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(54) **Title:** METHODS FOR TREATMENT OF B CELL RELATED CANCERS BY INHIBITION OF PKN1

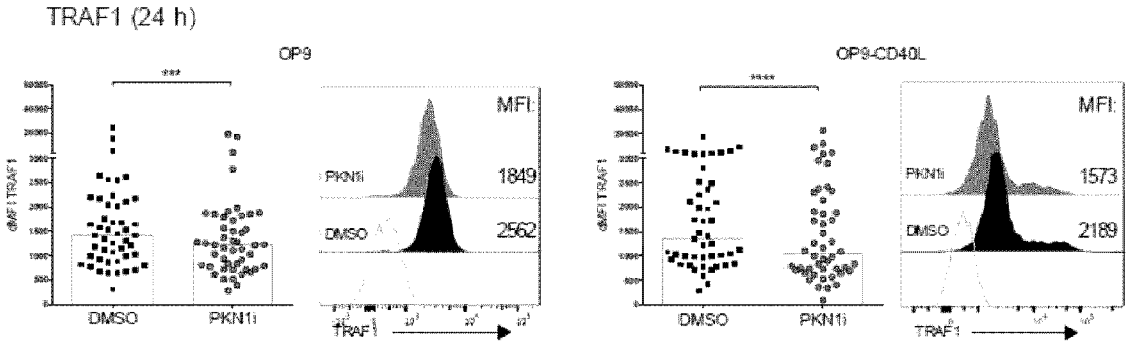

Figure 3a

(57) **Abstract:** The present application provides methods and compositions for treatment of B cell related cancers by inhibition of PKN1. The method for treating a B cell related cancer, such as Chronic Lymphocytic Leukemia (CLL) comprises administering a protein kinase C related kinase (PKN1) inhibitor or a pharmaceutically acceptable salt or solvate thereof. The method for treating the B cell related cancer can include administering the PKN1 inhibitor in combination with a Bcl-2 antagonist, such as venetoclax. Also provided is a method for determining whether a subject is susceptible to venetoclax resistance comprising measuring the level of TRAF1 expression in the subject's cancer cells.

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METHODS FOR TREATMENT OF B CELL RELATED CANCERS BY INHIBITION OF PKN1

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This present disclosure claims priority from U.S. Provisional Application Serial No. 62/855,275 filed May 31, 2019, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present disclosure pertains to the field of treatment of B cell-related cancers. More particularly, the present disclosure relates to inhibition of PKN1 in the treatment of TRAF1 expressing B-cell related cancers, and PKN1 inhibitors and uses thereof.

INTRODUCTION

[0003] Chronic lymphocytic Leukemia (CLL) is the most common human leukemia with 20,720 new cases and 3930 deaths expected in the US in 2019. CLL is considered to be largely a disease of the lymph node and bone marrow, where B-CLL cells receive survival signals, including tonic signals through their antigen specific B cell receptor (BCR)[1,2]. Several promising new therapies for CLL target BCR signaling, including the phosphatidyl inositol-3-kinase inhibitor Idelalisib, the Bruton's tyrosine kinase (BTK) inhibitor, Ibrutinib, as well as the Bcl-2 antagonist Venetoclax[3-8]. While these treatments are showing great promise, responses are not always durable and when relapse occurs there are limited treatment options available[3,9].

[0004] In addition to signaling through the BCR, B cells require signals through TNFR superfamily members such as CD40, to allow NF-κB mediated induction of prosurvival Bcl-2 family members, including Bcl-x_L, and Mcl-1[10,11]. Many human malignancies including B-CLL, B cell lineage NHL and Burkitt's lymphomas, exhibit constitutive signaling via TNFRs, such as CD40, CD30 or the EBV protein LMP1[12-14]. TNFR family members trigger NF-κB activation through recruitment of TRAF proteins[15]. TRAF1 is an NF-κB inducible protein whose expression is mainly limited to activated cells of the immune system. TRAF1 cannot induce NF-κB on its own, but forms a 1:2 heterotrimer with TRAF2[16]. The TRAF1/2_2

heterotrimer then recruits a single cellular inhibitor of apoptosis protein (cIAP)[16] to induce activation of the classical NF-κB signaling pathway downstream of a subset of TNFR family members, including CD40 on B cells[17]. TRAF1 is overexpressed in 48% of B cell related cancers with highest expression in the most refractory B-CLL[18]. Non-coding single nucleotide polymorphisms in *TRAF1* have also been linked to non-Hodgkin's lymphoma[19]. *TRAF1* expression is also required for lymphomagenesis in a spontaneous mouse tumor model induced by constitutively active NF-κB2[20], providing genetic evidence for the importance of TRAF1 in B cell malignancies. However, to date, the role of TRAF1 in human cancer remains unclear.

[0005] The above information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present disclosure. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present disclosure.

SUMMARY

[0006] The present disclosure provides methods and compositions for treatment of TRAF1 expressing B cell related cancers by inhibition of PKN1's activity on TRAF1 levels. In accordance with an aspect of the present disclosure, there is provided a method for treating a TRAF1 expressing B cell related cancer comprising administering a protein kinase C related kinase (PKN1) inhibitor to a subject in need thereof. Also provided is a use of a protein kinase C related kinase (PKN1) inhibitor for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Further provided is a use of a protein kinase C related kinase (PKN1) inhibitor in the manufacture of a medicament for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Even further provided is a protein kinase C related kinase (PKN1) inhibitor for use in treating a TRAF1 expressing B cell related cancer in a subject in need thereof. In an embodiment, the PKN1 inhibitor selectively inhibits PKN1 over protein kinase C θ (PKCθ).

[0007] In an embodiment, the PKN1 inhibitor is 4-(7H-purin-6-yl)-1-thia-4-azaspiro[5.5]undecane or a pharmaceutically acceptable salt or solvate thereof.

[0008] In another embodiment, the PKN1 inhibitor is 4-(4-(1H-pyrazol-4-yl)phenyl)-4-(4-chlorophenyl)piperidine (OICR 08727) or a pharmaceutically acceptable salt or solvate thereof.

[0009] In yet another embodiment, the PKN1 inhibitor is 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof. In an alternate embodiment, the PKN1 inhibitor is not 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

[0010] In accordance with another aspect of the present disclosure, there is provided a method for treating a TRAF1 expressing B cell related cancer comprising administering a protein kinase C related kinase (PKN1) inhibitor disclosed herein and a Bcl-2 antagonist, such as venetoclax, to a subject in need thereof. Also provided herein is a use of a protein kinase C related kinase (PKN1) inhibitor disclosed herein and a Bcl-2 antagonist for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Further provided herein is a use of a protein kinase C related kinase (PKN1) inhibitor disclosed herein and a Bcl-2 antagonist in the manufacture of a medicament for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Even further provided is a protein kinase C related kinase (PKN1) inhibitor disclosed herein and a Bcl-2 antagonist for use in treating a TRAF1 expressing B cell related cancer in a subject in need thereof.

[0011] In one embodiment, the B cell related cancer is non-Hodgkin lymphoma, or a Chronic Lymphocytic Leukemia (CLL, also known as small lymphocytic lymphoma, SLL). In another embodiment, the B cell related cancer is Burkitt's lymphoma. In yet another embodiment the B cell related cancer is B-cell lineage non-Hodgkins Lymphoma and Diffuse Large B cell Lymphoma (DCBL). In a particular embodiment, the B cell related cancer is Chronic Lymphocytic Leukemia (CLL).

[0012] In an embodiment, the subject has been identified as having a TRAF1 high expressing B cell related cancer. In another embodiment, the subject has been identified as having a TRAF1 expressing B cell related cancer, wherein at least a subfraction of cells express TRAF1.

[0013] In accordance with yet another aspect of the present disclosure, there is provided a method of reducing a subject's resistance to a Bcl-2 antagonist, such as venetoclax or navitoclax, comprising administering to the subject a PKN1 inhibitor disclosed herein. Also provided is a use of a PKN1 inhibitor disclosed herein for reducing a subject's resistance to a Bcl-2 antagonist, such as venetoclax or navitoclax. Further provided is a use of a PKN1 inhibitor in the manufacture of a medicament for reducing a subject's resistance to a Bcl-2 antagonist, such as venetoclax or navitoclax. Even further provided is a PKN1 inhibitor for use in reducing a subject's resistance to a Bcl-2 antagonist, such as venetoclax or navitoclax. In an embodiment, the subject has a B cell related cancer as disclosed herein. In an embodiment, the Bcl-2 antagonist is venetoclax.

[0014] In accordance with a further aspect of the present disclosure, there is provided a method for identifying a patient susceptible to venetoclax resistance comprising the step of determining whether the patient has a B cell related cancer, such as CLL, with a high or a low expression of TRAF1, wherein a high expression of TRAF1 is an indicator of venetoclax resistance in the patient.

[0015] In an embodiment, determining whether the B cell related cancer is TRAF1 low or TRAF1 high comprises measuring the expression of TRAF1 from a sample from the patient. In one embodiment, the sample is peripheral blood mononuclear cells or whole blood samples. In another embodiment, the sample is from a biopsy, such as from a diffuse large B cell lymphoma. High expression of TRAF1 may be determined by comparison to a TRAF1 high control or reference standard.

[0016] In an embodiment the method further comprises treating the subject with a PKN1 inhibitor disclosed herein and a Bcl-2 antagonist, such as venetoclax or navitoclax, if the level of TRAF1 is high. In another embodiment, the method further comprises treating the subject with a Bcl-2 antagonist, such as venetoclax or navitoclax, if the level of TRAF1 is low.

[0017] In another embodiment, the disclosure provides a method of selecting therapy for a subject having a B cell related cancer comprising measuring the level of TRAF1 expression in a sample of cancer cells (e.g., tumour cells) from the subject to determine if the subject's cancer is TRAF1 high or TRAF1 low and selecting a PKN1 inhibitor disclosed herein and a Bcl-2 antagonist, such as venetoclax or navitoclax, for treating a subject that has TRAF1 high

expression and selecting a Bcl-2 antagonist, such as venetoclax or navitoclax, for treating a subject that has TRAF1 low expression.

[0018] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating embodiments of the disclosure are given by way of illustration only, since various changes and modifications will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0019] For a better understanding of the disclosure, as well as other aspects and further features thereof, reference is made to the following description which is to be used in conjunction with the accompanying drawings and tables.

[0020] Figure 1. *PKN1 kinase activity and TRAF1 S146 are required for TRAF1 protein stability.* (a) RAJI or Daudi cells were stably transduced with lentiviruses expressing control shRNA (shCTL), with shRNA targeting TRAF1 (shTRAF1) or with shRNA targeting PKN1 (shPKN1) and whole cell lysates were subjected to western blot analysis for TRAF1, PKN1 or GAPDH. (b) shCTL or shPKN1 RAJI cells were treated with cycloheximide for the indicated times, then whole cell lysates were subjected to western blot to determine TRAF1, PKN1 or GAPDH levels as indicated on the figures (c,d). 293T cells stably transduced with shCTL (c) or shPKN1 (d) lentiviruses were transiently transfected with WT TRAF1 or with TRAF1 S146A expression plasmids. Cells were treated or not with cycloheximide (CHX) for the indicated times, then whole cell lysates were subjected to Western blot analysis of TRAF1, PKN1 or GAPDH levels. (e) shPKN1 293T cells were transiently co-transfected with TRAF1 as well as shPKN1-resistant WT or K644E (kinase dead) PKN1. Cells were then treated with cycloheximide (CHX) and then cell lysates were subjected to Western blot to analyze TRAF1, PKN1 or GAPDH levels. All experiments were repeated at least two times.

[0021] Figure 2. *PKN1 is required to protect TRAF1 from cIAP-mediated degradation during CD40 signaling in RAJI cells thereby contributing to TRAF1 dependent signaling.* (a) shCTL or shPKN1 RAJI cells were treated with cycloheximide (CHX) for the indicated times, with or without SMAC mimetic BV6 treatment, then the CD40 signaling complex was immunoprecipitated from whole cell lysates (IP1). TRAF1 was then immunoprecipitated

from the supernatants of the CD40 immunoprecipitates (IP2). IP1, IP2 or whole cell lysates were analyzed for levels of TRAF1 by Western blotting. (b) shCTL, shTRAF1 or shPKN1 RAJI cells were serum starved for 24 h to reduce constitutive signaling then serum was added back for the indicated times and whole cell lysates were analyzed by Western blotting for levels of p-ERK1/2, p-S6 or β-actin as indicated in the figure (c) RAJI shCTL, shPKN1 or shTRAF1 were analyzed for constitutive levels of p-NF-κB p65 by flow cytometry; left panel shows a representative histogram, right panel shows the average of 2 independent experiments. (d) RAJI cells were treated with 4 different doses of PKN1i (0.1, 1.0, 5.0 and 10 μM) for 24 h (left) and every 24 h up to 72 h (right) and then analyzed by flow cytometry for levels of intracellular TRAF1; average of 2 independent experiments. See also extended data in Figure 7.

[0022] Figure 3. *PKN1 inhibition lowers TRAF1 and signaling intermediates in CLL cells.* Primary CLL cells (from patients described in Table 1) were cultured on OP9 or OP9-CD40L stroma and treated with DMSO control or 10 μM PKN1i. Gating strategy for CLL cells is shown in Figure 7c. Intracellular markers were measured by flow cytometry: graphical representation of the median fluorescent intensities (left) and histograms from one representative donor comparing treated to untreated cells (right). TRAF1 expression after treatment with inhibitor for (a) 24 h and (b) 3 treatments up to 72 h. See Fig. 7e for before-after data for each patient. (c) phospho-ribosomal protein S6 (pS6), phospho-NF-κB (p-p65) and phopho-Erk at 72 h. Bars show the median and each symbol represents each individual donor; n = 48. dMFI refers to the MFI for the specific antibody stain minus the FMO (background) control. See Fig. 8 a,b, for before-after data for each patient. Statistical analysis was performed using a non-parametric (Wilcoxon) paired test. *P < 0.05, **P < 0.01, ***P <0.001 and ****P<0.0001; ns, not significant. See also extended data in Figures 7 and 8.

[0023] Figure 4. *Increased cell death and lower expression of Bcl-xL and Mcl-1 in CLL cells treated with PKN1 inhibitor*. Primary CLL cells were cultured on OP9 or OP9-CD40L stroma and treated with DMSO control or 10 μM PKN1i every 24 h up to 72 h and assessed by flow cytometry. (a) Representative plot showing gating on live cells (left) and summary plots of frequency of live cells (right). (b) Cell death, measured by frequency of cleaved caspase-3^+ cells. See Fig. 8d,e for before-after data for each patient. (c) Expression of survival markers Bcl-xL, Mcl-1 and Bcl-2. Bars show the median and each symbol represents each individual donor; n = 48. dMFI refers to the MFI for the specific antibody stain minus the FMO

(background) control. Statistical analysis was performed using a non-parametric (Wilcoxon) paired test. *P < 0.05, **P < 0.01, ***P <0.001 and ****P<0.0001; ns, not significant. See also extended data in Figure 8.

[0024] Figure 5. *Enhanced cell death and reduction of Bcl-2 family members with combined treatment with PKN1i and venetoclax.* Primary CLL cells were cultured on OP9 or OP9-CD40L stroma and treated with DMSO control, 10 μM PKN1i every 24 h up to 72 h ± 10 nm venetoclax (VEN) at 48 h or 10 nm VEN alone and assessed by flow cytometry. (a) Representative plot showing gating on live cells (left) and summary plots of frequency of live cells (right) in CLL incubated on OP9-CD40L. (b) TRAF1 expression. (c) Gating on $TRAF1^{lo}$ and $TRAF1^{hi}$ cells (left) in venetoclax-treated cells for analysis of cleaved caspase-3 in a representative donor. (d) Bcl-2 (e) Bcl-xL and Mcl-1 expression. Graphs show the mean ± SEM and each symbol represents each individual donor; n = 48. dMFI refers to the MFI for the specific antibody stain minus the FMO (background) control. Statistical analysis was performed using a non-parametric Dunn's multiple comparisons test. *P < 0.05, **P < 0.01, ***P <0.001 and ****P<0.0001; ns, not significant. See also Figures 9 and 10.

[0025] Figure 6. *Ibrutinib in combination with PKN1 inhibitor prevents selection of venetoclax-resistant cells.* (a) Schematic showing contribution of BCR and TNFR signaling in CLL and site of action of inhibitors. (b) TRAF1, (c) pNF-κB p65 and (d) Mcl-1 expression in primary CLL cells cultured on OP9 or OP9-CD40L stroma and treated with DMSO control, 10 μM PKN1i every 24 h up to 72 h ± 0.1 μM ibrutinib (IBR) at 48 h or 0.1 μM IBR alone as assessed by flow cytometry. (e) Mcl-1 expression in primary CLL cells cultured on OP9-CD40L stroma and treated with DMSO control, 0.1 μM IBR, 10 nM VEN or 10 μM PKN1i every 24 h up to 72 h ± 0.1 μM IBR and/or 10 nM VEN at 48 h as assessed by flow cytometry. Graphs show the mean ± SEM and each symbol represents an individual donor; n = 47. dMFI refers to the MFI for the specific antibody stain minus the FMO (background) control. Statistical analysis was performed using a non-parametric Dunn's multiple comparisons test. *P < 0.05, **P < 0.01, ***P <0.001 and ****P<0.0001; ns, not significant.

[0026] Figure 7. *TRAF1 expression in CLL cells.* (a) TRAF1 staining controls: shCTL, shPKN1 and shTRAF1 RAJI and MEC2 cells stained with primary anti-TRAF1 antibody (IF3, 2 μg/mL) and goat anti-rat PE secondary antibody. FMO- fluorescence minus one control (b) MEC2 cells treated with PKN1i for 24 and 72 h were analyzed by flow cytometry for levels of intracellular TRAF1; average of 3 and 2 experiments, respectively. (c)

Representative gating strategy for CLL cells (d) TRAF1 levels (dMFI) in primary CLL cells after 3 days of incubation on OP9 and OP9-CD40L plus DMSO (vehicle) control (e) TRAF1 expression in control (DMSO-treated) OP9 and OP9-CD40L cultures after 72 hours, for donors grouped by Rai stage (0-IV) defined at time of sample collection. Bars show the median and each symbol represents each individual donor; n = 46. (f) Before-after data showing primary CLL cell TRAF1 expression after co-culture with OP9 or OP9-CD40L stroma and 3 doses of DMSO control or 10 μM PKN1i at 72 hours, using the same data shown in Figure 3. Each symbol represents an individual donor; n = 46. dMFI refers to the MFI for the specific antibody stain minus the FMO (background) control. Statistical analysis was performed using a non-parametric (Wilcoxon) paired test.

[0027] Figure 8. *PKN1 inhibition lowers signaling intermediates and leads to increased cell death in CLL cells*. Before-after data replotted from Figure 3 showing primary CLL cell pS6 (a) and p-p65 (b) levels at 72 h after co-culture with OP9 or OP9-CD40L stroma and 3 doses of DMSO control or 10 μM PKN1i. Each symbol represents an individual donor; n = 48. Statistical analysis was performed using a non-parametric (Wilcoxon) paired test. Linear regression analysis of TRAF1 dMFI versus pS6 dMFI after co-culture OP9-CD40L stroma and 3 doses of DMSO control (c) or 10 μM PKN1i (d) at 72 h. Before after data showing frequency of live cells (d) and cell death, measured by frequency of cleaved caspase-3^{+} cells (e) at 72 h after co-culture with OP9 or OP9-CD40L stroma and 3 doses of DMSO control or 10 μM PKN1i, plotted using the same data shown in Figure 3 and 4. Each symbol represents each individual donor; n = 48. Statistical analysis was performed using a non-parametric (Wilcoxon) paired test.

