calcineurin and an anticancer drug. Preferably, the drug inhibiting calcineurin
10 is FK506. Such a pharmaceutical composition generally comprises a pharmaceutically acceptable carrier. By a pharmaceutically acceptable carrier is intended a carrier that is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the drug with which it is administered. For example, a pharmaceutically acceptable carrier can be physiological saline solution. Other pharmaceutically
15 acceptable carriers are known to one skilled in the art and described for instance in Remington: The Science and Practice of Pharmacy (20th ed., ed. A.R. Gennaro AR., 2000, Lippincott Williams & Wilkins).

Anticancer drugs interfere with cancer cells' ability to grow (multiply) or to survive. There are several types of drugs; each type interferes with the cell's ability to
20 grow or survive in a different way. A brief description of several examples of drug types that are used to treat people with cancer follows. These chemotherapies are well-known by one skilled in the art.

A first class of drugs is DNA-damaging drugs which react with DNA to alter it chemically and prevent it from permitting cell growth. For instance, this kind of drug
25 can be selected from the following group, but are not limited thereto : Busulfan (Myleran) ; Carboplatin (Paraplatin) ; Carmustine (BCNU) ; Chlorambucil (Leukeran) ; Cisplatin (Platinol) ; Cyclophosphamide (Cytosan, Neosar) ; Dacarbazine (DTIC-Dome) ; Ifosfamide (Ifex) ; Lomustine (CCNU) ; Mechlorethamine (nitrogen mustard, Mustargen) ; Melphalan (Alkeran) ; and Procarbazine (Matulane).

30 A second class of drugs is antitumor antibiotics which interact directly with DNA in the nucleus of cells, interfering with cell survival. For instance, this kind of drug can be selected from the following group, but are not limited thereto : Bleomycin (Blenoxane) ; Daunorubicin (Cerubidine) ; Doxorubicin (Adriamycin, Rubex) ;

Idarubicin (Idamycin) ; and Mitoxantrone (Novantrone).

A third class of drugs is antimetabolites which are chemicals that are very similar to the building blocks of DNA or RNA. They are changed from the natural chemical sufficiently so that when they substitute for it and block the cells' ability to
5 form RNA or DNA, preventing cell growth. For instance, this kind of drug can be selected from the following group, but are not limited thereto : 5-azacytidine (AZA-CR) ; Cladribine (Leustatin) ; Cytarabine (cytosine arabinoside, Ara-C, Cytosar-U) ; Fludarabine (Fludara) ; Hydroxyurea (Hydrea) ; 6-mercaptopurine (Purinethol) ; Etoposide; Methotrexate (Rheumatrex) ; and 6-thioguanine (Thioguanine).

10 A fourth class of drugs is DNA-repair enzyme inhibitors which act on enzymes in the cell nucleus that normally repair injury to DNA. These drugs prevent the enzymes from working and make the DNA more susceptible to injury. For example, such drugs can be Etoposide (VP-16, VePesid) ; Teniposide (VM-26, Vumon) ; and Topotecan (Hycamptin).

15 A fifth class of drugs is drugs that prevent cells from dividing by blocking mitosis. For example, such drugs can be Vinblastine (Velban) ; Vincristine (Oncovin) ; and Paclitaxel (Taxol).

A sixth class of drugs is hormones that can kill lymphocytes. In high doses, these synthetic hormones, relatives of the natural hormone cortisol, can kill malignant
20 lymphocytes. For example, such drugs can be Dexamethasone (Decadron) ; Methylprednisolone (Medrol) ; Prednisolone and Prednisone (Deltasone).

A seventh class of drugs is cell-maturing agents that act on a type of leukemia to induce maturation of leukemic cells. All-trans retinoic acid (ATRA) and Arsenic trioxide (Trisenox) can be cited as illustration.

25 An eighth class of drugs is biomodifiers based on natural products with exact mechanisms of action that are unclear, such as Interferon-alpha (Roferon A, Intron A).

A ninth class of drugs is monoclonal antibodies that target and destroy cancer cells with fewer side effects than conventional chemotherapy. Rituximab (Rituxan) and Gemtuzumab ozogamicin (Mylotarg) can be cited as illustration.

30 A tenth class of drugs is drugs with specific molecular targets. These agents are designed to block the specific mutant protein that initiates the malignant cell transformation, such as Imatinib mesylate (Gleevec, Glivec).

Of course, this list does not include every drugs being used or studied in clinical

trials. Combinations of these drugs and drug groups often form the basis of treatment. Certain of these drugs have been found to be more or less active in a particular subtype of cancer, in particular leukemia, lymphoma or myeloma.

In a particular embodiment, the calcineurin inhibitor is used in combination with
5 at least one anti-cancer drug selected from the group consisting of the second, third, fifth and sixth classes. For example, it can be combined with at least one drug selected from the group consisting of prednisolone, vincristine, daunorubicin, etoposide and cytarabine.

The compositions of the present invention may be administered orally,
10 parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. Preferably, the compositions are administered orally, intraperitoneally or intravenously. For
15 example, calcineurin inhibitor is administered orally and the anticancer drug is administered intravenously. Alternatively, the calcineurin inhibitor and the anticancer drug are both administered intravenously.

By a therapeutic amount is intended an amount of drug, alone or in combination with an anticancer drug, that is sufficient to inhibit cancer growth, progression or
20 metastasis in vivo. The effective amount of a drug for the treatment of cancer varies depending upon the administration mode, the age, body weight, sex and general health of the subject. It is an amount that is sufficient to effectively reduce cell proliferation, tumor size, cancer progression or metastasis. It will be appreciated that there will be many ways known in the art to determine the therapeutic amount for a given
25 application. For instance, the dose of FK506 can be from 0.001 mg/kg/day to 10 mg/kg/day, preferably between 0.01 and 10 mg/kg/day, more preferably between 0.1 and 1 mg/kg/day, by oral administration and between 0.001 and 1 mg/kg/day by intravenous injection, preferably between 0.01 and 0.5 mg/kg/day. In a particular embodiment, the blood FK506 level is comprised between 5 and 40 ng/ml, preferably
30 between 15 and 20 ng/ml. Accordingly, the administered dose of FK506 can be adapted in order to obtain the above-mentioned blood FK506 level. The dose of cyclosporin A as oral formulation (Neoral; Sandimmune) can be from 0.1 mg/kg/day to 10 mg/kg/day, preferably from 0.1 mg/kg/day to 1 mg/kg/day.

In a particular embodiment, the composition comprising the drug inhibiting calcineurin is administered for a short period of time. In a preferred embodiment, the calcineurin inhibitor is administered to the subject during a period of 2 to 10 weeks, preferably 3 to 8, more preferably 4 to 6 weeks. In a particular embodiment, the period
5 can be the period of the chemotherapy. Optionally, the period can be from one day to one month. Optionally, the period of treatment can be repeated, optionally with lower dose of calcineurin inhibitor. The drug inhibiting calcineurin can be administered once a day, twice a day or more. In a preferred embodiment, the drug inhibiting calcineurin and is administered so as to avoid an immunosuppressive effect. For example, this
10 immunosuppressive effect can be obtained by adapting the dose (e.g. lower dose) or the period of treatment (e.g. shorter period).

In a preferred embodiment, the calcineurin inhibitor is used to treat the subject during the remission (induction) treatment. Accordingly, it is preferably used alone or in combination with at least one anticancer drug used in the remission treatment. The
15 remission treatments are generally short (e.g., 6 weeks) and the length of this period is well adapted to have the antitumoral beneficial effect of the calcineurin inhibitors without the immunodeficient effect. FK506 has the advantage to cross the blood-brain barrier. Therefore, FK506 and the other calcineurin showing this capacity are particularly adapted the CNS invasion by the tumoral cells. In a alternative or additional
20 embodiment, the calcineurin inhibitor is used to treat the subject during the consolidation and/or continuation treatment.

The administration protocols for the cancer chemotherapy are well-known by one skilled in the art.