[0028] Figure 9. *Venetoclax treatment selects for TRAF^{hi} cells which have higher starting levels of TRAF1 than TRAF1^{lo} CLL cells*. Primary CLL cells were cultured on OP9 or OP9-CD40L stroma and treated with DMSO control or 10 nm venetoclax (VEN) for 24 h and assessed by flow cytometry. (a) Median TRAF1 expression and (b) before-after data of CLL cells classified as TRAF1^{lo} (dMFI 0-789, n=24) or TRAF1^{hi} (2282-116040, n=24). Statistical analysis was performed using a non-parametric (Wilcoxon) paired test. (c) TRAF1 expression at 24 h and 72 h. Bars show the median and each symbol represents each individual donor. Statistical analysis was performed using a non-parametric, non-paired (Mann-Whitney) test. (d) Histograms from one representative TRAF1^{hi} donor showing TRAF1 expression in CLL cells treated with DMSO control or 10 nM venetoclax.

[0029] Figure 10. *Staining intracellular TRAF1 and analysis by flow cytometry* (a) Controls for the specificity of TRAF1 staining. (b) The effect of the 5 selected inhibitors on the mean fluorescence intensity of TRAF1 measured by flow cytometry 24hrs after addition to the RAJI cells. dMFI refers to MFI measured for TRAF1 with anti-TRAF1 plus second step detection antibody minus the background when anti-TRAF1 is left out. **(c)** % initial TRAF1 versus concentration of inhibitor in nm. %Initial TRAF1 was calculated as ([TRAF1 dMFI of inhibitor treated cells]÷[TRAF1 dMFI of Raji DMSO control])*100. IC50 was calculated from a semi-log fit of the data.

DETAILED DESCRIPTION

[0030] Definitions

[0031] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0032] As used in the specification and claims, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

[0033] The term "comprising" as used herein will be understood to mean that the list following is non-exhaustive and may or may not include any other additional suitable items, for example one or more further feature(s), component(s) and/or ingredient(s) as appropriate.

[0034] The terms "B-cell related cancer" or "B cell lymphoma" are used herein to refer to cancers affecting B cells. B-cell related cancers include, but are not limited to non-Hodgkin lymphomas, Chronic Lymphocytic Leukemia (CLL, also known as small lymphocytic lymphoma, SLL), Burkitt's lymphoma, B-cell lineage non-Hodgkins Lymphoma and Diffuse Large B cell Lymphoma (DCBL).

[0035] The term "TRAF1" as used herein refers to TRAF1, or Tumour necrosis factor Receptor Associated Factor 1, from any species or source, optionally mammalian, such as human or mouse. For example, TRAF 1 has Genbank accession numbers: NM_005658, NM_001190945.

[0036] The term "PKN1" as used herein refers to PKN1, or Protein Kinase N-1, from any species or source, optionally mammalian, such as human or mouse. For example, PKN1 has

Genbank accession numbers: NM_213560, NM_002741. PKN1 is also referred to in the literature as PAK1, PKN, PRK1 or PRKCL1.

[0037] The term "PKN1 inhibitor" as used herein includes any substance that is capable of inhibiting/reducing the activity of PKN1 in stabilizing TRAF1 levels and in particular, its kinase activity, and thus, includes substances that inhibit PKN1 expression or activity and that also result in lowering of TRAF1 and that do not have non-specific effects that counteract the effect on TRAF1. Inhibition of TRAF1 activity is evidence of "on target" effects of the PKN1 inhibitor. Such inhibitors optionally include antisense nucleic acid molecules, small interfering RNA molecules, proteins, antibodies (and fragments thereof) that are internalized or expressed within the cell, small molecule inhibitors, peptide based inhibitors and other substances.

[0038] The term "selective PKN1 inhibitor" as used herein refers to an inhibitor that selectively inhibits PKN1 but does not or only minimally inhibits PKCθ, a member of the PKC family, which is the kinase family closest to PKN1.

[0039] The term "venetoclax", also known as ABT-199, as used herein refers to an orally bioavailable small molecule inhibitor of B cell lymphoma 2 (Bcl-2), that represses Bcl-2 activity by mimicking BH3 proteins to bind to Bcl2, thereby preventing its activity and leading to apoptosis in cancer cells.

[0040] The term "subject" is used herein interchangeably with the term "patient" to refer to a mammal, such as a human.

[0041] The following abbreviations used in this present disclosure have the following meanings:

CLL	Chronic Lymphocytic Leukemia
PKN1 from mouse	Protein kinase C related kinase N1, sometimes referred to as PRK1, any species or source, optionally mammalian, such as human or
shRNA	short hairpin or small hairpin RNA
TNFR	Tumor Necrosis Factor Receptor

TRAF1	TNRF associated factor 1 from any species or source, optionally mammalian, such as human or mouse

[0042] Methods/Uses

[0043] The present disclosure provides a method of treating a TRAF1 expressing B cell related cancer comprising administering an agent that lowers the levels of or inhibits the protein kinase C related kinase's, PKN1's, ability to stabilize TRAF1. TRAF1 is a target of phosphorylation by PKN1[21]. PKN1 phosphorylates TRAF1, but not other TRAF family members, at serine 146 in human or serine 139 in mouse[21]. The present inventors have found that certain PKN1 inhibitors induce TRAF1 protein degradation and reduce constitutive NF-κB signaling in B cell related cancers.

[0044] A previous method for treating B cell related cancers comprised administering a nutrient stress-inducing agent in combination with an agent that lowers the levels of or inhibits TRAF1 (International Publication No. WO 2015/131274, which is incorporated herein by reference). However, the present method of treating B cell related cancers advantageously relates to certain PKN1 inhibitors that reduce TRAF1 and does not require combination with a nutrient stress-inducing agent. Accordingly, in one embodiment, the methods and uses disclosed herein are in the absence of a nutrient stress-inducing agent.

[0045] In one embodiment, the B cell related cancer is non-Hodgkin lymphoma, or a Chronic Lymphocytic Leukemia (CLL, also known as small lymphocytic lymphoma, SLL). In another embodiment, the B cell related cancer is Burkitt's lymphoma. In yet another embodiment the B cell related cancer is B-cell lineage non-Hodgkins Lymphoma and Diffuse Large B cell Lymphoma (DCBL). In a particular embodiment, the B cell related cancer is Chronic Lymphocytic Leukemia (CLL).

[0046] In an embodiment, the subject has been identified as having a TRAF1 high expressing B cell related cancer. In another embodiment, the subject has been identified as having a TRAF1 expressing B cell related cancer, where at least a subfraction of cells express TRAF1.

[0047] The present disclosure further provides a method for treating a TRAF1 expressing B cell related cancer, such as CLL, in a subject by administering to the subject a PKN1 inhibitor in combination with another therapeutic agent, such as a chemotherapeutic agent. Also provided is use of a PKN1 inhibitor in combination with another therapeutic agent, such as a

chemotherapeutic agent, for treating a TRAF1 expressing B cell related cancer. Further provided is use of a PKN1 inhibitor in combination with another therapeutic agent, such as a chemotherapeutic agent, in the manufacture of a medicament for treating a TRAF1 expressing B cell related cancer. Even further provided is a PKN1 inhibitor in combination with another therapeutic agent, such as a chemotherapeutic agent, for use in treating a TRAF1 expressing B cell related cancer. The other chemotherapeutic agent can be, for example, a drug typically used in the treatment of cancer, such as a B cell related cancer. A non-limiting example of such a drug is Ibrutinib (brand name Imbruvica™, with the chemical name 1-[(3R)-3-[4-Amino-3-(4-phenoxyphenyl)pyrazolo[3,4-d]pyrimidin-1-yl]-1-piperidinyl]-2-propen-1-one).

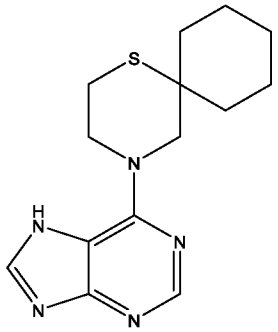
[0048] In certain embodiments treatment with a PKN1 inhibitor reduces or minimizes the subject's resistance to another therapeutic or chemotherapeutic agent. In one embodiment, there is provided a method for treating a TRAF1 expressing B cell related cancer, such as CLL, in a subject by administering to the subject a PKN1 inhibitor in combination with a Bcl-2 antagonist, such as a BH3 mimetic (for example, venetoclax or navitoclax). Also provided is use of a PKN1 inhibitor in combination with a Bcl-2 antagonist, such as a BH3 mimetic (for example, venetoclax) for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Further provided is use of a PKN1 inhibitor in combination with a Bcl-2 antagonist, such as a BH3 mimetic (for example, venetoclax or navitoclax) in the manufacture of a medicament for treating a TRAF1 expressing B cell related cancer in a subject in need thereof. Even further provided is a PKN1 inhibitor in combination with a Bcl-2 antagonist, such as a BH3 mimetic (for example, venetoclax or navitoclax) for use in treating a TRAF1 expressing B cell related cancer in a subject in need thereof. In such embodiments, the PKN1 inhibitor can also reduce the subject's resistance to the BH3 mimetic, such as venetoclax or navitoclax.

[0049] When the PKN1 inhibitor is administered or used with another therapeutic agent, the PKN1 inhibitor can be administered or used before, simultaneously with, or after administration or use of the other therapeutic agent.

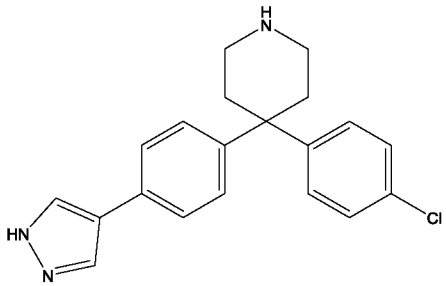
[0050] The PKN1 inhibitor or inhibitory agent includes any substance that is capable of inhibiting/reducing PKN1's activity in stabilizing TRAF1 levels and in particular, its kinase activity, and thus, includes substances that inhibit PKN1 expression or activity and that lower TRAF1 and that do not have non-specific effects that counteract the effect on TRAF1. Such

inhibitors optionally include antisense nucleic acid molecules, small interfering RNA molecules, proteins, antibodies (and fragments thereof) that are internalized or expressed within the cell, small molecule inhibitors, peptide-based inhibitors and other substances. In an embodiment, the PKN1 inhibitor of the present disclosure is effective in selectively inhibiting PKN1 and does not inhibit, or only minimally inhibits, PKCθ, a member of the PKC family, which is the kinase family closest to PKN1. Previously known PKN1 inhibitors such as staurosporin, lestaurtinib and tofactinib are not selective for PKN1 and/or have non-specific effects such that they are not effective for use as a PKN1 inhibitor in treating a B cell related cancer as described herein.

[0051] In specific embodiments, the PKN1 inhibitor is 4-(7H-purin-6-yl)-1-thia-4-azaspiro[5.5]undecane (PKN1i) or a pharmaceutically acceptable salt or solvate thereof. This PKN1 inhibitor has the following chemical formula:

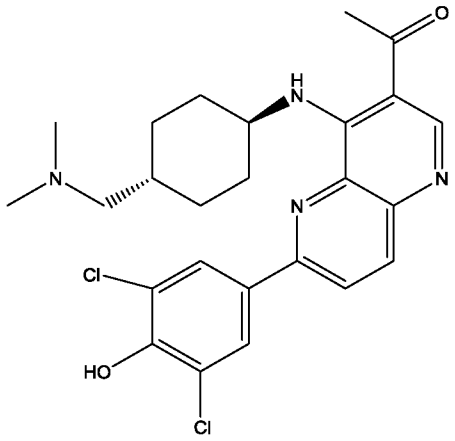


[0052] In another embodiment, the PKN1 inhibitor is 4-(4-(1H-pyrazol-4-yl)phenyl)-4-(4-chlorophenyl)piperidine (OICR 08727) or a pharmaceutically acceptable salt or solvate thereof. This PKN1 inhibitor has the following chemical formula:



[0053] In yet another embodiment, the PKN1 inhibitor is 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-

3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof. This PKN1 inhibitor has the following chemical formula:



[0054] In an alternate embodiment, the PKN1 inhibitor is not 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

[0055] The term "pharmaceutically acceptable salt" refers, for example, to a salt that retains the desired biological activity of a compound of the present disclosure and does not impart undesired toxicological effects thereto; and may refer to an acid addition salt or a base addition salt.

[0056] The term "solvate" as used herein means a compound or its pharmaceutically acceptable salt, wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. Examples of suitable solvents are ethanol, water and the like. When water is the solvent, the molecule is referred to as a "hydrate". The formation of solvates will vary depending on the compound and the solvate. In general, solvates are formed by dissolving the compound in the appropriate solvent and isolating the solvate by cooling or using an antisolvent. The solvate is typically dried or azeotroped under ambient conditions.

[0057] The term "antisense nucleic acid" as used herein means a nucleotide sequence that is complementary to its target transcription product. The nucleic acid can comprise DNA, RNA or a chemical analog, that binds to the messenger RNA produced by the target gene. Binding of the antisense nucleic acid prevents translation and thereby inhibits or reduces target protein expression. Antisense nucleic acid molecules may be chemically synthesized using naturally

occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

[0058] The term "siRNA" refers to a short inhibitory RNA that can be used to silence gene expression of a specific gene. The siRNA can be a short RNA hairpin (e.g. shRNA) that activates a cellular degradation pathway directed at mRNAs corresponding to the siRNA. Methods of designing specific siRNA molecules or shRNA molecules and administering them are known to a person skilled in the art. It is known in the art that efficient silencing is obtained with siRNA duplex complexes paired to have a two nucleotide 3' overhang. Adding two thymidine nucleotides is thought to add nuclease resistance. A person skilled in the art will recognize that other nucleotides can also be added.

[0059] Aptamers are short strands of nucleic acids that can adopt highly specific 3-dimensional conformations. Aptamers can exhibit high binding affinity and specificity to a target molecule. These properties allow such molecules to specifically inhibit the functional activity of proteins and are included as agents that inhibit PKN1.

[0060] The term "antibody" as used herein is intended to include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies. The antibody may be from recombinant sources and/or produced in transgenic animals. The term "antibody fragment" as used herein is intended to include without limitations Fab, Fab', F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and multimers thereof, multispecific antibody fragments and Domain Antibodies. Antibodies can be fragmented using conventional techniques. For example, F(ab')2 fragments can be generated by treating the antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques.

[0061] Antibodies to such proteins may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993, which are incorporated herein by reference. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference). Within the context of the present disclosure, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and $F(ab')_2$) and recombinantly produced binding partners.

[0062] For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

[0063] To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497, 1975) as well as other techniques such as the human B-cell hybridoma technique (Kozbor, D, and Roder, J: The production of monoclonal antibodies from human lymphocytes. *Immunology Today* 4:3 72-79, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96) and screening of combinatorial antibody libraries (Huse,W, Sastry,L, Iverson,S, Kang,A, Alting-Mees,M, Burton,D, Benkovic,S, and Lerner,R: Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246:4935 1275-1282, 1989). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the protein or fragment thereof and the monoclonal antibodies can be isolated.

[0064] For producing recombinant antibodies (see generally Huston et al, 1991; Johnson and Bird, 1991; Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-

lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complementary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

[0065] Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the disclosure. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the target (See, for example, Morrison et al. (Chimeric Human Antibody Molecules: Mouse Antigen-Binding Domains with Human Constant Region Domains. *PNAS* 81:21 6851-6855, 1984), and Takeda et al. (Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences. *Nature* 314:452-454), and the patents of Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B).

[0066] Monoclonal or chimeric antibodies specifically reactive with a target as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al. (Construction and Testing of Mouse--Human Heteromyelomas for Human Monoclonal Antibody Production. *PNAS* 80:12 7308-7312, 1983), Kozbor et al., *supra*; Olsson et al. (*Methods in Enzymol*, 92:3-16 1982) and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

[0067] The inhibitors described herein may also contain or be used to obtain or design "peptide mimetics". For example, a peptide mimetic may be made to mimic the function of an inhibitor. "Peptide mimetics" are structures which serve as substitutes for peptides in

interactions between molecules (See Morgan et al (1989), *Ann. Reports Med. Chem.* 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features. Peptide mimetics also include molecules incorporating peptides into larger molecules with other functional elements (e.g., as described in WO 99/25044). Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) *Proc. Natl. Acad, Sci USA* 89:9367) and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to an inhibitor peptide disclosed herein.

[0068] Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of the secondary structures of the proteins described herein. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

[0069] The terms “treatment” or “treating,” as used herein, mean an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable.

[0070] The term “therapeutically effective amount,” “effective amount” or “sufficient amount” of a compound of the present disclosure is a quantity sufficient to, when administered to the subject, including a mammal, for example a human, effect beneficial or desired results, including clinical results, and, as such, an “effective amount” or synonym

thereto depends upon the context in which it is being applied. An "effective amount" is intended to mean that amount of an agent that is sufficient to treat, prevent or inhibit such a B cell related cancer. The amount of a given agent that will correspond to such an amount will vary depending upon various factors, such as the given agent, the pharmaceutical formulation, the route of administration, the type or severity of condition, disease or disorder, the identity of the subject or host being treated, and the like, but can nevertheless be routinely determined by one skilled in the art. Also, as used herein, a "therapeutically effective amount" of an agent is an amount that prevents, inhibits, suppresses or reduces a B cell related cancer that benefits from the agents, as determined by clinical symptoms, in a subject as compared to a control. As defined herein, a therapeutically effective amount of an agent may be readily determined by one of ordinary skill by routine methods known in the art.

[0071] Moreover, a "treatment" or "prevention" regime of a subject with a therapeutically effective amount of an agent may consist of a single administration, or alternatively comprise a series of applications. For example, the agent may be administered at least once a week. However, in another embodiment, the agent may be administered to the subject from about one time per week to about once daily for a given treatment. The length of the treatment period depends on a variety of factors, such as the severity of the disease, the age of the patient, the concentration and the activity of the agent, or a combination thereof. It will also be appreciated that the effective dosage of the agent used for the treatment or prophylaxis may increase or decrease over the course of a particular treatment or prophylaxis regime. Changes in dosage may result and become apparent by standard diagnostic assays known in the art. In some instances, chronic administration may be required.

[0072] The term "administering" is defined as any conventional route for administering an agent(s) to a subject for use as is known to one skilled in the art. This may include, for example, administration via the parenteral (i.e., subcutaneous, intradermal, intramuscular, etc.) or mucosal surface route. In other embodiments this may include oral administration. The dose of the agent(s) may vary according to factors such as the health, age, weight and sex of the animal. The dosage regime may be adjusted to provide the optimum dose. One skilled in the art will appreciate that the dosage regime can be determined and/or optimized without undue experimentation.

[0073] To "inhibit" or "suppress" or "lower" or "reduce" or "down regulate" a function or activity, such as PKN1 expression or activity, is to reduce the function or activity when

compared to otherwise same conditions except for a condition or parameter of interest, or alternatively, as compared to another condition or control.

[0074] The PKN1 inhibitors for use in the methods and uses of the present disclosure are suitably formulated into pharmaceutical compositions for administration to subjects, for example human subjects, in a biologically compatible form suitable for administration *in vivo*. Such pharmaceutical compositions comprise a PKN1 inhibitor, as disclosed herein, and a pharmaceutically acceptable carrier or diluent. Also included is methods and uses of a pharmaceutical composition comprising a PKN1 inhibitor as disclosed herein and another chemotherapeutic agent as described herein (e.g., venetoclax or navitoclax), and optionally, a pharmaceutically acceptable carrier or diluent.

[0075] The compositions containing the agent(s) can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active agent(s) is combined in a mixture with a pharmaceutically acceptable vehicle (i.e. a carrier or diluent). Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (2003 - 20th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999. On this basis, the compositions include, albeit not exclusively, solutions of the agent(s) in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

[0076] The agents may be administered to a subject alone or in combination with pharmaceutically acceptable carriers, as noted above, and/or with other pharmaceutically active agents for the treatment of the B cell related cancer (e.g., CLL), the proportion of which is determined by the solubility and chemical nature of the agents, chosen route of administration and standard pharmaceutical practice. For example, the agents disclosed herein may be used or administered contemporaneously. As used herein, "contemporaneous administration" of two substances to an individual means providing each of the two substances so that they are both biologically active in the individual at the same time. The exact details of the administration will depend on the pharmacokinetics of the two substances in the presence of each other, and can include administering the two substances within a few hours of each other, or even administering one substance within 24 hours of administration of the other, if the pharmacokinetics are suitable. Design of suitable dosing regimens is routine for one skilled in the art. In particular embodiments, two substances will be administered

substantially simultaneously, i.e., within minutes of each other, or in a single composition that contains both substances.

[0077] The dosage of the agent(s) and/or compositions can vary depending on many factors such as the pharmacodynamic properties of the agent, the mode of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the frequency of the treatment and the type of concurrent treatment, if any, and the clearance rate of the compound in the animal to be treated. One of skill in the art can determine the appropriate dosage based on the above factors. The agents may be administered initially in a suitable dosage that may be adjusted as required, depending on the clinical response. For *ex vivo* treatment of cells over a short period, for example for 30 minutes to 1 hour or longer, higher doses of agent may be used than for long term *in vivo* therapy.

[0078] As PKN1 inhibition is thought to act through lowering TRAF1, the PKN1 inhibitor would be selected for B cell related cancers with TRAF1 expression in at least a subset of their B cells. Venetoclax treatment appears to enrich for TRAF1 hi (or positive) cells, so this treatment may be useful in conjunction with venetoclax, or for patients that do not respond to venetoclax alone. The present inventors suggest that having high levels of TRAF1 expression can be resistant to standard chemotherapy drugs. In particular, it has now been found that cancer cells that survive suboptimal venetoclax have high levels of TRAF1 (Fig. 5C).

[0079] Accordingly, TRAF1 may function as a biomarker for venetoclax resistance. Accordingly, the present disclosure further provides a method for determining whether a subject is susceptible to venetoclax resistance comprising measuring the level of TRAF1 expression in a sample of cancer cells (e.g., tumour cells) from the subject. The higher the level of TRAF1 expression the higher the possibility of the subject becoming resistant to venetoclax therapy.

[0080] In an embodiment, determining whether the B cell related cancer is TRAF1 low or TRAF1 high comprises measuring the expression of TRAF1 from a sample from the patient. In one embodiment, the sample is peripheral blood mononuclear cells or whole blood samples. In another embodiment, the sample is from a biopsy, such as from a diffuse large B cell lymphoma. High or positive expression of TRAF1 may be determined by comparison to a TRAF1 high or positive control or reference standard (RAJI control or RAJI shTRAF1).

[0081] TRAF1 expression levels can be measured using intracellular flow cytometry. Comparison to the levels measured from the subject's cells to control cells having a known level of TRAF1 expression allows identification of the subject's cancer as being TRAF1 high/positive or TRAF1 low/negative. Similarly TRAF1 expression from the subject's cells can be compared to a reference standard or reference value indicative of TRAF1 high/positive or TRAF1 low/negative expression. See Figure 5C, which shows that CLL cells can clearly fall into a TRAF1 hi and low group.

[0082] In an embodiment the method further comprises treating the subject with a PKN1 inhibitor disclosed herein if the level of TRAF1 is high or positive. In another embodiment, the method further comprises treating the subject with a PKN1 inhibitor disclosed herein and a Bcl-2 antagonist, such as venetoclax or navitoclax, if the level of TRAF1 is high or positive. As suboptimal venetoclax has been show to enrich for TRAF1 high or expressing cells, even patients with only a few TRAF1 positive cells would likely benefit from combined treatment.

[0083] In another embodiment, the disclosure provides a method of selecting therapy for a subject having a B cell related cancer comprising measuring the level of TRAF1 expression in a sample of cancer cells (e.g., tumour cells) from the subject to determine if the patient's cancer cells are TRAF1 positive or negative and selecting a PKN1 inhibitor disclosed herein and a Bcl-2 antagonist, such as venetoclax or navitoclax, for treating a subject that has TRAF1 expression and selecting a a Bcl-2 antagonist, such as venetoclax or navitoclax, for treating a subject that has TRAF1 low or negative expression.

[0084] The following non-limiting examples are illustrative of the present disclosure:

EXAMPLES

[0085] EXAMPLE 1

[0086] As described in more detail below, in this study, a PKN1 inhibitor, selective for PKN1 over PKCθ was used to demonstrate the role of PKN1 in CLL. PKN1 inhibition lowered the levels of TRAF1, phospho-NF-κB p65, Mcl-1, Bcl-x_L and phospho-ribosomal protein S6 (pS6) in primary human CLL cells. Treatment of CLL cells with suboptimal concentrations of venetoclax enriched for $TRAF1^{hi}$ cells among the surviving cells,

suggesting that TRAF1 may contribute to venetoclax resistance. Accordingly, when PKN1 inhibition was combined with venetoclax, increased cell death, associated with loss of the pro-survival Bcl-2 family members Bcl-2, Bcl-x_L and Mcl-1, was observed. These findings identify PKN1 as a target for inhibition in CLL, particularly in combination with venetoclax.

[0087] Materials and Methods

[0088] *Cell lines and cell line culture*: The human embryonic kidney 293 cell line (293 FT) was obtained from Invitrogen (Invitrogen, Carlsbad, United States) and was passaged in a 75cm^2 cell culture treated flask (Thermo Fisher Scientific, Waltham, United States) with Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, United States) at 37°C in a humidified atmosphere with 5% CO_2. The media was supplemented with 10% Fetal Calf Serum (Thermo Fisher Scientific) and 1% of 100X Glutamine-Penicillin-Streptomycin (Sigma-Aldrich, Oakville, Canada). The Burkitt's lymphomas RAJI and Daudi were obtained from ATTC. The CLL line MEC2[58] was provided by Mark Minden, UHN. RAJI, Daudi and MEC2 were maintained in RPMI1640 in 10% FCE with added glutamine, pyruvate, penicillin and streptomycin (GPPS). OP9 and OP9 cells transfected with CD40L (OP9-CD40L), generated as described[47], were kindly provided by Patrick Brauer and J.C. Zuniga-Pflücker, Sunnybrook Research Institute. OP9 or OP9-CD40L cells were plated at 10^5 cells/well of a 24-well plate in α-MEM (+GPPS) approximately 2 days before use and used when confluent.

[0089] *Human subjects*: Peripheral blood samples were obtained from CLL patients following informed consent in compliance with the Declaration of Helsinki and in agreement with the Sunnybrook Ethics Review Board and the University Health Network Research Ethics Review Board and approved by the University of Toronto Research Ethics board (Protocol 0030910). Peripheral blood mononuclear cells (PBMC) from 48 patients diagnosed with CLL were isolated using Ficoll-Paque Plus (GE Healthcare, Buckinghamshire, UK) density gradient and stored in liquid nitrogen until analysis. CLL cells were assessed by flow cytometry and characterized as $CD19^+/CD5^+$ cells, which generally accounted for >85% of analyzed cells. Cytogenetic alterations (17p13.1, 11q22.3 and 13q14.3 deletions and trisomy 12) were determined by fluorescent in situ hybridization. The characteristics of the patients are listed in Table 1. Patient data was obtained only after completion of the flow cytometry /inhibition analysis.

[0090] *shRNA knockdown*: 3 x 10^6 293FT cells (Invitrogen) were seeded in 10 cm plates overnight. Then cells were transfected with 0.8 µg of VSV-G, 5.4 µg psPAX2, and 6 µg of pLKO.1-shRNA plasmid (Openbiosystems, Chicago, United States) using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. shRNA mature antisense sequences for TRAF1 and PKN1 are AACAATGTTCTCAAACACACG (SEQ ID NO:1) and TATCCGCTTCTCACACATCAG (SEQ ID NO:2), respectively. 60 hours after transfection, the supernatant of the cultures was collected, filtered through a low protein binding 0.22 µM filter, and added to target cells for 24 hrs before selection with 4 µg/ml puromycin (Bio Basic, Markham, Canada). For TRAF1 knockdown in RAJI cells, cells were then seeded in 96 wells at 1 cell per well and cells were allowed to grow into clones from a single cell. Several clones were then tested for reduction in TRAF1 levels by Western Blotting and Flow cytometry, and those with the lowest TRAF1 levels were chosen for downstream assays. For PKN1 knockdown in RAJI and 293T cells, sufficient reduction in PKN1 protein level was observed in the pool knockdown by Western blotting so no cloning was necessary.

[0091] *Plasmid construction*: Human TRAF1 ORF was amplified from RAJI cells cDNA and cloned into pENTR-D-TOPO vector (Invitrogen) following manufacturer's instructions. S146A TRAF1 was derived from WT-TRAF1 in pENTR-D-TOPO using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, United States) following manufacturer's instructions. WT-TRAF1 and S146A-TRAF1 were cloned into a pCDNA3-c-Flag vector, a gift from Stephen Smale (Addgene plasmid # 20011)7. WT-TRAF1 in pCDNA-c-Flag was then modified where c-Flag tag was replaced with a 3x-HA tag. WT and K644E human PKN1 ORFs were codon optimized for mammalian gene expression and synthesized by GeneArt™ Gene Synthesis (Invitrogen). WT and K644E PKN1 were the cloned into a pCDNA3.1+ vector (Invitrogen).

[0092] *In vitro transfection*: One day before transfection, cells were seeded at 6x10^5 cells per well in a 12-well flat bottom culture plate (Corning Incorporated, New York, United States). Cells were then cultured overnight to reach 80-95% confluency. Prior to transfection, the growth media was replaced by 1X Opti-MEM Reduced Serum Media (Thermo Fisher Scientific). Cells were transfected with either 200ng of WT-TRAF1-HA, 200ng of S146A-TRAF1-FLAG, 50ng of WT PKN1, 50ng of K644E-PKN1 or 50ng of PCDNA 3.1+ empty vector with 1.5µl/well of Lipofectamine 2000 following manufacturer's instructions

(Invitrogen). After 6 hours of incubation, the media was then replaced with fresh growth medium and cells were cultured overnight until cycloheximide treatment on the next day.

[0093] *Cycloheximide Inhibition Assays*: Following overnight recovery from transfection, 293FT cells were treated or not with cycloheximide (Sigma-Aldrich) at 3µg/ml. Cells were then incubated for 2, 4 or 6 hours respectively. After treatment, the media was removed and cells were washed with PBS (Sigma-Aldrich) once then lysed for protein extraction. RAJI cells were treated or not with 3 µg/ml cycloheximide 3 or 6 hours with or without 5 µM BV6 (Sigma-Aldrich, St. Louis, USA). The solvents ethanol and DMSO were added in the controls for cycloheximide and BV6, respectively.

[0094] *Western blots*: Whole cells extracts were prepared by lysing cells with lysis buffer containing 0.5% Nonidet P-40 (source) with phosphatase and protease inhibitor mix (Roche, Basel, Switzerland). Total protein concentration was quantified by a colorimetric assay (Bio-Rad, Berkeley, United States), then subjected to SDS-PAGE (10% gel) and transferred to polyvinylidene difluoride membranes (semi-dry transfer; Bio-rad). After blocking with 5% non-fat milk in TBS-T, the membranes were probed with antibodies specific for TRAF1 (Cell Signaling, Danvers, United States), PKN1 (BD Biosciences, Franklin Lakes New Jersey, United States) and GAPDH (Thermo Fisher Scientific). The membranes were then incubated with HRP-conjugated anti-rabbit or anti-mouse (Jackson Immunoresearch, Baltimore, United States), and signals were detected with a chemiluminescence substrate (GE Healthcare, Baie D'Urfe, Quebec, Canada) and visualized by autoradiography.

[0095] *Flow cytometry analysis of RAJI, MEC2 and CLL cell lines*: Fc receptors were blocked with human Fc Block (eBioscience). For surface staining, live cells were stained with live/dead-e506 stain (eBioscience). CLL cells were stained with anti-CD19 BV605 (clone HIB19) from Biolegend (San Diego, CA) and anti-CD5 FITC (clone L17F12) or PE-Cy7 (clone UCHT2) purchased from eBioscience (La Jolla, CA). For intracellular staining, cells were fixed with Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). Purified TRAF1 antibody (clone 1F3, Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany) and anti-cleaved Caspase-3 AF647 (clone D3E9, New England Biolabs) were used prior to secondary staining with goat anti-rat PE (clone poly4054, Biolegend) for TRAF1. For PhosFlow, cells were fixed with formaldehyde and washed with BD PhosFlow Perm/Wash Buffer I (BD Biosciences). Cells were stained with anti p-p65-eFluor 660 (clone B33B4WP), anti-pS6 PE (clone cupk43k) and anti-pErk PerCP-

ef710 (clone MILAN8R) from eBioscience at 1:50 dilution for 60 min. at room temperature. Data were acquired on BD LSR Fortessa and analyzed with FLoJo software.

[0096] *Immunoprecipitation*: $6x10^6$ RAJI cells in 10 cm dishes were treated with Cycloheximide (see above) with or without BV6 and lysed in 800 µl 2% NP-40 buffer including protease and phosphatase inhibitor cocktails. 20 µl prewashed magnetic Protein G Dynabeads™ (Thermofisher) were incubated with 0.5 µg antibody for CD40 (IP1; G28.5, purified from hybridoma cell line using Protein G sepharose, hybridoma was originally provided by Diane Hollenbaugh, then at Bristol-Myers Squibb; the antibody is now available at BioXCell) for 10 minutes at room temperature, washed and then incubated with 100 µl of lysates overnight at 4°C. Unbound proteins were saved for a subsequent immunoprecipitation (IP2) by incubating them with 20 µl prewashed magnetic Protein G Dynabeads (Thermofisher) for 10 minutes at room temperature and 0.5 µg antibody for TRAF1 (clone 1F342; Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany). After three washes, immunoprecipitated proteins from IP1 and IP2 were fractionated on a 10% SDS-PAGE and immunoblotted for TRAF1 (clone 45D3; Cell Signaling).

[0097] *PKN1 inhibitor*: The PKN1 inhibitor 4-(7H-purin-6-yl)-1-thia-4-azaspiro[5.5]undecane (PKN1i) was identified using a high throughput screen using a commercial FRET-based kinase assay. A selection of compounds with pIC50>6 and selectivity for PKN1 over PKCθ were further tested and PKN1i was selected for this study, as it showed dose-dependent effects on TRAF1 levels in RAJI and MEC2 cells and was the most specific of the PKN1 inhibitors tested. PKN1i has a pIC50 for PKN1 of 6.1 and a pIC50 for PKCθ of <4. When tested against 360 kinases at 10µM, PKNi inhibited only 5 kinases at 90%. PKN1i was custom synthesized by Dalton Pharma Services, Toronto, Canada and its structure confirmed by Mass spectrometry and ^{1}H-NMR. Purity by HPLC was 99.2% by AUC at 280nm. The synthesis was a one-step synthesis from commercially available precursors.

[0098] *OP9 co-culture, CLL samples and treatment with inhibitors*: OP9 and OP9-CD40L cells were re-suspended at 10^5 cells/mL in OP9 Media (α-MEM, 20% Fetal Calf Serum) and seeded at 5 x 10^4 cells per well into a 24-well plate. CLL patient samples were thawed, washed to remove any DMSO present, and re-suspended at 8 x 10^6 cells/mL in high glucose RPMI 1640 medium (4.5 g/L total glucose), plated at 2 x 10^6 cells per well on a 24-well plate

containing confluent OP9 or OP9-CD40L cells and rested overnight. Some sample was taken directly post-thaw for baseline measurements. Samples were collected for flow cytometric analysis after 24 hours of treatment with 0.1% DMSO control or 10 μM PKN1i, and at 72 hours after 3 doses, every 24 hours, of 0.1% DMSO control or 10 μM PKN1i with or without one dose of 10 nM Venetoclax (EnzoLife Sciences, Farminggate, NY, ordered through Cedarlane, Ontario, Canada) or 0.1 μM ibrutinib (Selleck Chemical LLC, Houston Tx, distributed via Cedarlane, Ontario, Canada) at 48 hours. All CLL cells were treated with inhibitors at the indicated doses in a final concentration of 0.1% DMSO in high glucose RPMI.

[0099] *Flow cytometry analysis of patient samples*: Anti-human Fc Block (eBioscience) was used to block Fc receptors. For surface staining, live cells were stained with eBioscience Fixable Viability Dye eFluor® 506. CLL cells were stained with anti-CD19 BV605 (clone HIB19) from Biolegend (San Diego, CA) and PE-Cy7 (clone UCHT2) purchased from eBioscience (La Jolla, CA). For intracellular staining, cells were fixed and permeabilized using Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). Purified TRAF1 antibody (clone 1F3, Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany), anti-cleaved caspase-3 AF647 (clone D3E9, New England Biolabs), anti-human Bcl-2 BV421 (clone 100, Biolegend), anti-human Bcl-xL FITC (clone 7B2.5, Invitrogen) and anti-human Mcl-1 AF647 (clone D2W9E, New England Biolabs) were used prior to secondary staining with goat anti-rat PE (clone poly4054, Biolegend) for TRAF1. For phosphoflow, one volume of 2X Cytofix/Perm/Wash Buffer (3% PFA + BD Perm/Wash + ddH20) was added directly to cell culture and incubated at room temperature for 15 min and on ice for 60 min. Cells were washed two times with Perm/Wash buffer and re-suspended in Perm/Wash buffer with eBioscience anti-human Fc blocking antibody. Cells were stained with anti-human pNF-κBp65-eFluor 660 (clone B33B4WP), anti- pS6 PE (clone cupk43k) and anti-pErk PerCP-ef710 (clone MILAN8R) from eBioscience at 1:50 dilution for 60 min. at room temperature. Data were acquired on a BD LSR Fortessa and analyzed using FloJo™ software. Researchers were blind to patient demographics and clinical data until study experiments were complete. Statistics were calculated using Prism GraphPad™ 8 software.