The present invention further concerns a method for staging or characterizing a
25 hematopoietic tumor in a subject comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject. In a particular embodiment, the step of determining calcineurin activity is determined by assessing the phosphorylation of a substrate of calcineurin, preferably NFAT, a dephosphorylated substrate being indicative of a sustained calcineurin activity. In a preferred embodiment, the present
30 invention further concerns a method for staging or characterizing a hematopoietic tumor in a subject comprising determining the phosphorylation state of NFAT in cancer sample isolated from said subject. A dephosphorylated NFAT is an activated NFAT involved in cancer development whereas a phosphorylated NFAT is an inactivated

NFAT. In a particular embodiment, a sustained or increased activity of calcineurin, and for instance a dephosphorylated NFAT, is related to an invasive capacity, a metastatic potential, a relapse probability. The cancer sample from the patient is a body fluid, preferably a blood sample. In a preferred embodiment, the phosphorylation state of calcineurin substrate (e.g., NFAT) is assayed directly on the sample, preferably the
5 resected sample, without any culture step. Alternatively, the phosphorylation state of the calcineurin substrate (e.g., NFAT) is on a short culture of the sample, preferably less than one hour.

The present invention also concerns a method of assessing the responsiveness of
10 a subject having a haematopoietic tumor to a treatment with a calcineurin inhibitor, comprising determining the calcineurin activity in cells of the haematopoietic tumor isolated from said subject, a sustained calcineurin activity of said cells being indicative of a positive responsiveness to said treatment. By positive responsiveness is intended at least one effect selected from the group consisting of an inhibition of tumor load in
15 lymphoid organs, the disappearance of leukemic cells from the bone marrow, the restoration of normal hematopoiesis, the essentially complete disappearance of leukemic cells from invaded organs such as liver, spleen and kidney and a prolonged survival. In a preferred embodiment, the present invention also concerns a method of assessing the responsiveness of a subject to a treatment of a haematopoietic tumor with
20 a drug inhibiting calcineurin, comprising determining the phosphorylation state of NFAT in cancer sample isolated from said subject, a presence of a dephosphorylated NFAT being indicative of an efficiency of the drug inhibiting calcineurin for treating said haematopoietic tumor.

The present invention also concerns a method for selecting a subject having a
25 haematopoietic tumor to be treated by a calcineurin inhibitor comprising, determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject, and selecting the subject having tumoral cells with a sustained calcineurin activity.

The methods and uses described in the present invention should also be appropriate for solid tumors, in particular for metastasis from solid tumors. Therefore,
30 the present invention also contemplates such methods and uses.

The present invention concerns a method for screening, identifying or selecting a drug for treating a haematopoietic tumor, comprising contacting in vitro or in vivo a test compound with a calcineurin substrate, preferably a NFAT polypeptide, under

conditions in which calcineurin is able to dephosphorylate said calcineurin substrate, preferably the NFAT polypeptide, and determining whether said test compound affects the phosphorylation state of calcineurin substrate, preferably the NFAT. In a particular embodiment, a calcineurin substrate, preferably a NFAT polypeptide, under conditions
5 in which calcineurin is able to dephosphorylate said substrate is comprised into isolated cells or into cells of a test non-human animal.

For example, calcineurin activity can be determined by the phosphorylation state of a calcineurin substrate. The phosphorylation state can be assayed by different methods known by one skilled in the art. In a first embodiment, calcineurin activity can
10 be determined by a biochemistry assay, (i.e. activity in a cellular extract with a specific peptidic substrate). Such a peptidic substrate for phosphorylation by calcineurin is commercially available (e.g., Calcineurin Colorimetric Assay Kit, Calbiochem, San Diego, U.S.A.; ref 31; calcineurin substrate : RII Phosphopeptide (BIOMOL international, American Peptide Company), LKT-C0248-M001 (Axxora platform)). In a
15 second and preferred embodiment, calcineurin activity is determined by analysis of in vivo phosphorylation of a calcineurin substrate. Such a substrate can be for example NFAT, NF- κ B, Transducer Of Regulated CREB (TORC), ELK1 or MEF2. For example, for NFAT, the two forms of NFAT (phosphorylated and un-phosphorylated) show a different migration. Accordingly, the NFAT can be analyzed by western blot as
20 detailed in the example. For instance, a total cellular extract can be prepared for cells of the sample, resolved by a SDS-PAGE electrophoresis and submitted to immunoblot analysis with NFAT antibodies for changes in mobility shifts directly associated with phosphorylation levels. Alternatively, the calcineurin substrate can be immuno-precipitated, resolved by a SDS-PAGE gel and submitted to immunoblot analysis with
25 an antibody specific for said substrate. In a third embodiment, calcineurin activity can be determined by immunocytochemistry with a substrate having a different sub-cellular localization depending on its calcineurin-dependent phosphorylation state. For example, a dephosphorylated NFAT will be observed in the nucleus whereas a phosphorylated NFAT will be observed in the cytoplasm. In a preferred embodiment, the
30 phosphorylation state of the calcineurin substrate, in particular NFAT, is assayed directly on the removed sample, without any culture step. Alternatively, the phosphorylation state of the calcineurin substrate, in particular NFAT, is on a short culture of the sample, preferably less than one hour.

In order to determine whether calcineurin has dephosphorylated substrate, (e.g., NFAT), a radioactively labelled phosphate group may also be used, e. g. in the form of ^{32}P -orthophosphate. This will provide a direct signal on the substrate (e.g., NFAT) which may be determined by counting incorporated radiolabel or other means, such as
5 immuno- precipitating substrate (e.g., NFAT), separating substrate (e.g., NFAT) on a gel and subjecting the gel to autoradiography to determine the signal from substrate (e.g., NFAT).

In an other aspect, the methods can use a conformational antibody which distinguishes between phosphorylated substrate (e.g., NFAT) and un-phosphorylated
10 substrate (e.g., NFAT). Such antibodies, which may be polyclonal, monoclonal or binding fragments of complete antibody molecules (e. g. single chain Fv fragments) may also be used in determining the extent to which the residue has been phosphorylated. Kits comprising such antibodies form another aspect of the invention. When available, antibodies specific of the phosphorylated calcineurin substrate will be
15 preferred in the western blot.

EXAMPLES

The following example illustrates the invention.

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EXAMPLE 1

The Inventors have characterized a fusion between TEL and the 3' part of the gene encoding the JAK2 protein kinase in a case of childhood T cell ALL carrying a t(9;12) chromosomal translocation. The resulting chimeric gene encodes a TEL-JAK2
25 fusion protein in which the 336 amino-terminal residues of TEL are fused to the catalytic domain of JAK2, resulting in the constitutive activation of TEL-JAK2 tyrosine kinase activity. TEL-JAK2 is a strong oncogene in vivo since its targeted expression in the lymphoid lineage of transgenic mice results in a highly invasive lymphoma/leukemia (14).

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The inventors have now found that the calcineurin/NFAT pathway is activated in TgTEL-JAK2 leukemic cells. Further analyses have shown that (i) the calcineurin/NFAT pathway is activated in a number of mouse models of lymphoma/leukemia induced by other human oncogenic proteins including activated

Notch, overexpressed Myc and in a xenograft model of EBV-associated Hodgkin-like B cell lymphoma; (ii) that activation of the calcineurin/NFAT pathway is observed when tumour cells are maintained in vivo but is generally lost in vitro, suggesting that it is not under sole control of the primary oncogene activated in these haematopoietic malignancies; (iii) using the well-characterized model of TEL-JAK2-induced T cell leukemia/lymphoma, that in vivo inhibition of calcineurin by treatment of mice with CsA or FK506 result in the complete inactivation of NFAT and inhibition of tumor cell expansion and invasion.