[00100] Results

[00101] *Protein Kinase 1 is required for TRAF1 protein stability*

[00102] To demonstrate the effect of PKN1 on TRAF1 biology, PKN1 was knocked down in lymphoma cell lines using lentiviral delivery of either a control small hairpin (sh) RNA (shCTL) or an shRNA targeting PKN1 (shPKN1). Knockdown of PKN1 led to a concomitant reduction in the level of TRAF1 protein in RAJI and Daudi cells (Fig. 1a). To demonstrate the effect of PKN1 on protein stability, RAJI cells expressing control shRNA (shCTL) or shRNA targeting PKN1 (shPKN1) were incubated with cycloheximide at 37°C to block new protein synthesis and analyzed for levels of PKN1 and TRAF1 by western blotting. The results show that the absence of PKN1 decreases the stability of TRAF1 protein (Fig. 1b).

[00103] PKN1 was previously shown to phosphorylate TRAF1 on serine 146[21]. To test the importance of TRAF1 S146 in TRAF1 stability, mutant human TRAF1 constructs were generated in which serine 146 was replaced with alanine (S146A). Upon transient transfection into 293T cells, TRAF1 S146A showed reduced stability compared to WT TRAF1 (Fig. 1c). In contrast, when TRAF1 WT or S146A were expressed in 293T cells that had been knocked down for PKN1, both WT and TRAF1S146A showed a similar half-life, which was decreased compared to that of WT TRAF1 in PKN1 sufficient cells (Fig. 1d).

[00104] To test whether the kinase activity of PKN1 was required for TRAF1 stability, TRAF1 as well as shRNA-resistant WT or kinase dead (K644E) PKN1 were expressed in 293T cells that had been stably transduced with shPKN1 (Fig. 1e). In the absence of PKN1, as before, TRAF1 protein showed a half-life of between 2 and 4 hrs. Overexpression of WT PKN1 in the PKN1 knockdown cells restored TRAF1 protein half-life to at least 6 hrs. In contrast, kinase dead PKN1 failed to increase TRAF1 stability. Although higher expression of WT compared to kinase dead PKN1 was observed in 293T cells, the level expressed was still in excess of the physiological level of PKN1 observed in cells before knockdown and in another experiment, super-physiological levels of mutant PKN1 also failed to increase TRAF1 stability (data not shown). Thus, reduced expression of PKN1 K644E is unlikely to account for its lack of efficacy in conferring TRAF1 protein stability. These results show that the kinase activity of PKN1 as well as TRAF1 S146 are required for TRAF1 protein stability in B lymphomas.

[00105] *PKN1 protects TRAF1 from cIAP-mediated degradation in the CD40 signaling complex.*

[00106] This study was performed to investigate the mechanism of TRAF1 degradation. TRAF1 is important for the recruitment of cIAPs leading to NF-κB activation. However, cIAPs are dual function E3 ligases. cIAPs can add K63-linked polyubiquitin to RIP, thereby activating NF-κB signaling[24], but can also add K48- linked ubiquitin to TRAF proteins, thereby inducing TRAF protein degradation[25]. Therefore, without wishing to be bound by theory, it was hypothesized that PKN1 is required to protect TRAF1 from cIAP mediated degradation during CD40 signaling. To test this hypothesis, RAJI cells were treated with cycloheximide with or without the SMAC mimetic BV6 and then CD40 was immunoprecipitated from control RAJI cells or from RAJI cells knocked down for PKN1. A second immunoprecipitation of TRAF1 was used to analyze the effect of SMAC mimetics on the cytosolic, non-CD40 associated TRAF1 pool. In the presence of PKN1, CD40 associated TRAF1 was decreased at 6 hrs, but was rescued by BV6 (Fig. 2a). In the absence of PKN1, TRAF1 was less stable, already degrading by 3 hrs, and again partially rescued by BV6 treatment. In contrast, the cytosolic pool of TRAF1 (IP2 in Fig. 2a) was not sensitive to BV6 treatment. These results suggest that cIAPs contribute to degradation of TRAF1 in the CD40 signaling complex, and that PKN1 at least partially protects TRAF1 from cIAP-induced degradation.

[00107] *TRAF1 and PKN1 contribute to constitutive NF-κB, MAPK and mTOR signaling in RAJI cells.*

[00108] This study was performed to demonstrate the contribution that PKN1 and TRAF1 have on constitutive signaling in RAJI cells. RAJI cells were deprived of serum and amino acids and then serum / amino acids were added back to synchronize constitutive signaling. Knockdown of TRAF1 dramatically reduced the level of pERK1/2 and pS6, a downstream target of mTOR (Fig 2b), as well as reducing NF-κB signaling as measured by flow cytometry for pNF-κB p65 (Fig 2c). Knockdown of PKN1 had an intermediate effect compared to direct TRAF1 knockdown, correlating with its partial effect on TRAF1 levels (Fig 1a, Fig. 7a). These findings demonstrated that inhibition of PKN1 can be used to reduce constitutive TRAF1-dependent survival signaling in B cell cancers.

[00109] *PKN1 inhibitor 4-(7H-purinyl-6-yl)-1-thia-4-azaspiro[5.5]undecane treatment of CLL recapitulates the effects of PKN1 knockdown on TRAF1 levels in cell lines*

[00110] As it is difficult to knockdown genes in primary cells, demonstrating the role of PKN1 in primary human B cell cancers necessitated the identification of a PKN1 inhibitor. Although the inhibitors staurosporin, lestaurtinib and tofacitinib can inhibit PKN1, they have no selectivity for PKN1 and in fact were initially identified as PKC and JAK inhibitors, respectively[26-29]. Therefore, a high throughput screen for PKN1 inhibitors was conducted, using a commercial, *in vitro* FRET-based kinase assay, for compounds that selectively blocked PKN1 over PKCθ, as the PKC family is the nearest kinase family to the PKN family. A series of compounds were tested for effects on TRAF1 levels on the cell lines RAJI and MEC2. Of the inhibitors tested, one inhibitor, 4-(7H-purinyl-6-yl)-1-thia-4-azaspiro[5.5]undecane (PKN1i) was chosen as a tool compound, because it showed dose response-dependent decreases in TRAF1 after 24 or 72 hrs of treatment of RAJI or the CLL line MEC2 (Fig. 2d and Fig. 7b) as well as a >100-fold selectivity for PKN1 over PKCθ (data not shown). Although this first generation PKN1i inhibits other kinases (data not shown), it showed substantially more selectivity than two other first-generation inhibitors tested in the kinase screen (data not shown) and was therefore selected as a tool compound for demonstrating the effect of PKN1 inhibition.

[00111] *PKN1 inhibition lowers TRAF1 and signaling intermediates in primary CLL cells*

[00112] A total of 48 CLL patient samples, the majority of which represent high risk stages of disease, were obtained from two centers (Table 1). To test the effects of PKN1 inhibition on primary CLL cells, cells were incubated on OP9 stromal cells or with OP9 cells transfected with CD40L. CLL cells have been reported to exhibit autocrine signaling through CD40L[30,31]. The level of TRAF1 on CLL can also be further upregulated on CLL through CD40 ligation *ex vivo*[32]. Consistently, inclusion of CD40L on the stromal cells resulted in increased TRAF1 protein levels in 28 out of 48 donors (58.3%) (Fig. 7c, 7d). Therefore, both culture conditions were compared throughout the study. CLL cells incubated on OP9 or OP9-CD40L maintained their viability for at least 6 days, whereas without the stromal cell layer, the CLL cells showed almost 50% loss of viability after overnight culture post-thaw (data not shown). While there was no apparent effect of Rai stage on TRAF1 levels, for Rai stage 4 patients, there appeared to be two separate patient populations whose leukemia cells exhibited either high or low levels of TRAF1 when cultured on OP9 cells (Fig. 7e).

[00113] Addition of PKN1 inhibitor (PKN1i) significantly lowered the levels of TRAF1 in the CLL cells after 24 hours of treatment, with slightly more significant effects for CLL cells in the OP9-CD40L co-cultures than the OP9 co-cultures (Fig. 3a, $P<0.0001$ and $P<0.001$, respectively and Fig. 7f). Similar results were obtained after 3 doses of PKN1i added every 24hr and read out at 72hrs (Fig. 3b, $P<0.0001$)).

[00114] The study further considered how PKN1i affected constitutive signaling in the CLL cells. After 3 doses of PKN1i, there was a significant reduction in levels of pS6 and pNF-κB p65 on CLL cells incubated on OP9 or OP9-CD40L, recapitulating the effects seen on Raji cells (Fig. 3c and Fig. 8a,b). Although the link between TRAF1 and pS6 levels is not known, there was a weak correlation between TRAF1 levels and pS6 before and after treatment with PKN1 at 72 hours when cells were incubated on OP9 stroma (Fig. 8c), although this was not observed for cultures of CLL with OP9 -CD40L (data not shown). In contrast to the effects on pNF-κB and pS6, PKN1 inhibition had no impact on levels of pErk (Fig. 3c, lower panels), suggesting that other factors have a dominant effect on Erk activation in the primary CLL cell cultures. Taken together these results show that inhibition of PKN1 results in lower levels of TRAF1 in primary CLL cells with concomitant reductions of pNF-κB and pS6.

[00115] *Increased cell death and lower expression of survival markers in CLL cells treated with PKN1 inhibitor*

[00116] Elevated expression of TRAF1 in hematopoietic malignancies, combined with the evidence in the literature that TRAF1 protects lymphocytes from apoptosis, suggests that TRAF1 may contribute to apoptosis-resistance in CLL.[18,33-35] Therefore, the study considered whether PKN1 inhibition resulted in increased cell death of the CLL cells by assessing cell viability using a live/dead stain as well as monitoring levels of activated caspase-3 by flow cytometry. Indeed, there was a significantly lower percentage of viable cells and a higher frequency of CLL cells expressing cleaved caspase-3 in cells treated for 3 days with PKN1i (Fig. 4a,b and Fig. 8d,e). Bcl-xL and Mcl-1 are two anti-apoptotic Bcl-2-family members known to be regulated by NF-κB and transcriptionally induced by microenvironmental survival signals in CLL[10,36]. Consistent with the decreased survival of CLL cells after PKN1i treatment, Bcl-xL and Mcl-1 were significantly reduced in CLL cultures after 3 doses of PKN1i in both culture systems (Fig. 4c). In contrast, Bcl-2 was slightly increased in the

surviving CLL cells after treatment with PKN1i, suggesting that Bcl-2 might represent a mechanism of resistance to the PKN1 inhibition (Fig. 4c).

[00117] *PKN1 inhibition in combination with venetoclax results in increased CLL death correlating with reduced TRAF1 and Bcl-2 family members*

[00118] Venetoclax, a BH3 mimetic that antagonizes Bcl-2 and induces cellular apoptosis is approved for the treatment of patients with relapsed CLL and 17p deletion[7,37]. Although showing great promise, mechanisms of venetoclax resistance are emerging[38,39]. The finding that PKN1i treatment led to down regulation of Mcl-1 and Bcl-xL, but an increase in Bcl-2, suggested that PKN1i and venetoclax (or other Bcl-2 antagonists or BH3 mimetics) might be complementary in collectively targeting three pro-survival Bcl-2 family members and thereby inducing CLL death. A suboptimal dose of venetoclax that killed about 1/3 of the CLL cells in culture when added alone, was selected to reveal potential venetoclax resistance and to allow observation of additive effects with PKN1i.

[00119] PKN1i in combination with venetoclax affected cell death, with significantly lower numbers of viable cells after treatment with both inhibitors (Fig. 5a). Of note, for about half the CLL patient samples, cells that survived venetoclax treatment were enriched for those high in TRAF1, with some cultures showing an apparent increase in TRAF1 of as much as 68-fold (Figure 5b and Fig. 9a,b). In contrast, the combination of venetoclax and PKN1i together resulted in a greater loss of $TRAF1^{hi}$ cells (i.e., cells with high levels of TRAF1) than PKN1i alone (Fig. 5b). Of note, those CLL cultures that were enriched for $TRAF1^{hi}$ cells after venetoclax treatment were those with higher TRAF1 levels to start with (Fig. 9c). These data showed that the $TRAF1^{hi}$ cells selectively survive venetoclax treatment, and thus that TRAF1 represents a venetoclax resistance mechanism. Accordingly, when the study gated on the $TRAF1^{hi}$ or $TRAF1^{lo}$ cells in venetoclax treated CLL samples with a mixed population, the $TRAF1^{hi}$ cells were largely viable whereas the $TRAF1^{lo}$ cells have high levels of cleaved caspase-3, consistent with the hypothesis that venetoclax treatment selects for $TRAF1^{hi}$ CLL cells (Fig. 5c). Moreover, in a patient sample with heterogeneous TRAF1 expression before venetoclax, an enrichment for the $TRAF1^{hi}$ cells after venetoclax treatment was observed (Fig. 9d). These data show that suboptimal venetoclax allows selective survival of $TRAF1^{hi}$ cells in the CLL cultures.

[00120] As had been shown in figure 4, treatment with PKN1i alone led to selection of CLL with slightly higher Bcl-2 levels. Conversely, CLL cells that survived venetoclax treatment had higher Bcl-xL and Mcl-1 (Fig. 5d). However, treatment with both inhibitors together resulted in a decrease in all three pro-survival Bcl-2 family members in the surviving CLL cells (Fig. 5d,e). Taken together, these data show that by targeting TRAF1, Bcl-xL and Mcl-1, PKN1 inhibition has complementary effects with venetoclax (or other BH3 mimetics) on CLL cells.

[00121] *Ibrutinib does not affect TRAF1 levels but has additive effects with PKN1i on Mcl-1 levels*

[00122] The BTK inhibitor ibrutinib targets a key step in the BCR signaling pathway (Fig. 6a) and has shown promising results in reducing tumor burden. Moreover, ibrutinib in combination with venetoclax has shown remarkable effects in CLL treatment[40,41]. Therefore, it was important to compare the effects of PKN1i with those of ibrutinib. For these experiments a dose of ibrutinib previously shown to completely block BTK activity[42] was chosen. Although BTK is upstream of NF-κB in the BCR signaling pathway, no effect of ibrutinib on levels of TRAF1 or pNF-κB p65 was observed (Fig. 6b,c). Ibrutinib did not produce observable effects on Bcl-xL, pS6 or cell death either alone or in combination with PKN1i and/or venetoclax (data not shown). However, consistent with the literature[43,44], ibrutinib treatment resulted in reduced levels of Mcl-1 in CLL cells and these effects were additive with PKN1i in both OP9 and OP9-CD40L cultures (Fig. 6de). While ibrutinib in combination with venetoclax reduced the overall level of Mcl-1, the combination of Venetoclax plus PKN1i was more effective in eliminating the Mcl-1^{hi} population (Fig. 6e).

[00123] Discussion

[00124] There has been much recent interest and success in targeting antigen receptor signaling in B cell malignancies[45]. However, to date there has been minimal attention paid to TNFR-mediated survival signaling in B cell malignancy, despite the fact that TRAF-binding TNFR family members provide critical survival signals to lymphocytes[2,42,46]. Moreover, there is accumulating evidence for the importance of CD40L in promoting drug resistance and contributing to tumor cell survival in the lymphoid microenvironment[47,48].

[00125] TRAF1 plays a key role in sustaining CD40-mediated signaling in both normal and malignant cells[49,50]. TRAF1, itself an NF-κB induced gene, contributes to a feedback survival loop in the CD40 signaling complex leading to enhanced NF-κB signaling. The present Example has shown that the kinase PKN1 is important to maintain TRAF1 protein levels in cells, at least in part through mediating TRAF1 resistance to cIAP-mediated degradation in the CD40 signaling complex. By lowering the levels or activity of PKN1, TRAF1 protein levels were reduced, thereby breaking the NF-κB dependent feedback survival loop, as evidenced by reduced levels of pNF-κB p65, Bcl-xL and Mcl-1. A strong effect of PKN1i or PKN1 knockdown on levels of pS6 in the malignant B cells was also demonstrated. Although the link between PKN1-TRAF1 and pS6 is not presently known, pS6 is a downstream target of mTOR, and is an important mediator of cell size, and thus highly relevant to cancer signaling[51,52].

[00126] As many CLL cells overexpress both TRAF1 and Bcl-2, it has been suggested that the combination of Bcl-2 and TRAF-induced survival pathways contributes to the development of this disease[53]. Here it has been found that just 24 hours of treatment with a suboptimal dose of venetoclax *in vitro*, was sufficient to enrich for $TRAF1^{hi}$ cells among the surviving cells. These $TRAF1^{hi}$ cells were observed in CLL samples with higher TRAF1 level to start with, consistent with the increased levels of TRAF1 being due to selective survival of $TRAF1^{hi}$ over $TRAF1^{lo}$ cells, rather than due to de novo induction of TRAF1. Consistently, TRAF1 expression in mixed cultures of $TRAF1^{hi}$ and $TRAF1^{lo}$ cells after venetoclax treatment showed that the $TRAF1^{lo}$ cells expressed activated caspase-3, whereas the $TRAF1^{hi}$ cells did not. Treatment of PKN1i and venetoclax in combination allowed reduction of TRAF1, Bcl-xL, Mcl-1 (by PKN1i) and reduced Bcl-2 levels (due to venetoclax) and led to increased cell death compared to either inhibitor alone. Without wishing to be bound by theory, these findings suggest that TRAF1-dependent TNFR superfamily signaling in B-CLL contributes to survival of CLL cells and that overexpression of TRAF1 may represent a mechanism of venetoclax resistance. As TRAF1 levels are readily measured by intracellular flow cytometry, this can be a useful tool to monitor venetoclax resistance.

[00127] Conversely, treatment of CLL cultures with PKN1i alone led to an apparent, albeit modest, increase in Bcl-2, suggesting that PKN1i by lowering TRAF1, Bcl-xL and Mcl-1, selects for CLL that have higher Bcl-2, highlighting the complementarity of the two

pathways. Bcl-2 is a homeostatic pro-survival molecule in normal lymphocytes[54,55] but is often overexpressed in malignancy through chromosomal translocations, gene amplification or dysregulation of transcriptional mechanisms[56]. In contrast, Bcl-xL and Mcl-1 are induced downstream of costimulatory receptors and particularly by TRAF-binding TNFR family members in lymphocytes, and their overexpression in cancer is thought to be due to dysregulated NF-κB activation and/or constitutive signaling[2]. Thus, targeting TRAF1-dependent Bcl-xL and Mcl-1 induction through PKN1 inhibition, provides a complementary approach to inhibition of Bcl-2.

[00128] Although the target of ibrutinib, BTK, is upstream of NF-κB signaling in CLL cells, no effect of ibrutinib on TRAF1 was observed. Moreover, ibrutinib did not reduce pNF-κB p65 levels, although a decrease in Mcl-1 was observed that was additive with the effects of PKN1i. *Ex vivo* analysis of CLL cells has shown that the de novo resistance that arises in ibrutinib and venetoclax treated-cells in response to microenvironmental agonists, such as CD40L, is largely due to NF-κB -dependent overexpression of antiapoptotic proteins[42]. By targeting the multiple signaling pathways, including NF-κB, downstream of TRAF1-dependent TNFR signaling, PKN1i has unique effects compared to BCR signaling inhibitors. PKN1i, by decreasing the TNFR-TRAF1-signaling axis, will block CD40L or other TNF family survival signals from the microenvironment and may therefore be superior to Ibrutinib in reducing NF-κB-mediated apoptosis-resistance in patients.

[00129] This study showed that patient samples with highest starting levels of TRAF1 were those that were resistant to a suboptimal venetoclax dose. Taken together, this data demonstrates that inhibitors of PKN1 are useful to avoid TRAF1-dependent venetoclax resistance. Further, B cell malignancies can express a variety of TNFRs including TNFR2, CD30, 4-1BB, or the EBV protein, LMP1, all of which exhibit TRAF1-dependent signalling[57]. Therefore, this Example demonstrates that PKN1 inhibition functions to inhibit TRAF1-dependent signaling in B-CLL and other TRAF1-positive B cell malignancies.

[00130] EXAMPLE 2: Additional Selective PKN1 inhibitors

[00131] To identify additional PKN1 inhibitors, a screen was performed on 700 known kinase inhibitors from the Ontario Institute for Cancer Research (OICR) collection. Inhibitors were tested at a single dose of 1μM and then 25 of these that showed inhibition of PKN1 were selected for further titrations. From this secondary screen, 5 compounds (see Table 2) were

selected for further study based on low IC50 for PKN1 and other considerations. The 5 inhibitors were added to the B lymphoma RAJI at the concentrations indicated in the figures for 24hrs, then cells were permeabilized for staining intracellular TRAF1 and analysis by flow cytometry. The compounds are available from OICR and/or commercially through multiple sources such as Medchem Express, Selleckchem and Cayman Chemical.

[00132] Purified TRAF1 antibody (clone 1F3, Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany) was used at 2 μg/ml, prior to secondary staining with goat anti-rat PE (clone poly4054, Biolegend) added at a 1:100 dilution. TRAF1 staining was first tested on RAJI cells expressing an shRNA to knockdown TRAF1 (shTRAF1), in RAJI cells with shRNA to knockdown PKN1 or control RAJI cells or RAJI cells expressing a control shRNA. The results show lowered staining compared to control RAJI cells when TRAF1 or PKN1 were knocked down (Fig. 10a). The effect of the 5 selected inhibitors from Table 2 on the mean fluorescence intensity of TRAF1 measured by flow cytometry 24hrs after addition to the RAJI cells is shown in Fig. 10b. Of these, two compounds showed substantial dose-dependent reduction in TRAF1 protein levels. The %initial TRAF1 versus concentration of inhibitor is shown in Fig. 10c, which shows that 2 of the 5 PKN1 inhibitors substantially reduced TRAF1 levels in the lymphoma cells.

[00133] The inhibitor OICR 00019288 (a MELK inhibitor, previously noted to have effects on CLL[59]) has an IC50 for PKN1 of 11nm, and an IC50 for lowering TRAF1 in RAJI cells of 88nm (see Table 3). The inhibitor OICR 08727 (an AKT inhibitor) has an IC50 for PKN1 of 49 nM and an IC50 for lowering TRAF1 in RAJI cells of 3.8 μM (see Table 3). The other PKN1 inhibitors had only weak effects on TRAF1.

[00134] The previous inhibitor, PKN1i (also known as GSK3367291A) has an IC50 for PKN1 of 0.8μM and an IC50 for lowering TRAF1 in RAJI of approximately 5μM (see Table 3). Therefore, the order of potency of inhibition of PKN1 by direct measurements matches the order of potency in reducing TRAF1 protein levels, suggesting, without wishing to be bound by theory, that this is due to an on-target effect on PKN1. Therefore, these results demonstrated that PKN1 inhibition may be useful to lower TRAF1 in lymphomas or CLL cells to treat cancer.

[00135] All publications, patents and patent applications mentioned in this Specification are indicative of the level of skill of those skilled in the art to which this invention pertains and are herein incorporated by reference to the same extent as if each

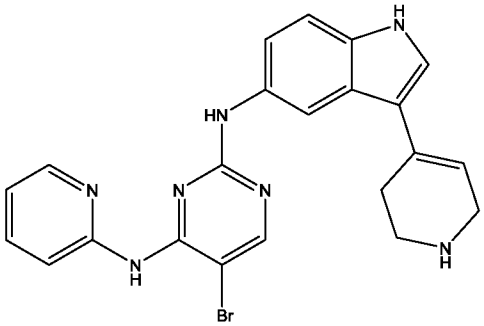
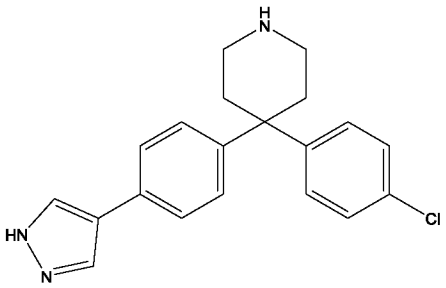
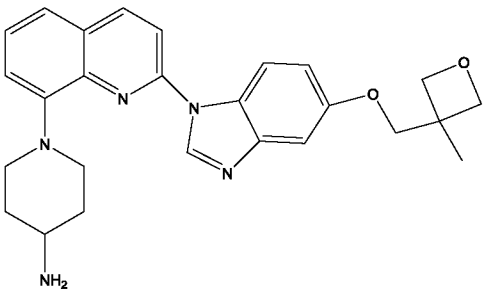
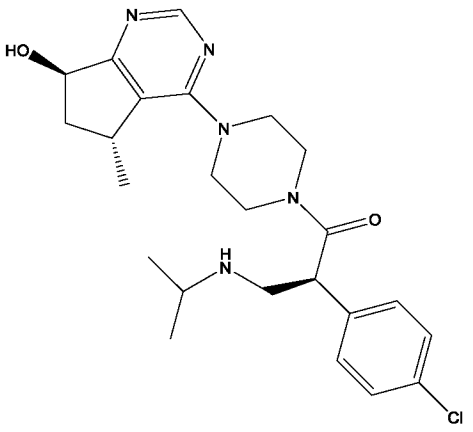
individual publication, patent, or patent applications was specifically and individually indicated to be incorporated by reference.

[00136] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

Table 1: Patient Demographic and Clinical Characteristics for CCL samples used in Example 1.

Cohort	Sunnybrook	PMH	Total
Age			
Median - *yr*	69.1	59.1	64.9
Range - *yr*	44-86	39-82	39-86
		No. (%) of patients	
n=	28 (58.3)	20 (41.7)	48 (100)
Sex			
Male	15 (53.6)	14 (70.0)	29 (60.4)
Vital Status			
Deceased	0 (0)	5 (25.0)	5 (10.4)
Lost to follow-up	0 (0)	1 (5.0)	1 (2.1)
Rai Stage			
0	2 (7.1)	0 (0.0)	2 (4.2)
I	3 (10.7)	1 (5.0)	4 (8.3)
II	5 (17.9)	2 (10.0)	7 (14.6)
III	2 (7.1)	1 (5.0)	3 (6.3)
IV	16 (57.2)	15 (75.0)	31 (64.5)
Not tested	0 (0)	1 (5.0)	1 (2.1)
β2-microglobulin			
>3.5 mg/liter	14 (50.0)	Not tested	N/A
FISH			
Not tested	6 (12.5)	5 (25.0)	11 (22.9)
		No. (%) of tested patients	
del(17p)	3 (13.6)	1 (6.7)	4 (10.8)
del(13q)	14 (63.6)	9 (60.0)	23 (62.1)
del(11q)	2 (9.0)	1 (6.7)	3 (8.1)
Trisomy 12	2 (7.9.0)	1 (6.7)	3 (8.1)

Table 2:

Expt ID	OICR ID	OICR Name	Chemical Formula	PKN1 IC50 (nM)
1	OICR0007450A01	PERK inhibitor, CP-22, CP-941222, PF-941222		18
2	OICR0008727A01	AT7867, AT-7867, AT-867		49
3	OICR0008764A01	Crenolanib, ARO-002, CP-868596		22
4	OICR0011116A01	GDC-0068, RG-7440, Ipatasertib		30

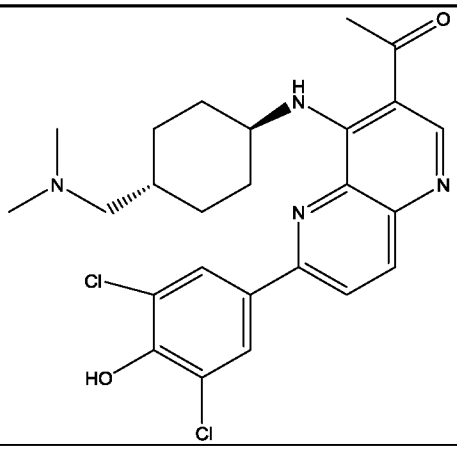
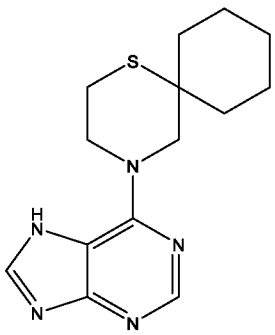
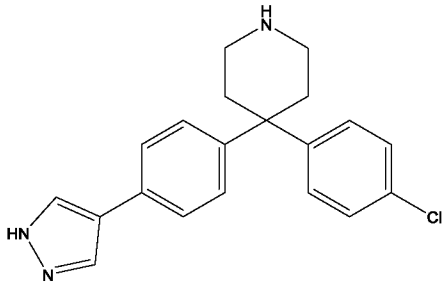
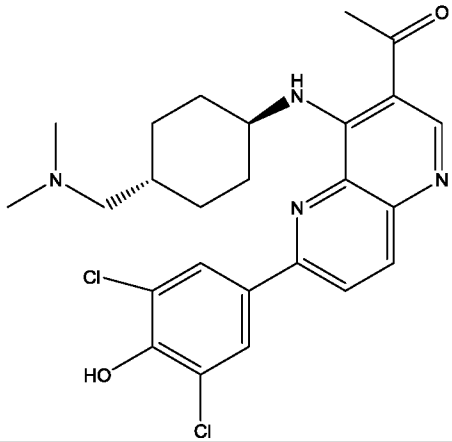
Expt ID	OICR ID	OICR Name	Chemical Formula	PKN1 IC50 (nM)
5	OICR001928 8	OTSSP167, OTS-167, OTS-167PO, OTSSP-167		11

Table 3:

Compound	OICR or GSK name	Target	IC50 PKN1 (μM)	IC50 To lower TRAF1 RAJI (μM)
PKN1i 	GSK3367291A	PKN1	0.8	5
OICR0008727A01 	AT7867, AT-7867, AT-867	AKT1 (PKBa), AKT2 (PKBb), AKT3 (PKBg), p70S6K (S6K, S6K1)	0.049	3.6
OICR0019288 	OTSSP167, OTS-167, OTS-167PO, OTSSP-167	Maternal Embryonic Leucine Zipper Kinase (MELK, PK38) Inhibitor	0.011	.088

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WE CLAIM:

1. A use of a protein kinase C related kinase (PKN1) inhibitor for treating a TRAF1 expressing B cell related cancer in a subject in need thereof.

2. The use of claim 1, wherein the PKN1 inhibitor is used in the absence of a nutrient stress-inducing agent.

3. The use of claim 1 or 2, wherein the TRAF1 expressing B cell related cancer is Chronic Lymphocytic Leukemia (CLL).

4. The use of any one of claims 1 to 3, wherein the PKN1 inhibitor selectively inhibits PKN1 over protein kinase C θ (PKCθ).

5. The use of any one of claims 1 to 4, wherein the PKN1 inhibitor is 4-(7H-purin-6-yl)-1-thia-4-azaspiro[5.5]undecane or a pharmaceutically acceptable salt or solvate thereof.

6. The use of any one of claims 1 to 3, wherein the PKN1 inhibitor is is 4-(4-(1H-pyrazol-4-yl)phenyl)-4-(4-chlorophenyl)piperidine or a pharmaceutically acceptable salt or solvate thereof.

7. The use of any one of claims 1 to 3, wherein the PKN1 inhibitor is 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

8. The use of any one of claims 1 to 4, wherein the PKN1 inhibitor is not 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

9. The use of any one of claims 1 to 8, wherein the PKN1 inhibitor is used in combination with another chemotherapeutic agent.

10. The use of claim 9, wherein the other chemotherapeutic agent is a Bcl-2 antagonist.

11. The use of claim 10, wherein the Bcl-2 antagonist is a BH3 mimetic.

12. The use of claim 11, wherein the BH3 mimetic is venetoclax.

13. The use of claim 9, wherein the other chemotherapeutic agent is a BTK inhibitor.

14. The use of claim 13, wherein the BTK inhibitor is ibrutinib.

15. Use of a protein kinase C related kinase (PKN1) inhibitor to reduce a subject's resistance to a Bcl-2 antagonist.

16. The use according to claim 15, wherein the Bcl-2 antagonist is venetoclax.

17. The use of claim 15 or 16, wherein the PKN1 inhibitor is used in the absence of a nutrient stress-inducing agent.

18. The use of any one of claims 15 to 17, wherein the leukemia is Chronic Lymphocytic Leukemia (CLL).

19. The use of any one of claims 15 to 18, wherein the PKN1 inhibitor selectively inhibits PKN1 over protein kinase C θ (PKCθ).

20. The use of any one of claims 15 to 19, wherein the PKN1 inhibitor is 4-(7H-purin-6-yl)-1-thia-4-azaspiro[5.5]undecane or a pharmaceutically acceptable salt or solvate thereof.

21. The use of any one of claims 15 to 18, wherein the PKN1 inhibitor is 4-(4-(1H-pyrazol-4-yl)phenyl)-4-(4-chlorophenyl)piperidine or a pharmaceutically acceptable salt or solvate thereof.

22. The use of any one of claims 15 to 18, wherein the selective PKN1 inhibitor is 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

23. The use of any one of claims 15 to 19, wherein the selective PKN1 inhibitor is not 1-(6-(3,5-dichloro-4-hydroxyphenyl)-4-(((1*r*,4*r*)-4-((dimethylamino)methyl)cyclohexyl)amino)-1,5-naphthyridin-3-yl)ethan-1-one (OICR 00019288) or a pharmaceutically acceptable salt or solvate thereof.

24. A method for identifying a patient susceptible to venetoclax resistance comprising the step of determining whether the patient has a B cell related cancer with a high or a low expression of TRAF1, wherein a high expression of TRAF1 is an indicator of venetoclax resistance in the patient.

25. The method of claim 24, wherein the B cell related cancer is Chronic Lymphocytic Leukemia (CLL).

26. The method of claim 24 or 25, wherein the step of determining whether the patient has a B cell related cancer with a high or a low expression of TRAF1, comprises comparing a sample of cells from the patient with a control sample of cells from a tumor of the same B cell related cancer and having a known expression level of TRAF1.

27. The method of any one of claims 24 to 26, wherein the method comprises intracellular flow cytometry to measure TRAF1 levels.

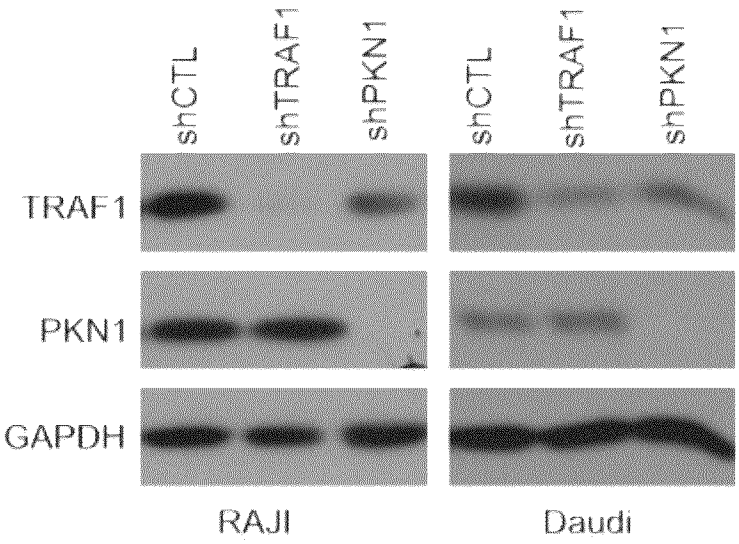


Figure 1a

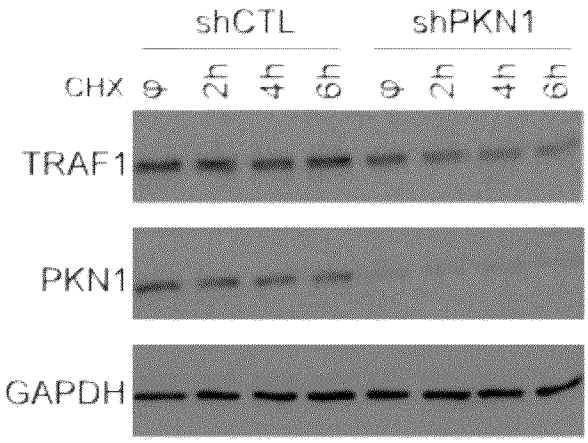


Figure 1b

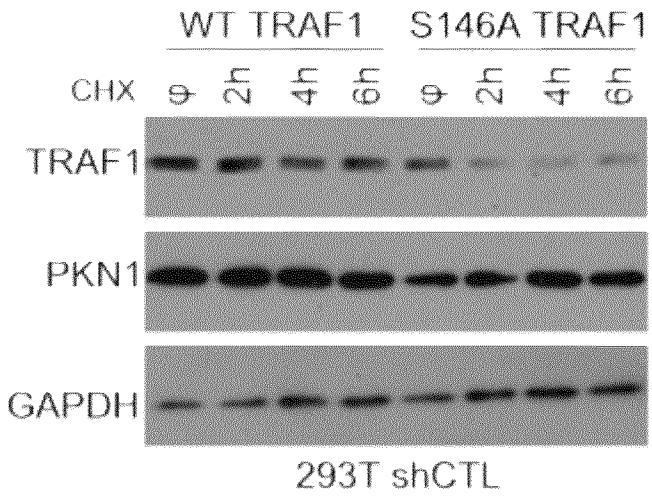


Figure 1c

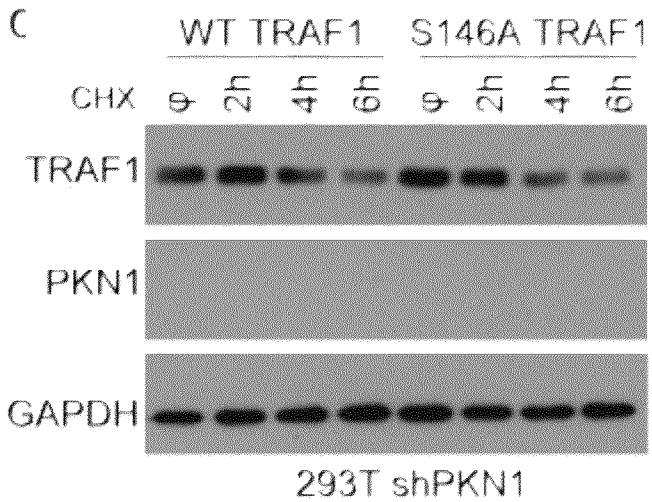
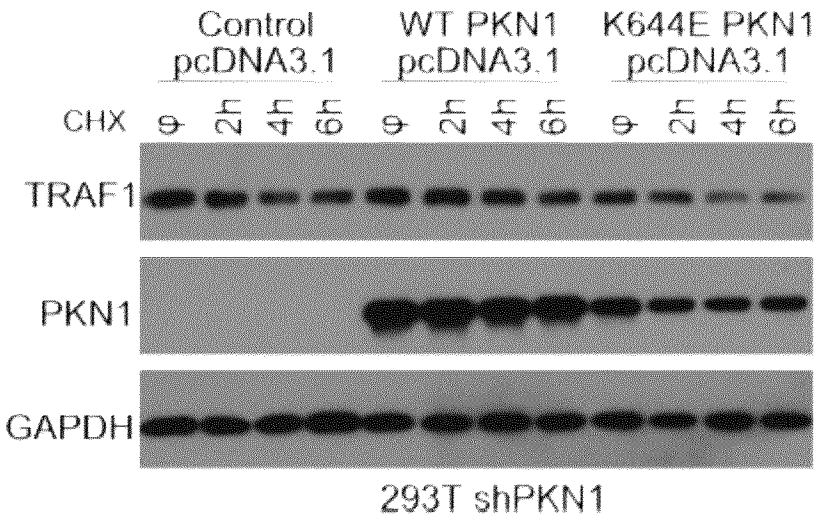