10 MATERIALS AND METHODS

Mouse models:

The following mouse models of human lymphoma/leukemia were used in the present study. The generation of transgenic mice that constitutively express TEL-JAK2 under control of the lymphoid lineage specific E μ SR α promoter have been previously described by the inventors' group (18). TEL-JAK2 transgenic mice develop a fatal T-cell acute lymphoblastic leukemia (T-ALL) and T cell lymphoma at 2 to 22 weeks of age with specific amplification of the SP CD8 and DP CD4/CD8 lymphoid T-cells. Leukemogenic potential of activated Notch1 was formally proven in studies published by Pear and colleagues using a bone marrow reconstitution assay with cells containing a retrovirally-transduced, activated-form of Notch1, ICN1 (15). 100% of the animals reconstituted with cells expressing ICN1 allele developed DP CD4/CD8 T-ALL/lymphoma by 3 to 8 weeks post transplantation. Retroviral-mediated transfer of ICN1 in mouse bone marrow HSCs followed by adoptive transfer in irradiated recipient mice was as described. T cell leukemia/lymphoma developed in spleen and lymph nodes of recipient mice within 1 month as originally described. The transgenic line in which a tamoxifen inducible Myc fusion protein (c-Myc-ER) is expressed in the T cell lineage under control of the CD2 promoter has been previously described. In the present study, the inventors have used a cell line derived from a c-Myc-ER-induced thymic lymphoma in p53 $\pm$ mice, ERP15-14 (kindly provided by Dr. J. Neil). These cells were maintained either in tissue culture, or transplanted in nu/nu mice where they formed tumors at the site of injection. The tumor cells obtained from a human EBV-associated non Hodgkin B cell lymphoma have been propagated in SCID mice and were kindly provided by Dr. D. Decaudin (Institut Curie, Paris).

Cell culture

Leukemia-derived primary cells and leukemia cell lines (TEL-JAK2, ICN1 and ERP15-14) were maintained in RPMI 1640 supplemented with 10% foetal calf serum, 100U/ml penicillin, 100µg/ml streptomycin, 2mM glutamine (all from Life Technologies) and $5 \cdot 10^{-5}$ 2-βmercaptoethanol (Sigma). The ERP15-14 cell line was maintained in the absence of 4-hydroxy-tamoxifen (4OHT, from Sigma), suggesting that the Myc-ER transgene shows basal expression activity in the absence of exogenous stimulation with 4-OHT. Cyclosporin A (CsA) and ionomycin (Iono) used for in vitro experiments have been purchased from Sigma and used at 1µg/ml for the period of time indicated (CsA cat. C1832; Iono cat. I0634).

In vivo drugs administration

Cyclosporin A (Neoral100mg/ml, Novartis) or FK506 (PROGRAF, for intravenous injection 5mg/ml, Fujisawa Laboratories) have been diluted in 10% Cremophor (BASF). C57BL6 mice at 8 weeks of age were injected via the caudal vein with $5 \cdot 10^6$ TEL-JAK2 leukemic cells. One week after injection, one mouse was sacrificed to ascertain that leukemic cells had invaded the spleen of recipients mice. At that time 3 groups of mice were randomly selected and implanted with osmotic pumps (ALZET) containing either CsA (0.6mg/mouse/day), Prograf (0.06 mg/mouse/day) or left untreated. The osmotic pump system have been used to insure continous delivery of the drugs and to avoid the toxic effects observed with acute delivery via daily i.p injections. Finally, 5 or 10 days post-treatment, mice were sacrificed and subjected to analysis.

Analysis of NFAT activation in leukemic cells by EMSA.

A [^{32} P]dCTP end-labeled probe corresponding to the mouse IL2 -45 promoter region (+ strand : 5'-cgagaatgctGGAAAaataatatgggggtg-3' (SEQ ID No 1) was used to evaluate NFAT DNA-binding activity by Electrophoresis Mobility Shift Assay (EMSA), as described previously, using 2 µg proteins from nuclear extracts prepared from TEL-JAK2 leukemic T cells obtained from invaded thymuses and, as control, thymocytes from non transgenic littermates.

Analysis of calcineurin/NFAT activation by western blot.

Total cellular extracts prepared from cells obtained directly from diseased animals or control littermates or harvested at the indicated times following culture, were resolved by SDS-PAGE and subjected to immunoblot analysis with the indicated

antibody (Ab). The NFAT1(sc-7296), NFAT2 (sc-7294), NFAT4 (sc-8321), STAT5 (C-17; sc-835) antibodies were purchased from Santa Cruz Biotechnology. The phosphotyrosine- STAT5 antibody (05-495) was purchased from Upstate Biotechnology. The pan-NFAT Ab (796) was kindly provided by Dr. Nancy Rice.

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RESULTS and DISCUSSION.

The status of NFAT protein expression and calcineurin activation in TEL-JAK2 leukemic cells was analyzed by western blot, using antibodies specific for NFAT1, NFAT2 and NFAT4 and compared to control thymocytes. The state of NFAT activation can be easily assessed by SDS/PAGE since the fully dephosphorylated form (activated form) of the respective NFATs migrate faster in these conditions than the phosphorylated NFAT isoforms, the fully phosphorylated form displaying the slowest migration. The results of Figure 1 show that TEL-JAK2 leukemic cells obtained from an invaded thymus of a diseased TgTEL-JAK2 mouse express higher levels of NFAT1 as compared to normal thymocytes obtained from a non transgenic littermate control (Fig.1A). Furthermore, NFAT1 was essentially stoichiometrically present in its fully dephosphorylated (activated) state in leukemic cells as shown by its rapid electrophoretic migration. For comparison, Fig. 2B displays the relative migration of the fully dephosphorylated and fully phosphorylated NFAT1 isoforms, obtained from TEL-JAK2 leukemic cells maintained in culture for 1 hours in the presence of CsA to inhibit calcineurin to basal levels of activity, or in the presence of ionomycin to optimally activate calcineurin. As can be observed from this analysis, the NFAT1 isoform observed in TEL-JAK2 leukemic cells migrates at the same position as the fully activated NFAT induced in ionomycin-treated cells (Fig 1B, compare lanes 2 to 4). The use of antibodies specific for NFAT2 and NFAT4 similarly demonstrated the activation of these NFAT proteins in TEL-JAK2 leukemic cells as compared to normal thymocytes control (data not shown). However, unlike NFAT1, the expression levels of NFAT2 and NFAT4 were found to be similar in TEL-JAK2 leukemic cells as compared to control thymocytes (data not shown). To independently demonstrate the activation of the calcineurin/NFAT pathway in TEL-JAK2 leukemic cells, the inventors compared the NFAT DNA binding activity by electrophoretic mobility shift assay (EMSA) in nuclear extracts obtained from TEL-JAK2 leukemic cells and normal thymocyte, as control. The probe used in these experiments was a high affinity [³²P]-labelled DNA

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oligonucleotide corresponding to the - 45 NFAT binding site of the mouse IL2 promoter. As shown in Fig.2A, almost no retarded complex could be detected in thymocyte nuclear extracts, reflecting the low, steady-state levels of NFAT activation in developing thymocytes. In contrast, TEL-JAK2 nuclear extracts displayed a high level of DNA binding activity to the NFAT probe (Fig.2A, compare lanes 2 and 3). This difference did not result from a difference in nuclear protein concentration between leukemic and control cells, since the same level of DNA binding activity to an Sp1-specific probe was observed in both types of extracts (Fig.2A, bottom panel). The NFAT/probe complex was specific as its formation was inhibited by the addition to the reaction mixture of a 100 fold molar excess of unlabeled NFAT oligonucleotide used as competitor, but was unaffected in the presence of the same molar excess of a mutant NFAT oligonucleotide carrying a mutation in the NFAT binding site core sequence (data not shown). The NFAT/probe complex was quantitatively super-shifted by the addition to the reaction mixture of an antibody specific to an epitope common to NFAT1-4 (pan-NFAT antibody), but not by a control antibody (Fig.2B, compare lanes 2, 5 and 6). In line with the fact that NFAT1 is overexpressed in TEL-JAK2 leukemic cells, most -but not all- of the NFAT/probe complex was supershifted by addition of an excess of NFAT1-specific antibody (Fig.2B, compare lanes 2 and 3). In contrast, the antibody directed against NFAT4 only slightly affect the complex, suggesting that NFAT4 is contributing to NFAT DNA binding activity observed in TJ2 leukemic cells but to a lesser extend as compared to NFAT1. Similar observations were made in several pairwise comparison between control thymocytes and independent TEL-JAK2 leukemias arising in different TgTEL-JAK2 mouse individuals (data not shown). The inventors conclude from these experiments that TEL-JAK2 leukemic cells both upregulate the expression of NFAT1 and display the constitutive dephosphorylation, nuclear accumulation and DNA binding activation of NFAT1, NFAT2 and NFAT4.