Figure 1d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WT PKN1
pcDNA3.1
K644E PKN1
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CHX
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2h
4h
6h
TRAF1
PKN1
GAPDH
293T shPKN1

Figure 1e

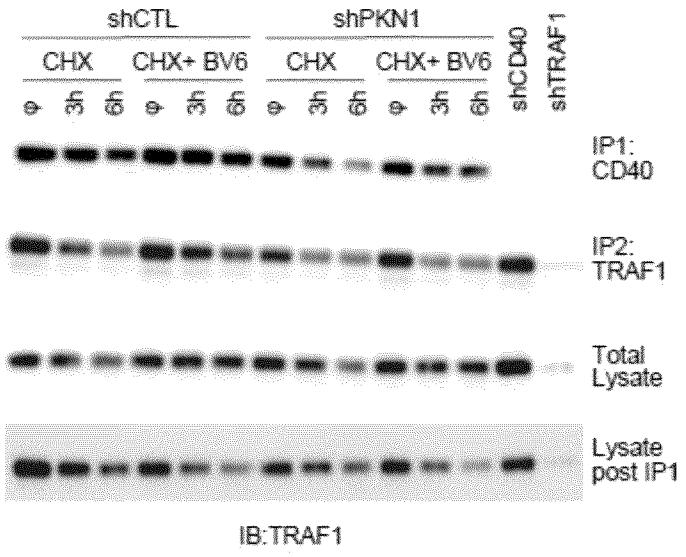

Figure 2a

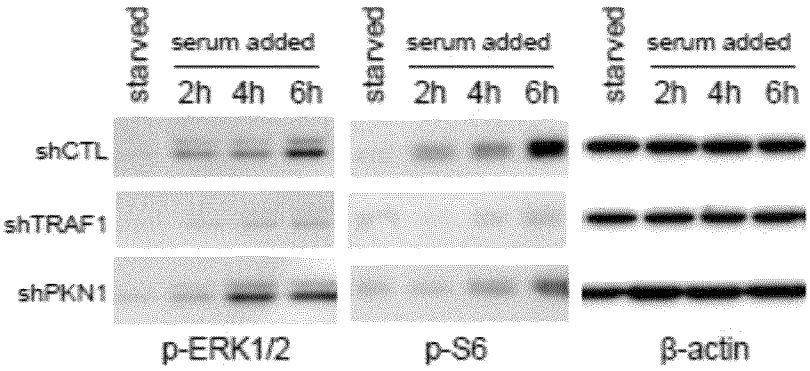

Figure 2b

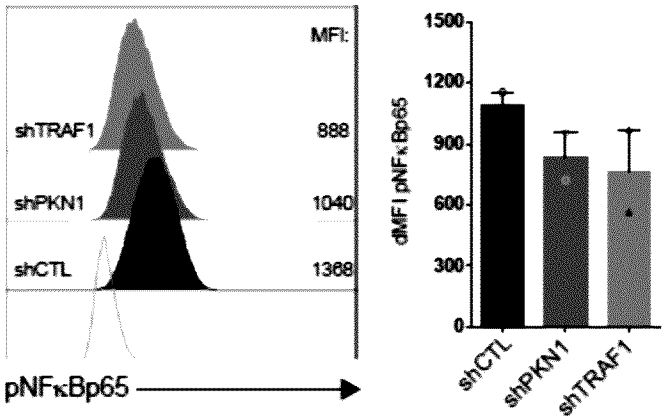

Figure 2c

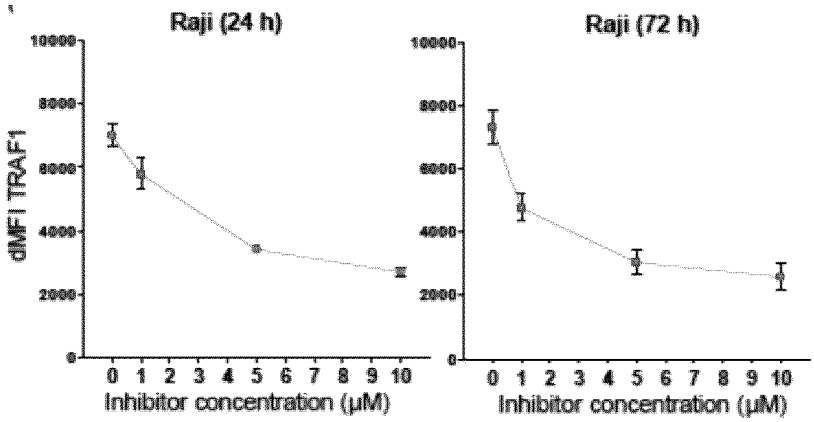

Figure 2d

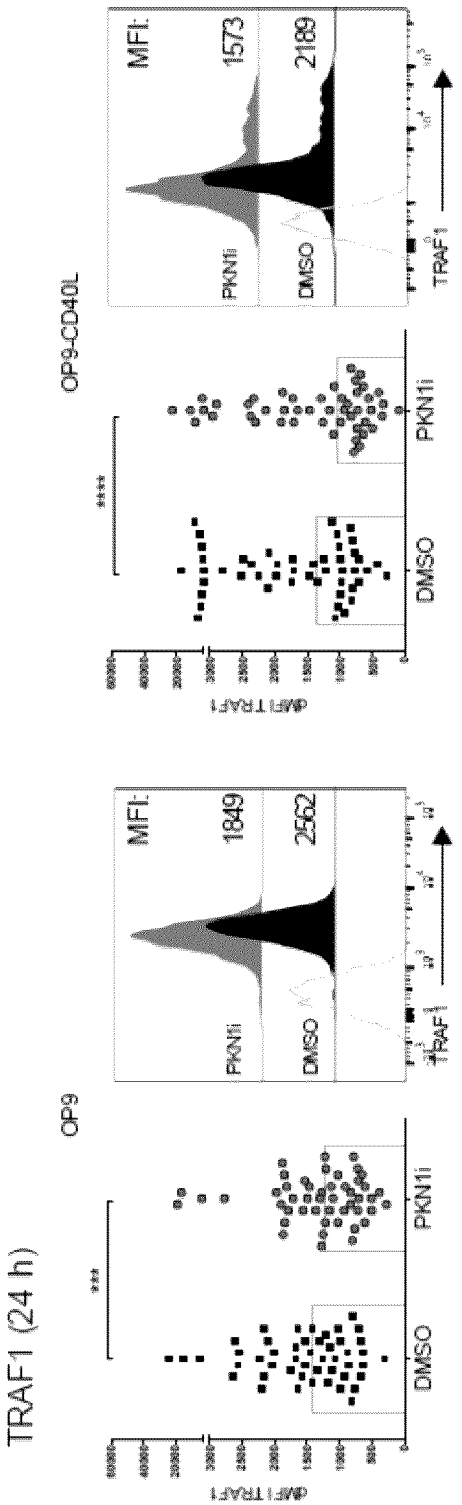
TRAF1 (24 h)
OP9
OP9-CD40L
dMFI TRAF1
***
DMSO
PKN1i
MFI:
1849
2562
1573
2189
TRAF1

Figure 3a

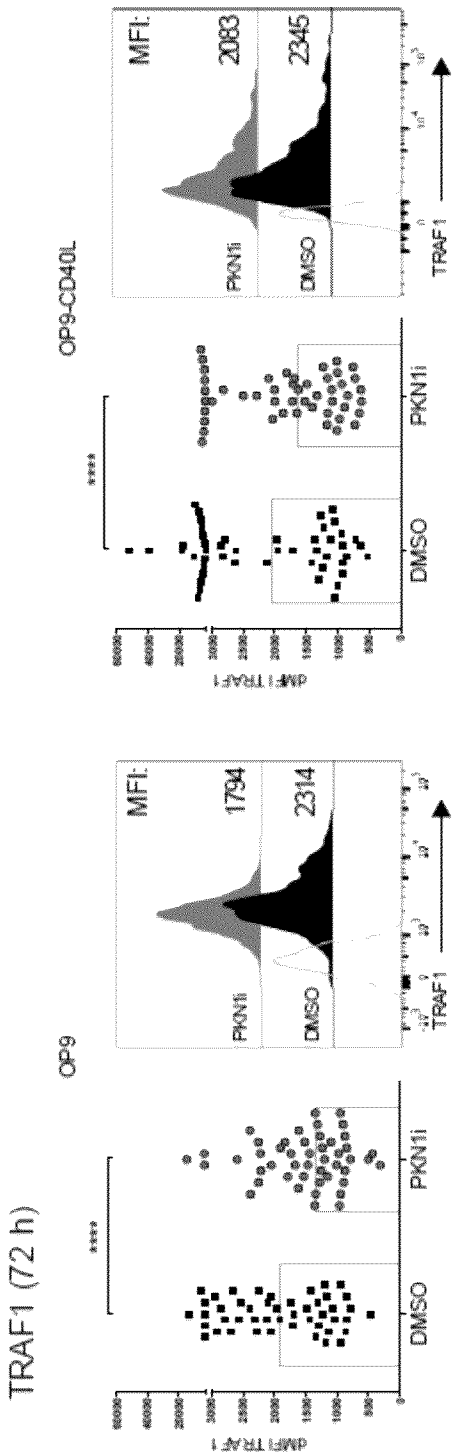
TRAF1 (72 h)
OP9
OP9-CD40L
DMSO
PKN1i
****
dMFI TRAF1
MFI:
1794
2314
2083
2345
TRAF1

Figure 3b

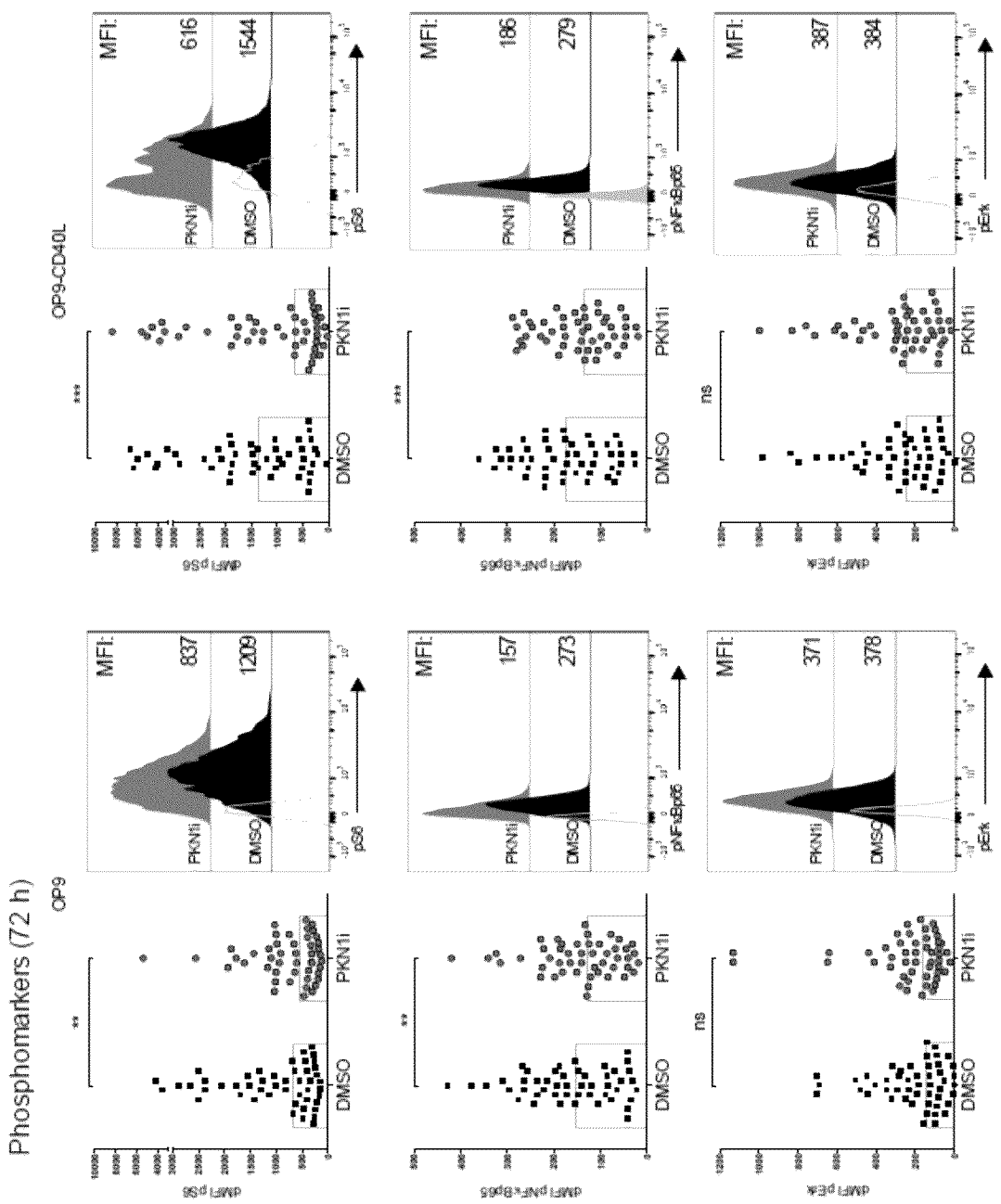
Phosphomarkers (72 h)
OP9
OP9-CD40L
dMFI pS6
dMFI pNFκBp65
dMFI pErk
MFI:
837
1209
157
273
371
378
616
1544
186
279
387
384
PKN1i
DMSO
pS6
pNFκBp65
pErk
**
***
ns

Figure 3c

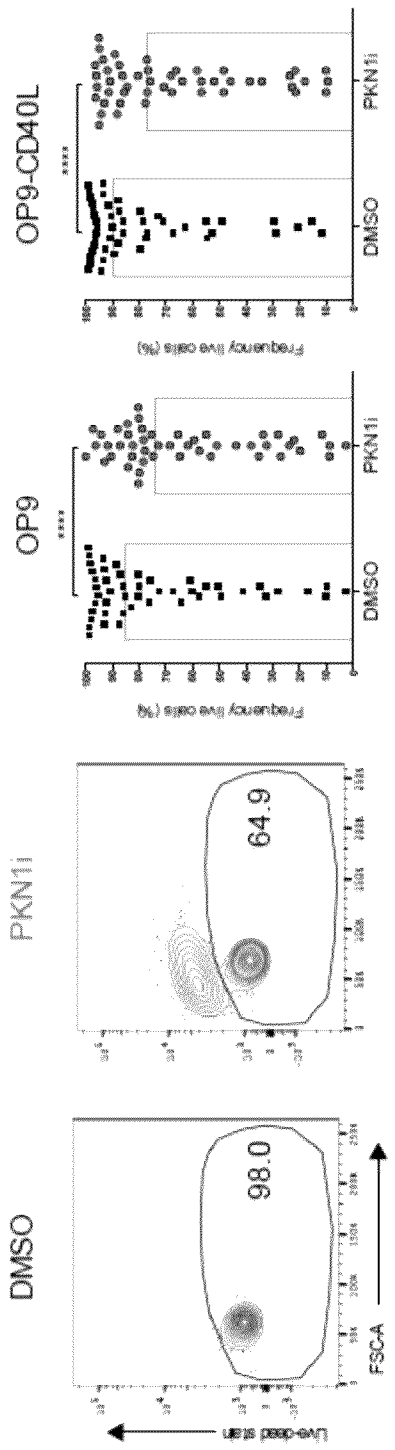
DMSO
PKN1i
98.0
64.9
Live-dead stain
FSCA
OP9
****
DMSO
PKN1i
Frequency live cells (%)
OP9-CD40L
****
DMSO
PKN1i
Frequency live cells (%)

Figure 4a

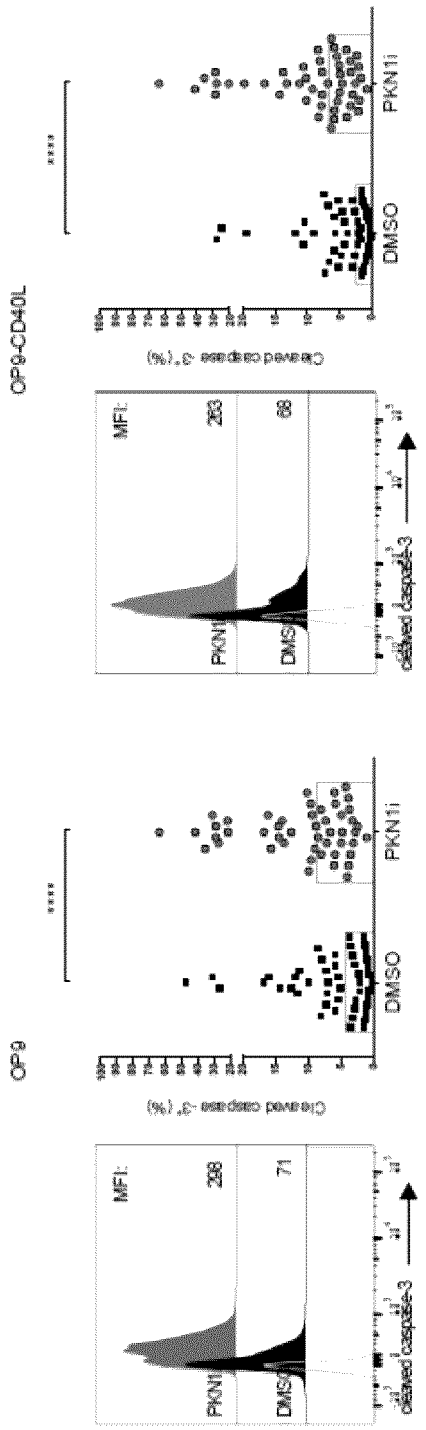
OP9-CD40L
OP9
Cleaved caspase-3+ (%)
DMSO
PKN1i
****
MFI:
263
68
298
71
PKN1i
DMSO
cleaved caspase-3