In order to investigate whether constitutive NFAT activation was specific to TEL-JAK2 leukemia or whether it is a more general property of leukemic cells, the inventors analyzed NFAT protein expression and activation in other mouse models of human leukemia. Mutation of Notch1 by either point mutation or as the result of the t(7;9)(q34;q34.3) chromosomal translocation is observed in a majority of human T cell leukemia. Previous studies have shown that retroviral-mediated transduction of mouse bone marrow cells with an activated Notch mutant (Intracellular Notch1= ICN1)

followed by adoptive transfer of transduced cells in irradiated syngeneic hosts resulted in a T cell lymphoma/leukemia that faithfully reproduced the human disease (15). ICN1-induced leukemia were generated using this protocol and analyzed for NFAT activation as described above. As shown in Fig. 3A, ICN1-induced leukemic cells expressed the dephosphorylated (activated) isoforms of NFAT1 (Fig3A, upper panel, compare lanes 1 to 7), NFAT2 (Fig.3A, bottom panel, compare lanes 1 to 7) and NFAT4 (data not shown). Cyclosporin A treatment of ICN1 leukemic cells lead to the appearance of hyperphosphorylated (inactivated) isoforms of NFAT2 at the expense of the non phosphorylated isoforms that are not observed in non-treated ICN1 leukemic cells (Fig.3A bottom panel, compare lanes 1-7 to lane 8 ; see Fig. 3B for a scheme). In contrast, ionomycin-treated ICN1 leukemic cells show an NFAT2 migration profile which is indistinguishable from that observed in ICN1 non-treated leukemic cells (Fig. 3A, bottom panel, compare lanes 1-7 to lane 9; see Fig. 3B for a scheme). These results show that NFAT proteins are in their fully activated state in ICN1 leukemic cells. Similar observations were made in transplanted T cell leukemia obtained following inoculation of CD2-Myc-induced T cell leukemia to nu/nu recipient mice as well as in a mouse xenograft model of a human Hodgkin-like B cell lymphoma (Fig.5B).

The fact that NFAT activation is observed in a large panel of lymphoid malignancies, induced by primary oncogenes acting in distinct signaling networks suggested to us that it was unlikely to result solely from the activity of the initiating oncogene. To investigate this in further detail, the inventors compared NFAT1 activation in extracts of leukemic cells obtained directly from diseased animals, or from the same cells maintained in culture in the absence of growth factors and serum (Fig. 4A and B, compare lanes 2 to 3). Under these conditions, TEL-JAK2 tyrosine kinase activity is not affected as shown by the maintenance of STAT5 in its tyrosine-phosphorylated state (Fig. 4B). In line with the results described above, NFAT1 was in its dephosphorylated (activated) state in TEL-JAK2 leukemic cells obtained directly from diseased animals. In contrast, maintenance of these cells in culture resulted in their stoichiometric re-phosphorylation (inactivation) by the endogenous, NFAT protein kinases (Fig. 4A compare lanes 2 and 3). As expected, re-phosphorylation of NFAT1 lead to a decrease in DNA binding activity in leukemic cells to the levels normally observed in normal thymocytes, as analyzed by EMSA (data not shown). Similar to the results described above for TEL-JAK2 leukemic cells (Fig. 4A), ICN1-induced

leukemias (Fig. 5A) and human EBV-associated non Hodgkin B cell lymphoma (Fig. 5B) displayed the activated isoform of NFAT1 when leukemic cells were obtained directly from diseased animals and NFAT activation was lost when cells were maintained in culture for a period of time as short as one hour. These results indicate
5 that NFAT activation is not under the sole control of the initiating oncogene of these leukemia and appears to require the presence of a proper in vivo tumor microenvironment.

To investigate whether activation of calcineurin/NFAT pathway is important for tumor maintenance in vivo and to test whether the well characterized calcineurin
10 inhibitors currently used in human medicine could be of therapeutic value, the inventors analyzed the in vivo effects of both CsA and FK506 on TEL-JAK2 leukemia progression. Primary TEL-JAK2 leukemic cells were grafted by i.v. inoculation to syngeneic recipient mice. Under these conditions, leukemia corresponding to the expansion of the original leukemic clone efficiently transplanted in secondary hosts to
15 invade their spleen and lymph nodes and to induce their death within 20-30 days. Recipient mice were transplanted with TgTEL-JAK2 leukemic cells and maintained for one week to allow moderate leukemic cell expansion. After that period of time, three cohorts were generated. The first was left untreated, the second group was implanted with an osmotic pump delivering a continuous amount of CsA and the third implanted
20 with osmotic pumps delivering FK506 (see Materials and methods).

When compared to non-treated control mice, 10 days CsA and Prograf-treated mice exhibited a statistically reduced spleen weight (Fig. 6A and 6B; p value <0.05), suggesting that CsA treatment either induced apoptosis of leukemic cells and/or inhibited their proliferation. To analyze this in further details, bone marrow imprints
25 from untreated or CsA- or Prograf-treated leukemia were morphologically analyzed. Histopathological analysis of the liver parenchyme of these mice was also carried out. Figure 8 show that bone marrow from leukemic TgTEL-JAK2 mice was exclusively composed of an homogenous population of T lymphoblastic cells (Fig. 8B) while normal bone marrow is mainly composed of granulocytic cells. Interestingly, treatment
30 of mice with either CsA or Prograf resulted in the severe decrease in the number of leukemic blasts and in the recovery of a cell composition close from that of normal bone marrow (Fig. 8C and 8D). The process of tumor metastasis was also strongly inhibited by CsA or Prograf treatment. Indeed, whereas leukemic blasts efficiently invaded the

liver sinusoids and parenchyma of non-treated mice (Fig. 9, panels A and B), leukemic blasts were severely reduced in numbers in the livers from CsA- or Prograf-treated mice (Fig. 9C and 9D).

Biochemical analysis of leukemic cells isolated from the spleen of non-treated mice and from mice treated with either CsA or Prograf showed the loss of NFATs activation as shown by the appearance of slowly migrating, heavily phosphorylated (inactive) isoforms of NFAT1 and NFAT4 (Fig. 7A and 7B), demonstrating that NFAT activation in TEL-JAK2 leukemia is under control of calcineurin and that inhibition of this process by CsA or Prograf (FK506) is associated with the inhibition of tumor growth progression. Of note, constitutive activation of NFkB, another REL superfamily member activated in these leukemic cells (N. dos Santos and JG, unpublished obs.) was not affected by treatment with CsA or Prograf, demonstrating the specificity of these compounds for calcineurin (data not shown).

In conclusion, these results show (i) that calcineurin is activated in a variety of mouse models for T and B cell lymphoma/leukemia, a property which begins to be recognized in human lymphoid malignancies as well (inventors' unpublished observations; (13)(29); (ii) that activation of this pathway is observed in lymphoid malignancies initiated by a wide spectrum of initiating oncogenes and depends upon the presence of a specific in vivo environment; (iii) that activation of calcineurin is important for leukemia progression in vivo; (iv) that pharmacological inhibitors of calcineurin activity, namely cyclosporin A and Prograf (FK506) are of therapeutic benefit in mouse models of human leukemia. The inventors propose that targeting calcineurin for inhibition by treatment by CsA or/and FK506 (Prograf), two calcineurin inhibitors commonly used in transplantation medicine can be of therapeutic benefit in curative treatment of human lymphoid malignancies by affecting the leukemic cell itself and/or its microenvironment (stroma; angiogenesis). Since activation of this pathway is not necessarily under control of the primary (initiating) oncogenic, activation of the calcineurin/NFAT pathway may be a valuable marker of tumor evolution (stage) and/or lymphoma/leukemia classification significant to prognosis and/or diagnosis. Finally the animal models and approaches used here paves the way to identify and study novel inhibitory compounds of calcineurin and/or NFATs in hematopoietic malignancies.