Figure 4b

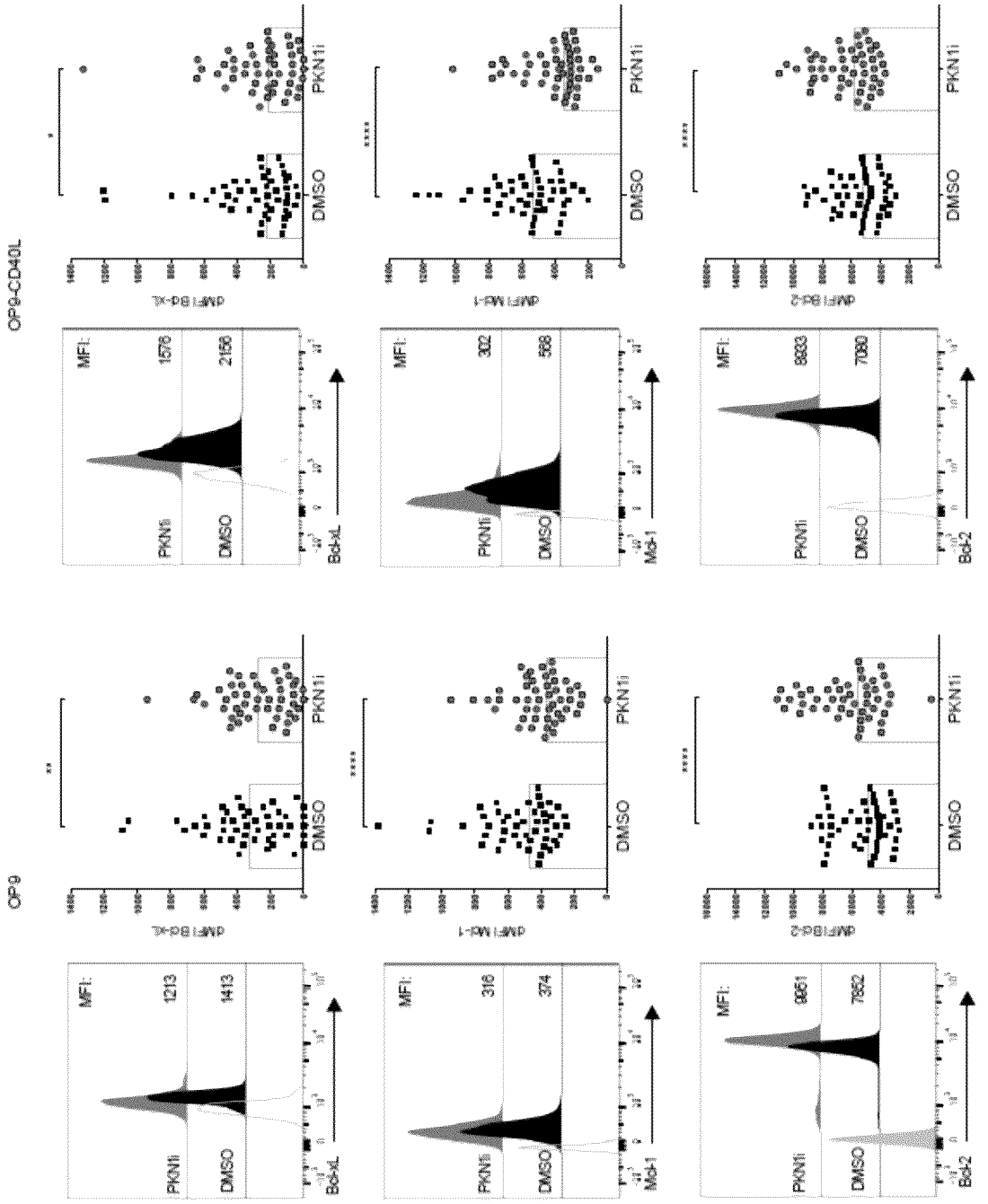
OP9
OP9-CD40L
dMFI Bcl-xL
dMFI Mcl-1
dMFI Bcl-2
DMSO
PKN1i
MFI:
1213
1413
316
374
9661
7852
1578
2156
302
568
8833
7080
Bcl-xL
Mcl-1
Bcl-2

Figure 4c

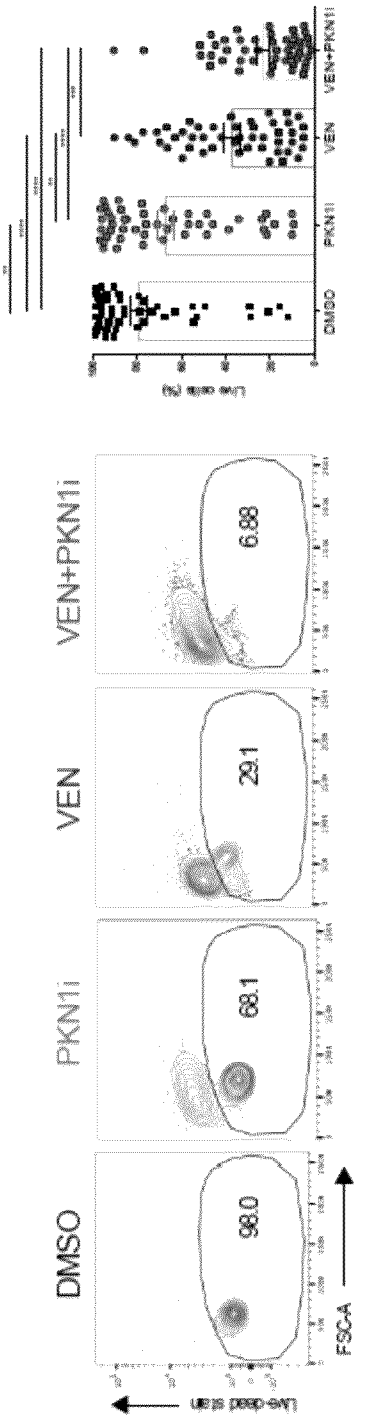
DMSO
PKN1i
VEN
VEN+PKN1i
98.0
68.1
29.1
6.88
FSC-A
DMSO
PKN1i
VEN
VEN+PKN1i

Figure 5a

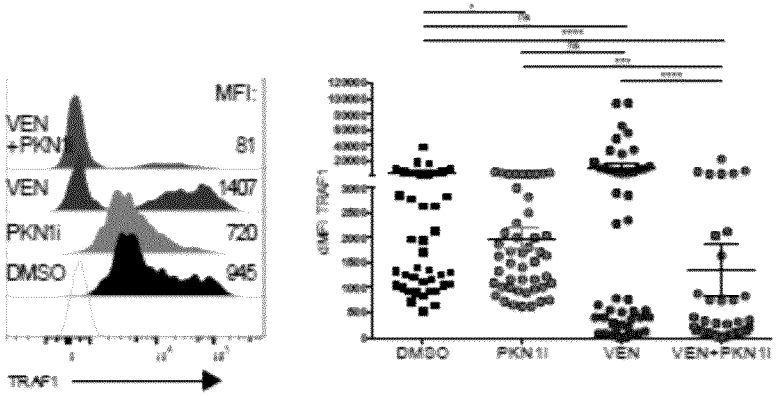

Figure 5b

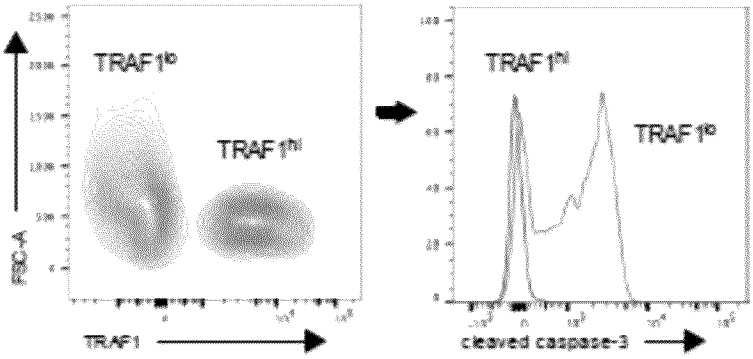

Figure 5c

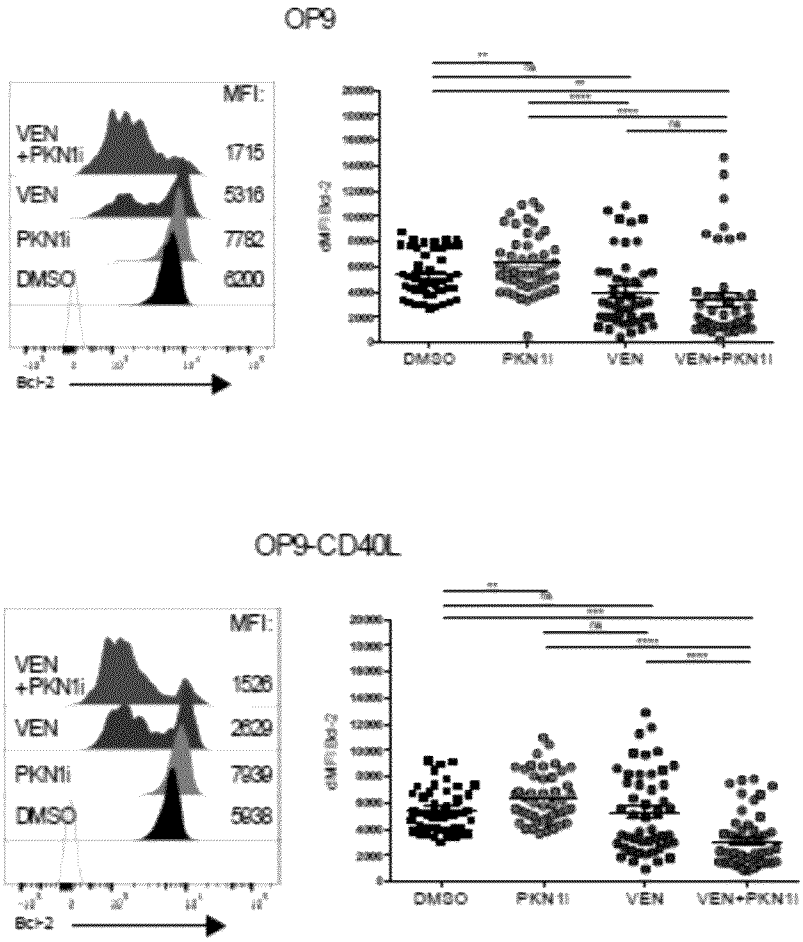
OP9
MFI:
VEN +PKN1i
1715
VEN
5316
PKN1i
7782
DMSO
6200
Bcl-2
gMFI Bcl-2
DMSO
PKN1i
VEN
VEN+PKN1i
OP9-CD40L
MFI:
VEN +PKN1i
1526
VEN
2629
PKN1i
7839
DMSO
5838
Bcl-2
gMFI Bcl-2
DMSO
PKN1i
VEN
VEN+PKN1i

Figure 5d

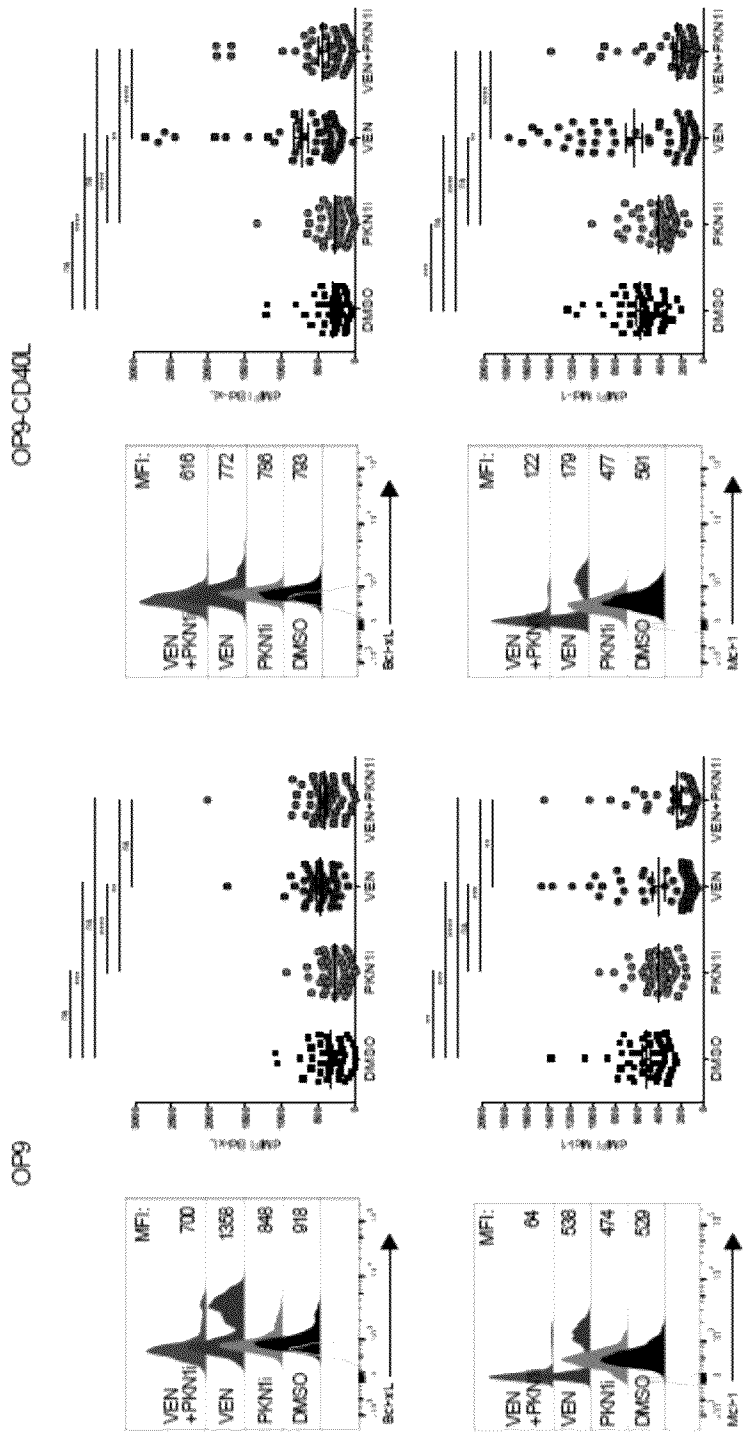
OP9
OP9-CD40L
MFI:
VEN +PKNi
VEN
PKNi
DMSO
700
1336
848
918
616
772
798
783
64
538
474
629
122
179
477
591
Bcl-xL
Mcl-1
DMSO
PKNi
VEN
VEN+PKNi