EXAMPLE 2

Materials and methods

Mice

The transgenic mouse model for TEL-JAK2-induced T-cell leukemia/lymphoma has been described previously¹⁴. To generate E μ SR α -TEL-JAK2/CD3 ϵ ^{-/-} and E μ SR α -TERL-JAK2/Rag^{-/-}, TEL-JAK2 mice were bred with the CD3 ϵ ²¹ and Rag2²² knock-out mice according to standard procedures. All mice used were in a C57BL6 genetic background (Charles River Laboratories, L'Arbresle, France). T-cell acute lymphoblastic leukemia induced by constitutively activated NOTCH 1 were generated as previously described¹⁵. Wild-type bone marrow cells obtained from 5-FluoroUracil-treated C57B6 mice (150 mg/kg) were grown for two days in serum-free medium in the presence of 10ng/ml IL6, 10ng/ml Flt3L, 10ng/ml IL3, and 100ng/ml SCF (Stem Cell Technologies, Vancouver, BC) and then spin-infected with a retrovirus encoding the entire *Notch1* intracellular domain (ICN1; amino acids 1760–2555) using the pMig-ICN1 construct kindly provided by Dr Warren Pear¹⁵. Transduced cells were intravenously injected to reconstitute lethally irradiated (8,125 Gy) C57BL6 recipient mice. The animals were maintained under specific pathogen-free conditions in the animal facilities of Institut Curie (Orsay, France). Live animal experiments were carried out in accordance with the guidelines of the French Veterinary Department.

Immunoprecipitation and Western blot analysis

Whole-cell extracts were processed for Western blots as described previously¹⁴ using antibodies to: NFATc1 (SC-7194; Santa Cruz), NFATc2 (SC-7296; Santa Cruz), NFATc3 (SC-8321; Santa Cruz), ERK2 (C-14; Santa Cruz), STAT5 A+B (SC-835; Santa Cruz), CalcineurinA (AB1695; Chemicon), the HA epitope tag monoclonal antibody (AB16918; Abcam), tyrosine phosphorylated-STAT5 (05-495; Upstate). Immunoprecipitation assays were carried out using the anti-HA tag antibody as previously described¹⁴.

Retroviral-mediated gene transfer

The cDNA encoding the constitutively activated HA tagged-calcineurin A α mutant²³ was kindly provided by Dr Neil Clipstone in the pBJ5 vector and was subcloned in the MSV-Puro vector (Clontech). Retroviral virus stocks were obtained following transfection of the PlatE packaging cell line²⁴ using the calcium phosphate coprecipitation method. After over-night incubation, medium (DMEM + 10% fetal calf serum) was changed and viral stocks were collected between 24H later and titrated on

NIH3T3 cells and normalized to 10^6 infectious units/ml. To transduce leukemic cells, spin-infections were performed at 3000 rpm for 2h at 30°C with retroviral supernatants complemented by 4 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Viral supernatant corresponding to the MSCV-Puro empty vector and the MSCV-Puro-CnA* were used
 5 at the same multiplicity of infection (MOI) to infect leukemic cells.

Treatment of leukemic mice with CsA and Prograf

The cells from invaded spleen of either ICN1 or TJ2 induced primary leukemia were collected by gentle disruption of the organ in serum-free RPMI medium (Invitrogen) and injected intravenously in the tail vein of 6-10 weeks old C57BL6 mice
 10 (Charles River Laboratories, L'Arbresle, France). When spleen weight reached 200mg, mice were randomized and subjected to treatment with either vehicle alone (PBS plus 10% Cremophor EL[®]), CsA (Néoral[®], Novartis, Rueil-Malmaison, France) at a dose of 30mg/kg/day or Prograf (Prograf[®], Astellas, Ireland) at a dose of 3mg/kg/day. CsA was diluted in PBS plus 10% Cremophor EL[®] (Sigma-Aldrich Chemie, Steinheim,
 15 Germany). Alzet[®] osmotic pumps were loaded and then primed at 37°C in PBS 0,9% NaCl 24h prior to their subcutaneous implantation (ALZET compagny, Cupertino, CA, USA), following the manufacturer instructions. Statistical analysis, survival curves and organ weights were calculated using Prism 4 (GraphPad, San Diego, CA, USA).

Assesement of apoptosis and proliferation *in vivo*

20 Single cell suspensions were prepared from invaded livers and stained with fluorochrome-labeled antibodies, as previously described¹⁴. AnnexinV staining was performed using the AnnexinV-PE Apoptosis detection kit following the manufacturer instructions (Abcam, Cambridge, UK). BrdU staining was performed using the FITC or APC BrdU flow kit following the manufacturer instructions (BD Biosciences, France).
 25 Briefly, two hours before sacrifice, mice were intraperitonealy injected with 2mg/mouse of BrdU and cells were stained with fluorochrome-labeled anti-BrdU antibodies and analyzed using a FACSCalibur cytometer (BD Biosciences, France). In order to evaluate the proportion of cycling or apoptotic leukemic cells specifically, BrdU and AnnexinV analyses were performed on the GFP-positive cells for the ICN1 model, and
 30 on the THY1.2-positive cells for the TEL-JAK2 model (anti-Thy1.2-FITC; BD Pharmingen, San Diego, CA, USA). The data were analyzed using the CellQuest (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) softwares. Cell apoptosis was confirmed by *in situ* detection of fragmented DNA, using Terminal dUTP Nick-End

Labeling (TUNEL) assays²⁵, on deparaffinized 5- μ m-thick sections, treated with proteinase K (20 μ g/mL) for 15 minutes at room temperature.

Pathology and electron microscopy

Morphology and differentiation of the ICN1 and TJ2 mice bone marrow were evaluated on May-Grünwald Giemsa-stained cytopins. Histochemical analyses were performed on paraffin-embedded tissue sections (5 μ m thick) of organs invaded by leukemic cells. Sequential sections were obtained on a microtome with water flow (HM 350 Niagara, Microm, Francheville, France). The subsequent sister sections were used for H&E staining and TUNEL assay analysis. For electron microscopy analysis, samples fixed in 2% glutaraldehyde-buffered 0.1M. cacodylate were embedded in epoxyresin. Semi-thin sections were stained with 2% toluidine blue, and ultra-thin sections with uranylacetate lead as previously described²⁶.

RESULTS and DISCUSSION.

When the calcineurin phosphatase is inactive, the NFAT (Nuclear Factor of Activated T cells) transcription factors are hyperphosphorylated and located in the cytoplasm. T cell activation results in the calcium- and calmodulin-dependent activation of calcineurin, which induces the dephosphorylation of NFATs and a conformational switch that allows their translocation to the nucleus where they play a critical role in many aspects of T cell function. The ratio between the fully phosphorylated (slow migrating in SDS/PAGE) and fully dephosphorylated (fast migrating) forms of NFATs thus provides a convenient index to assess calcineurin activity. Thus, in unstimulated thymocytes maintained *ex vivo*, NFATc1 (NFAT2), NFATc2 (NFAT1) and NFATc3 (NFAT4) are hyperphosphorylated, insensitive to exposure to the calcineurin inhibitor cyclosporine A (CsA), but become fully dephosphorylated upon stimulation by ionomycin (Fig. 10a, lanes 2-4 and data not shown). *In vivo*, thymocytes displayed a combination of phosphorylated and non-phosphorylated NFATc1 and NFATc2 (Fig. 10a, lane 1), likely reflecting the activation of calcineurin in cells asynchronously responding to several developmental cues. Strikingly, independent primary T-cell tumors induced by activated intracellular NOTCH1 (ICN1)¹⁵ or the TEL-JAK2 fusion protein¹⁴ displayed fully dephosphorylated NFATc1 and NFATc2 (Fig. 10a, lanes 5 and 8). *Ex vivo* CsA- or ionomycin-treated tumor cells were used as controls for NFAT phosphorylation status (Fig. 10a, lanes 6, 7, 9, 10). These observations imply that

calcineurin is activated in a sustained fashion in these T-cell malignancies. Calcineurin activation did not result from the hypersensitivity of leukemic cells to pre-T-cell receptor (TCR)- or TCR-derived signals, two well characterized receptors coupled to the calcium-dependent activation of the calcineurin/NFAT pathway, as fully dephosphorylated NFAT was also observed in T-cell lymphoma/leukemia obtained from TEL-JAK2/CD3 ϵ ^{-/-} and TEL-JAK2/Rag^{-/-} compound mice in which these receptors are either non-functional or absent²¹ (Fig. 10b). Importantly, sustained calcineurin activity was also observed in mouse models of T-cell lymphoma/leukemia induced by the loss-of-function of Ikaros²² or the overexpression of c-Myc²⁷ and in a xenograft model of human EBV-associated non Hodgkin B cell lymphoma²⁸ (Fig. 10e and data not shown). Interestingly, calcineurin activation in leukemic cells required specific signal(s) from the tumor micro-environment, as it was rapidly and constantly lost when cells were maintained in culture (Fig. 10c), precluding any *ex vivo* study of the significance of calcineurin activation in this setting. Of note, TEL-JAK2 remained active under these *ex vivo* conditions, as shown by the maintenance of the constitutive activation of STAT5 in these leukemic cells (Fig. 10d). This indicates that the mere activation of the initiating oncogene is not sufficient for the sustained calcineurin activation in these tumor cells.

To investigate whether calcineurin activation participates in T-cell leukemogenesis, ICN1 or TEL-JAK2 leukemic mice were treated with CsA or Prograf. The inhibitory activity of these structurally unrelated compounds is mechanistically distinct as it depends upon their binding to different immunophilins. Primary ICN1 and TEL-JAK2 tumor cells were transplanted into syngeneic mice, resulting in the synchronous engraftment of these oligo/monoclonal diseases to recipient mice. In accordance with the pathological features of the original ICN1 and TEL-JAK2 mouse leukemia models^{14,15}, the transplanted leukemias effaced the normal bone marrow (BM) architecture to replace it with an homogeneous population of monomorphous lymphoblasts (Fig. 11a and Fig. 11e) and invaded the peripheral lymphoid organs (Fig. 11b), as well as several non-hematological organs such as the liver (Fig. 11c and data not shown). Mice at an early stage of leukemia progression were treated with either 30mg/kg/day CsA, or 3mg/kg/day Prograf, or solvent vehicle as control and compared for further disease evolution. Strikingly, CsA or Prograf treatments restored normal hematopoiesis in both leukemia models, with a severe reduction of leukemic blasts in

the bone marrow, associated with the re-appearance of mature granulocytes and megakaryocytes (Fig. 11a and Fig. 11e). In addition, these inhibitors induced a dramatic reduction in splenic tumor load (Fig. 11b) and a near complete suppression of tumor cells from the hepatic perivascular spaces and sinusoids (Fig. 11c). These anti-leukemic effects were associated with the inhibition of calcineurin activation in the regressing tumors, as evidenced by the nearly complete NFAT rephosphorylation under these conditions (Fig. 12a and data not shown), formally demonstrating that constitutive NFAT dephosphorylation in leukemic cells *in vivo* is indeed the consequence of calcineurin activation and implying that calcineurin enzymatic activity plays an essential role in T-cell leukemogenesis. Therapeutic treatment with CsA or Prograf induced tumor cell death, as shown by the appearance of cells with the structural (Fig. 12b and data not shown) and ultrastructural (Fig. 12c) features of apoptotic cells in the regressing tumors. The inventors also used TUNEL and Annexin V staining to show a striking increase in the number of apoptotic cells in CsA- and Prograf-treated tumors (Fig. 12d and 12e). Besides this effect on cell death, *in vivo* calcineurin inhibition in ICN1 and TEL-JAK2 leukemic cells also impinged on cell cycle progression as evidenced by the significant decrease in the proportion of BrdU-positive proliferating tumor cells (Fig. 12e and f). Importantly, these cellular responses and the strong effect on tumor growth induced by Prograf treatment were associated with a statistically significant prolongation of survival of Prograf-treated tumor-bearing mice (Fig. 11d and data not shown).

Since calcineurin activation in ICN1- and TEL-JAK2-induced leukemias depends upon exogenous signals specific to the *in vivo* tumor micro-environment (Fig. 10c), the inventors sought to bypass this requirement and studied whether expression of a constitutively activated mutant of calcineurin in leukemic cells would favor disease progression. Deletion of the carboxy-terminal autoinhibitory domain of the catalytic subunit of calcineurin (PP3CA, referred to as CnA) results in its constitutive, calcium-independent activation²³. ICN1 and TEL-JAK2 leukemic cells were transduced with a retrovirus encoding the constitutively activated mutant of calcineurin (CnA*) or the MSCV control retrovirus (Fig. 13a) and intravenously injected into syngeneic mice immediately after transduction. The kidney, liver and spleen weight of mice injected with CnA*-transduced ICN1 or TEL-JAK2 leukemic cells was significantly increased as compared to mice engrafted with mock-transduced cells (Fig. 13b, c and e).

Moreover, histopathological analysis of sternum and kidney sections clearly showed that the CnA*-transduced leukemia exhibited a significantly more invasive phenotype as compared to mock-transduced cells (Fig 13c and d).

The ICN1 and TEL-JAK2 mouse models used in this study are highly relevant to human malignancies, as activating NOTCH1 mutations are observed in over 50% of T-ALL patients and constitutive activation of the JAK/STAT signaling pathway is frequently observed in ALL. Using these mouse models, the inventors identified calcineurin activation as a key signaling pathway in T cell lymphoma-/leukemogenesis and showed that calcineurin targeting by specific inhibitors is of therapeutic value in the treatment of these malignancies. The molecular mechanisms that account for the sustained activation of calcineurin in these leukemic cells remain to be identified, but appear to require signal(s) from the tumor micro-environment and to be, at least in the TEL-JAK2 mouse model, independent of TCR and pre-TCR expression. NFAT transcription factors are critical mediators of calcineurin activation in T cells where they play either redundant, specific or even antagonistic role. Therefore, they are possible candidates as downstream effectors of calcineurin in leukemic cells. However, other calcineurin targets may also contribute to the proliferative and anti-apoptotic functions of this phosphatase. In different animal and cellular models, NFAT factors have been proposed to contribute in a positive or negative fashion to oncogenesis. More recently, CsA-sensitive nuclear accumulation of NFATc1 was described in a subset of human aggressive B-cell lymphoma and in pancreatic carcinoma^{13,29,30}. These observations raise hopes that calcineurin inhibitors may also have therapeutic benefit in non-hematopoietic malignancies.

The pre-clinical data reported here warrant a comprehensive analysis of the activation of calcineurin in human lymphoid malignancies. As many T-cell leukemias/lymphomas are only partially sensitive to existing therapies, treatment of haematopoietic tumor with available calcineurin inhibitors alone or associated to current chemotherapies in a combined or a sequential regimen is of high interest, at least in those molecular subtypes where calcineurin activation can be demonstrated.

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reference.

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CLAIMS

1- Use of a calcineurin inhibitor for the preparation of a medicament for treating
5 a haematopoietic tumor having a sustained calcineurin activity.

2- Use according to claim 1, wherein said calcineurin inhibitor is used in
combination with a cancer therapy.

10 3- Use according to claim 2, wherein said cancer therapy is selected from the
group consisting of a cancer chemotherapy, an immunotherapy, a radiotherapy, a
hormone or cytokine therapy, any other therapeutic method used for the treatment of a
haematopoietic tumor and a combination thereof, preferably a cancer chemotherapy.

15 4- Use according to any one of claims 1-3, wherein said calcineurin inhibitor is
selected from the group consisting of cyclosporin A, FK506, FK520, L685,818,
L732,731, ISATX247, FK523 and 15-0-DeMe-FK-520.

20 5- Use according to claim 4, wherein said calcineurin inhibitor is FK506.

6- Use according any one of claims 1 to 5, wherein said hematopoietic tumor is a
lymphoma and/or a leukemia.

25 7- Use according any one of claims 1 to 6, wherein the subject to be treated
presents dephosphorylated substrate of calcineurin, preferably NFAT, in cells of the
haematopoietic tumor isolated from said subject.

30 8- Use according any one of claims 1 to 7, wherein said calcineurin inhibitor is
administered to the subject during a period of 2 to 7 weeks, preferably 4 to 6 weeks.

9- Use according any one of claims 1 to 8, wherein the medicament is used for a
remission treatment.

10- A method for selecting a subject having a haematopoietic tumor to be treated by a calcineurin inhibitor comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject, and selecting the subject having tumoral cells with a sustained calcineurin activity.

5

11- A method of assessing the responsiveness of a subject having a haematopoietic tumor to a treatment with a calcineurin inhibitor, comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject, a sustained calcineurin activity of said cells being indicative of a positive
10 responsiveness to said treatment.

12- Method according to claim 10 or 11, wherein said calcineurin inhibitor is selected from the group consisting of cyclosporin A, FK506, FK520, L685,818, L732,731, ISATX247, FK523 and 15-0-DeMe-FK-520.

15

13- Method according to claim 12, wherein said calcineurin inhibitor is FK506.

14- Method according to any one of claims 10-13, wherein said hematopoietic tumor is a lymphoma and/or a leukemia.

20

15- Method according to any one of claims 10-14, wherein calcineurin activity is determined by assessing the phosphorylation of a substrate of calcineurin, preferably NFAT, a dephosphorylated substrate being indicative of a sustained calcineurin activity.

25

16- A method for staging or characterizing a hematopoietic tumor in a subject comprising determining calcineurin activity in cells of the haematopoietic tumor isolated from said subject.

17- Method according to claim 16, wherein a sustained calcineurin activity is
30 indicative of an invasive capacity, a metastatic potential, and/or a relapse probability.

18- Method according to claim 16 or 17, wherein calcineurin activity is determined by assessing the phosphorylation of a substrate of calcineurin, preferably

NFAT, a dephosphorylated substrate being indicative of a sustained calcineurin activity.

19- A pharmaceutical composition comprising FK506 and an anticancer drug.

- 5 20- Product containing FK506 and an anticancer drug as a combined preparation for simultaneous, separate or sequential use in the treatment of a hematopoietic tumor having a sustained calcineurin activity.

Application number / numéro de demande: EP06/68576

Figures: 1a^b, 2a^b, 3a^b, 4a^b, 5a^b, 6a^b, 7a^b-10abcd, 11a-ce, 12a-df, 13ac-e

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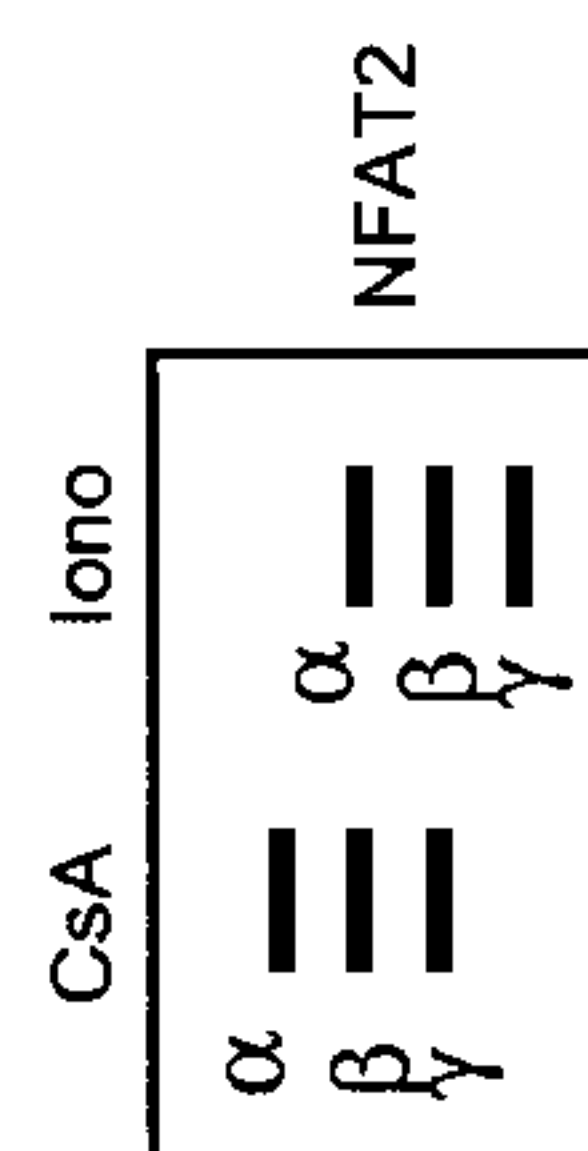
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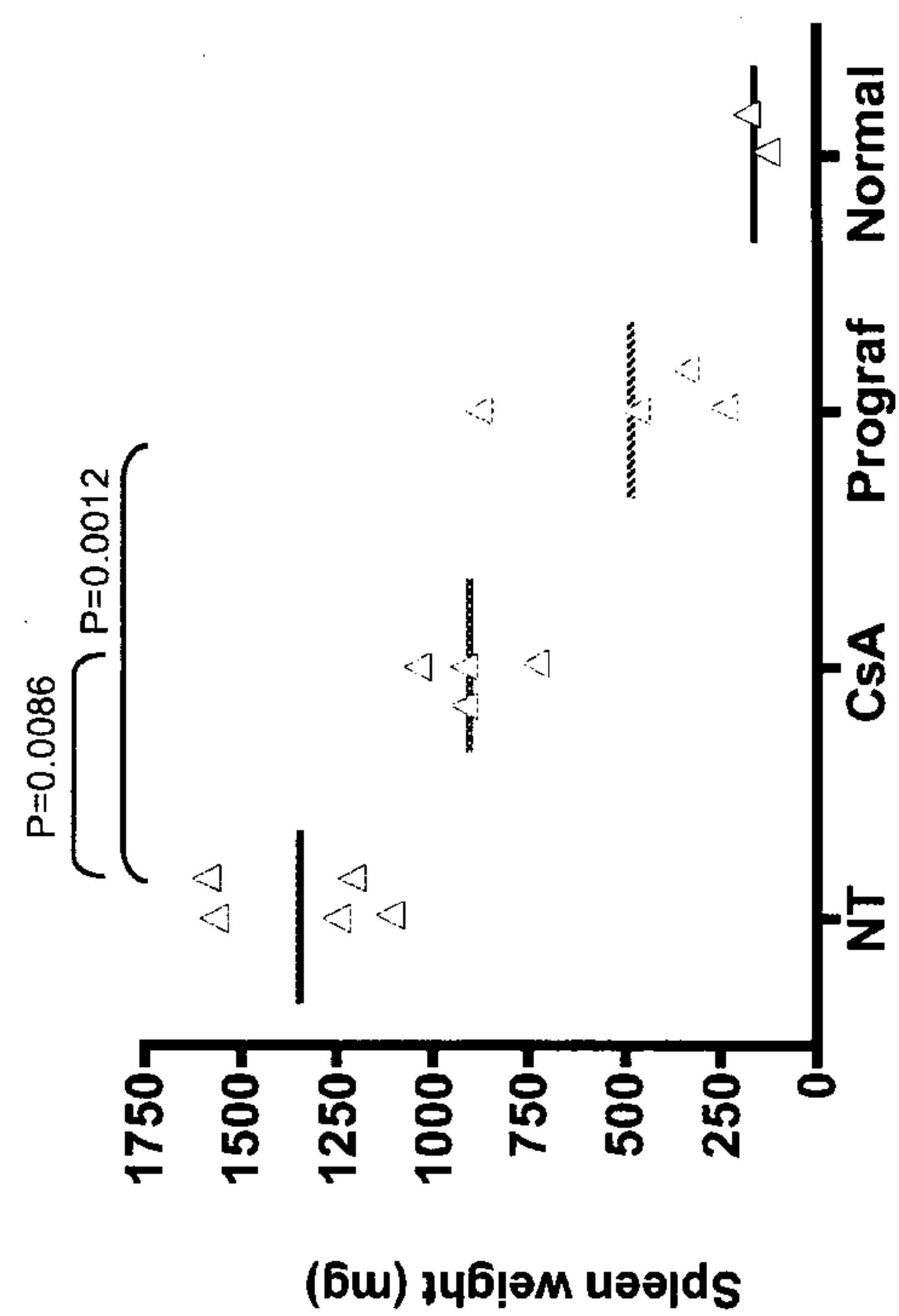
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Fig. 3B



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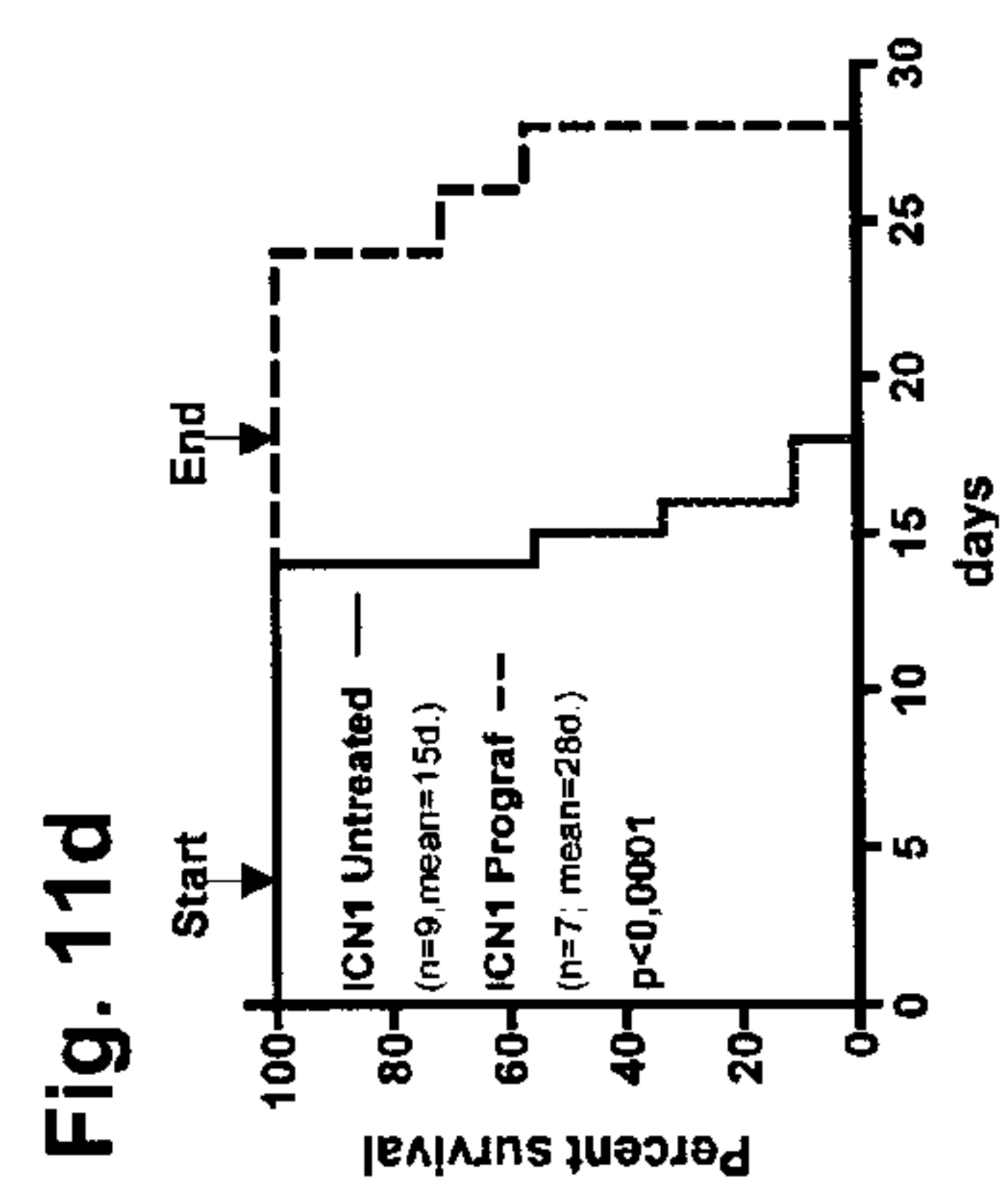
Fig. 6A



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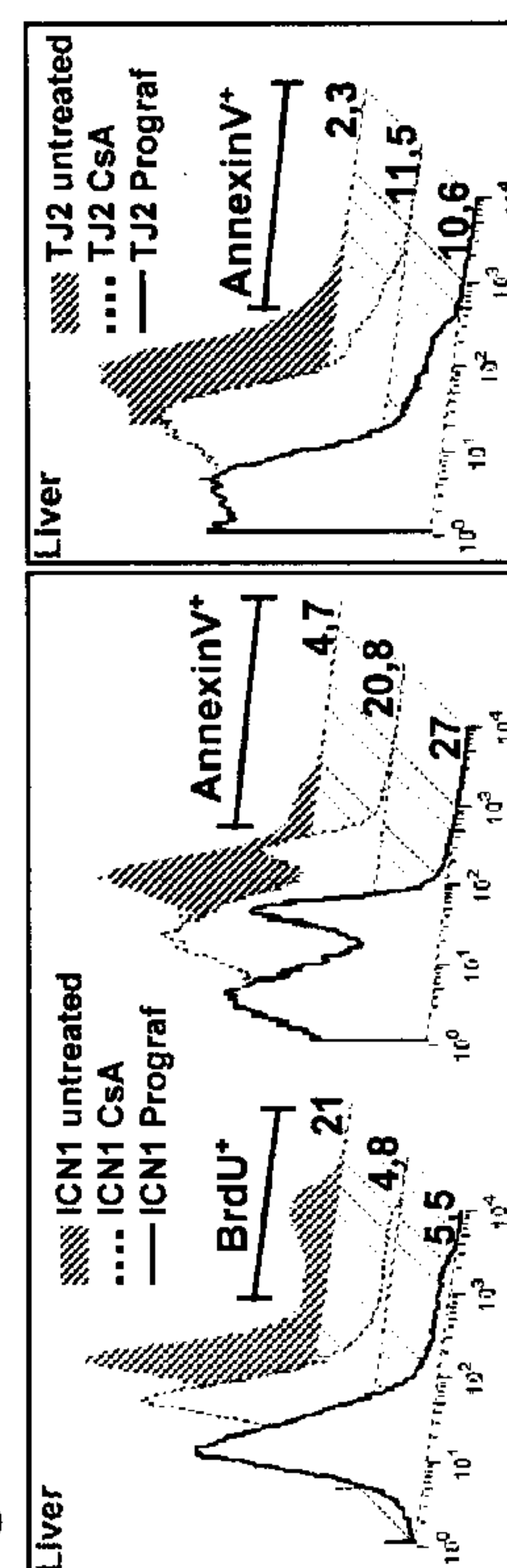
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Fig. 12e



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