Figure 5e

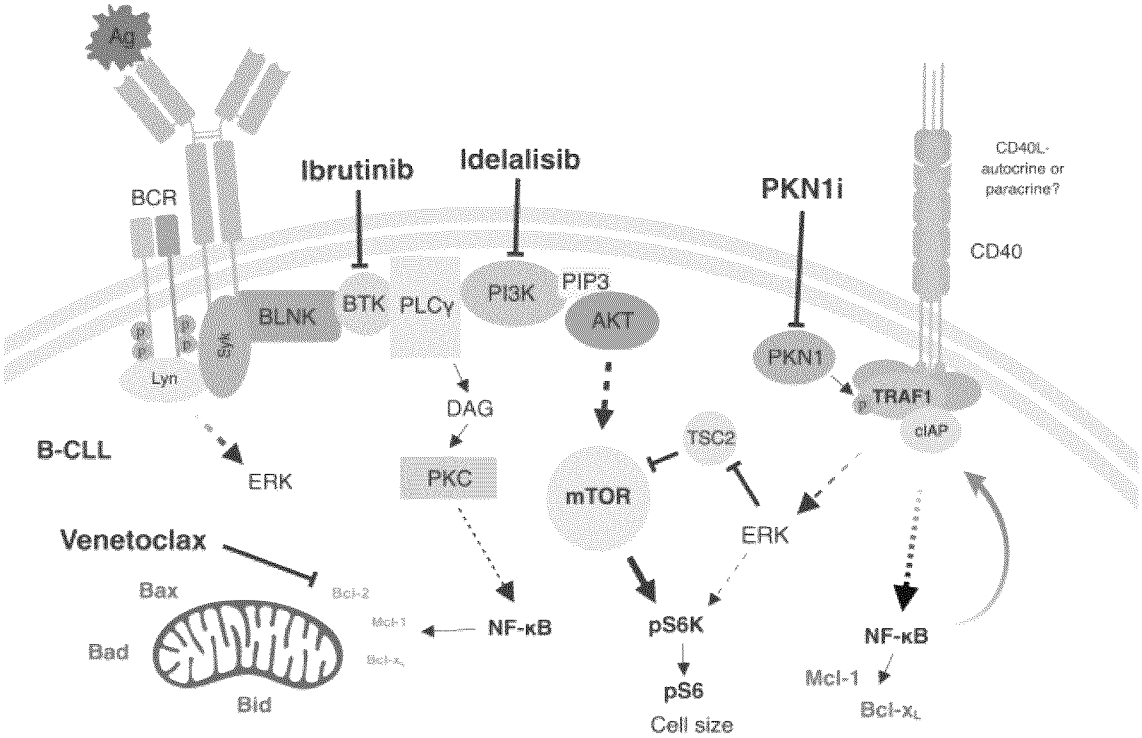

Figure 6a

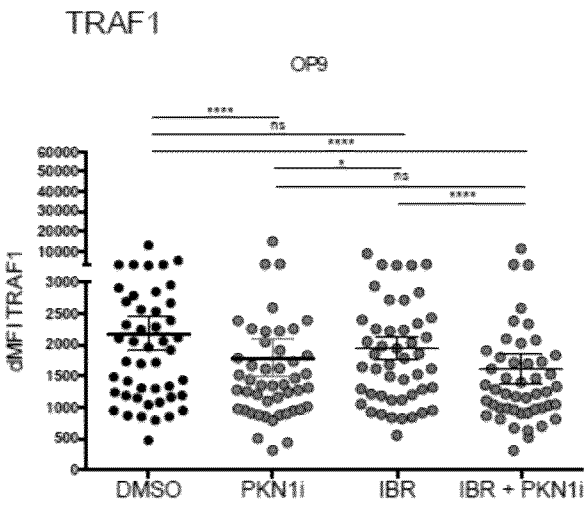

Figure 6b

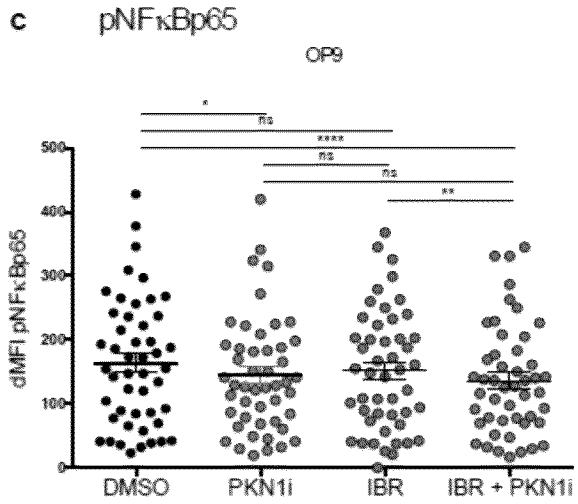

Figure 6c

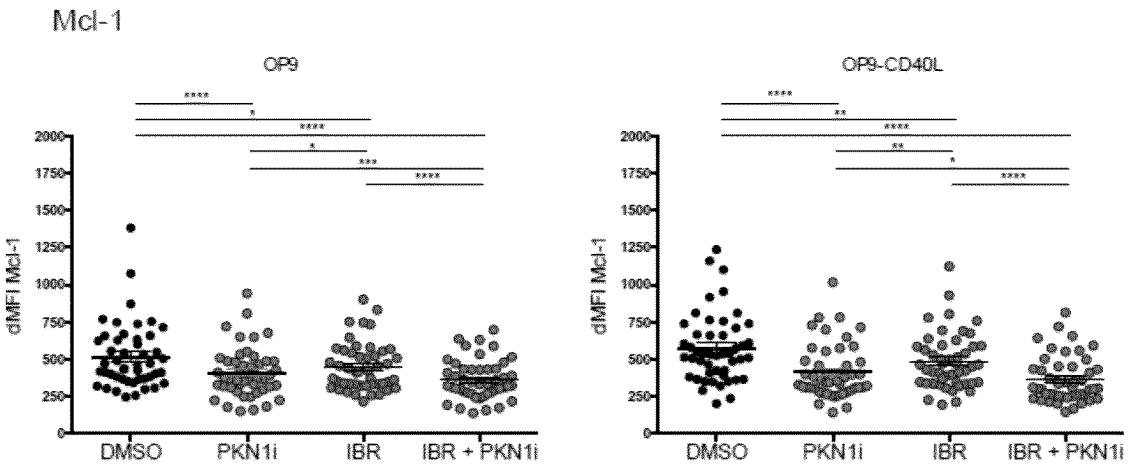

Figure 6d

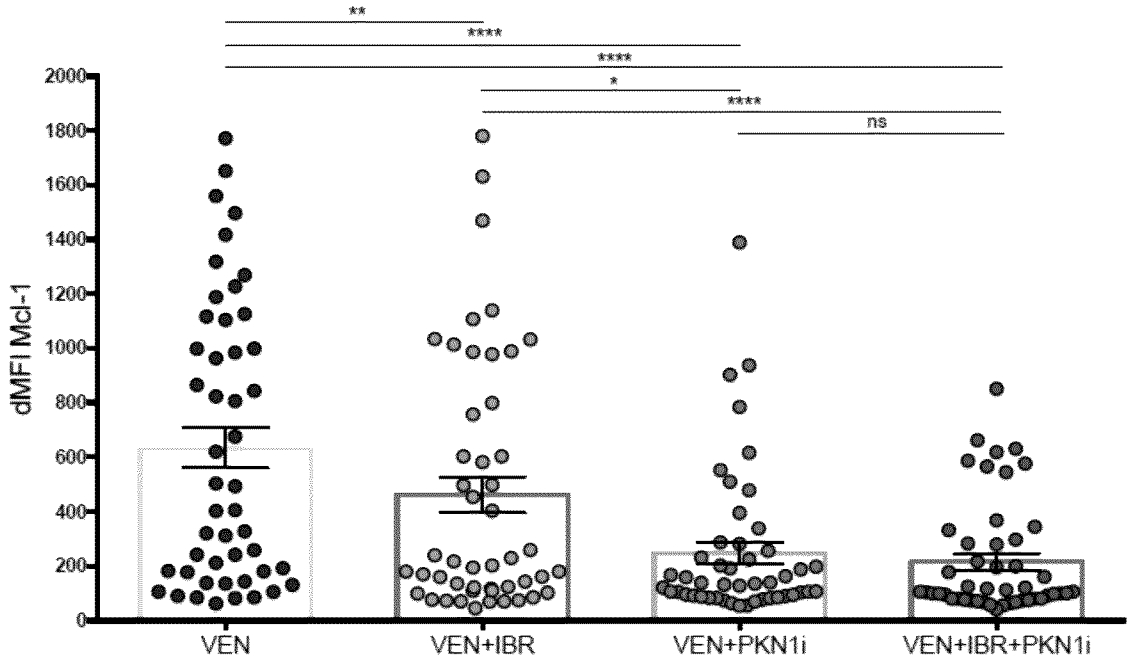
**
****
****
*
****
ns
2000
1800
1600
1400
1200
1000
800
600
400
200
0
dMFI Mcl-1
VEN
VEN+IBR
VEN+PKN1i
VEN+IBR+PKN1i

Figure 6e

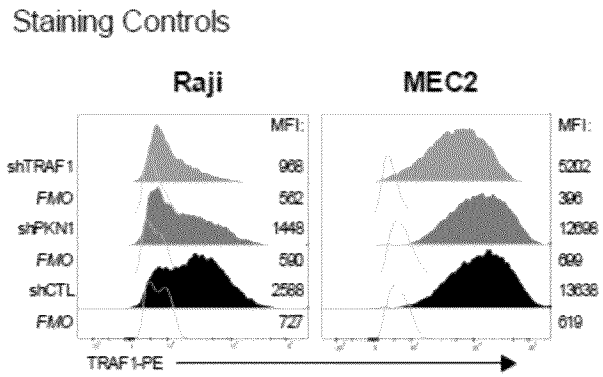

Figure 7a

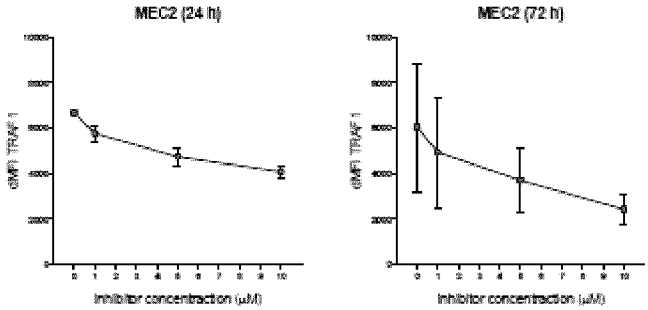

Figure 7b

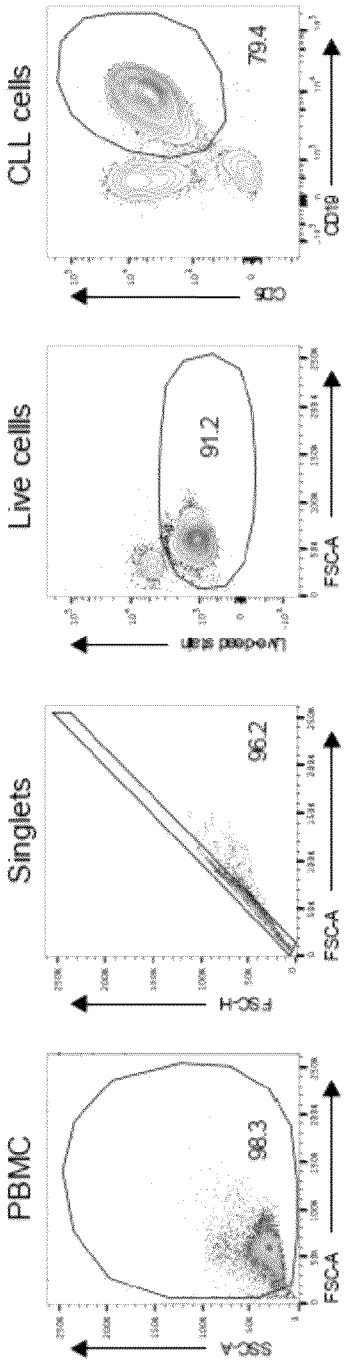
PBMC
98.3
SSC-A
FSC-A
Singlets
96.2
FSC-H
FSC-A
Live cells
91.2
Livedead stain
FSC-A
CLL cells
79.4
CD5
CD19

Figure 7c

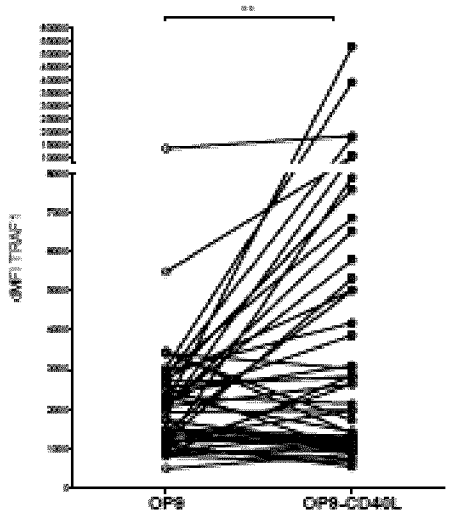

Figure 7d

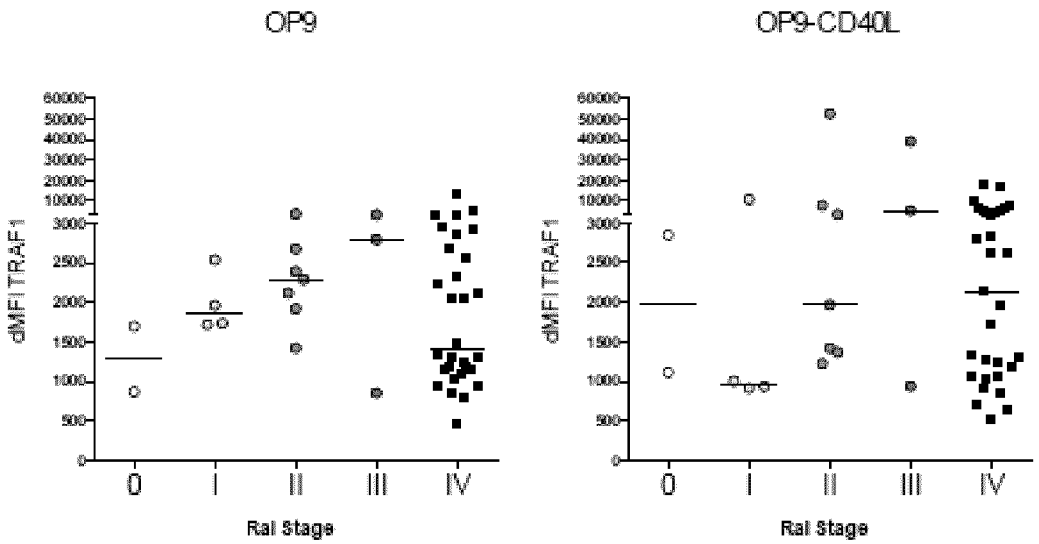

Figure 7e

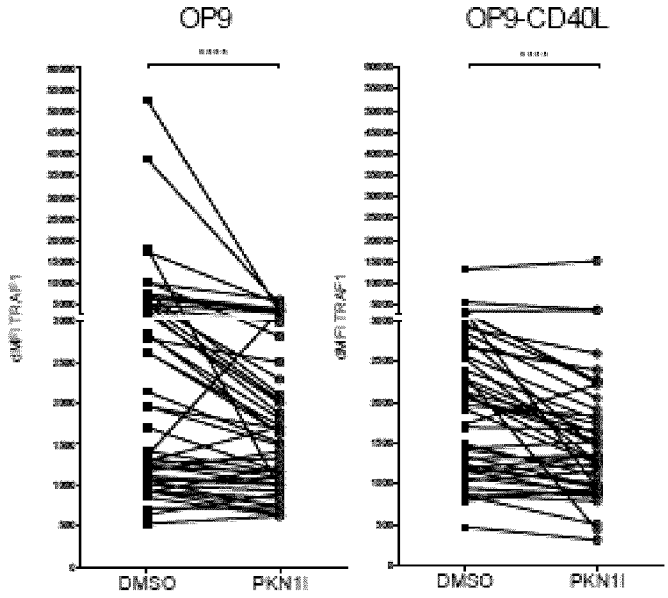

Figure 7f

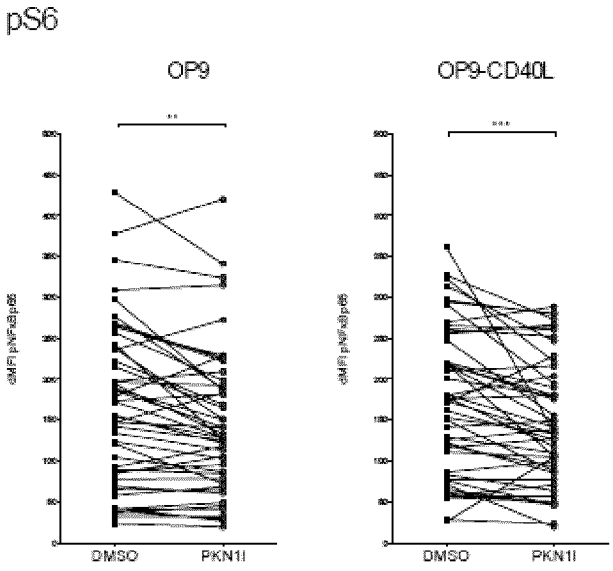

Figure 8a

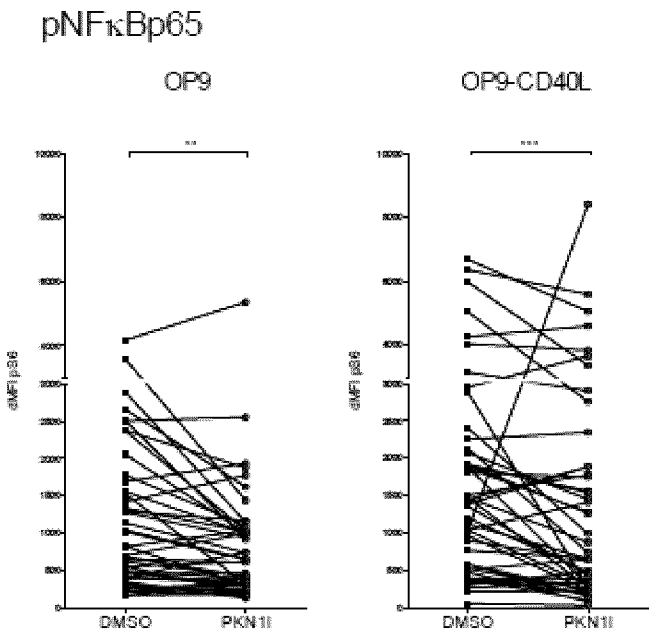

Figure 8b

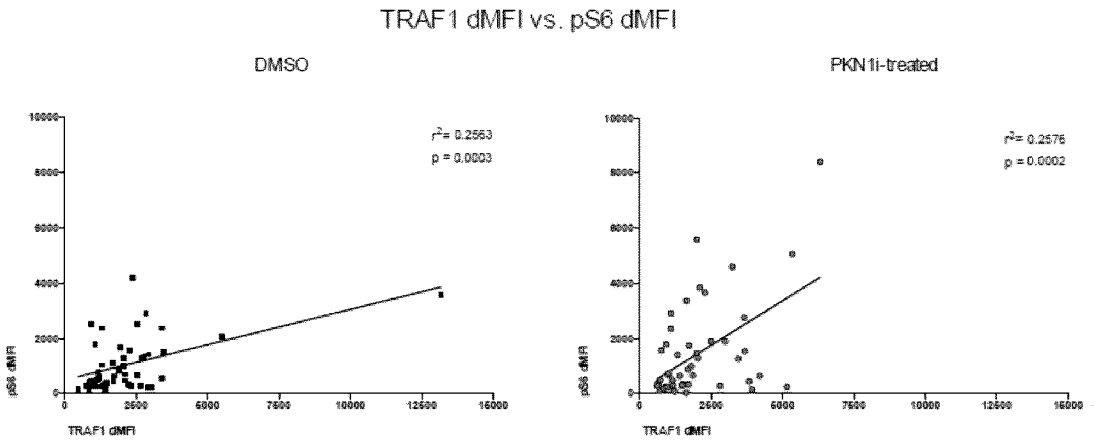

Figure 8c

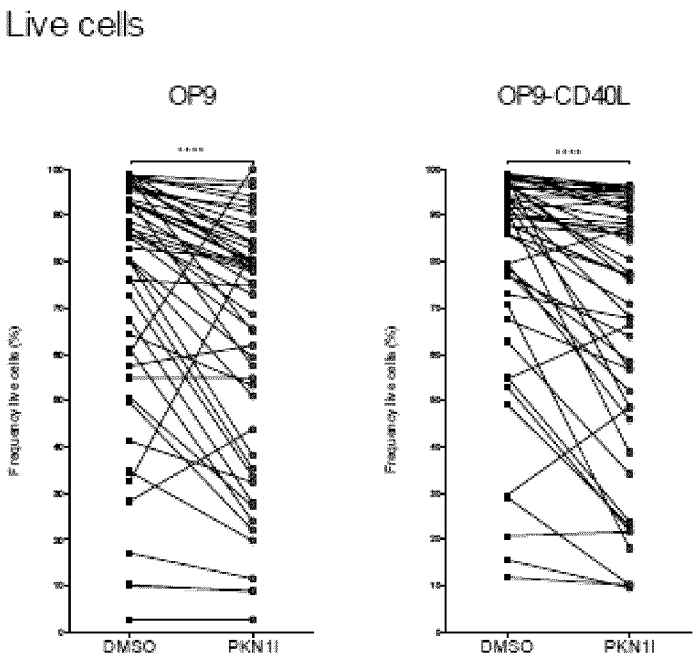

Figure 8d

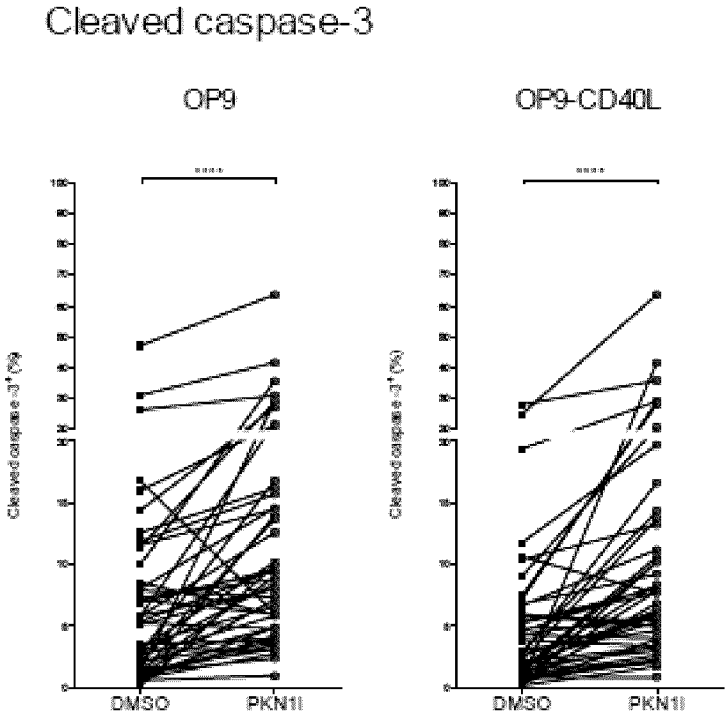

Figure 8e

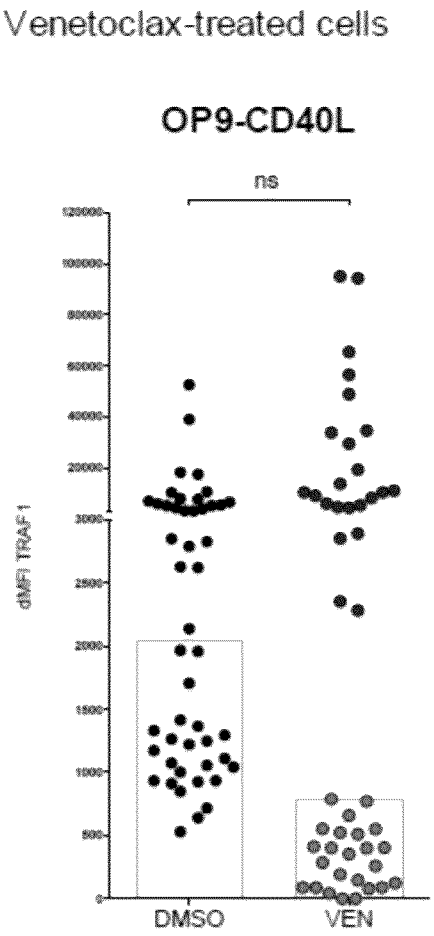

Figure 9a

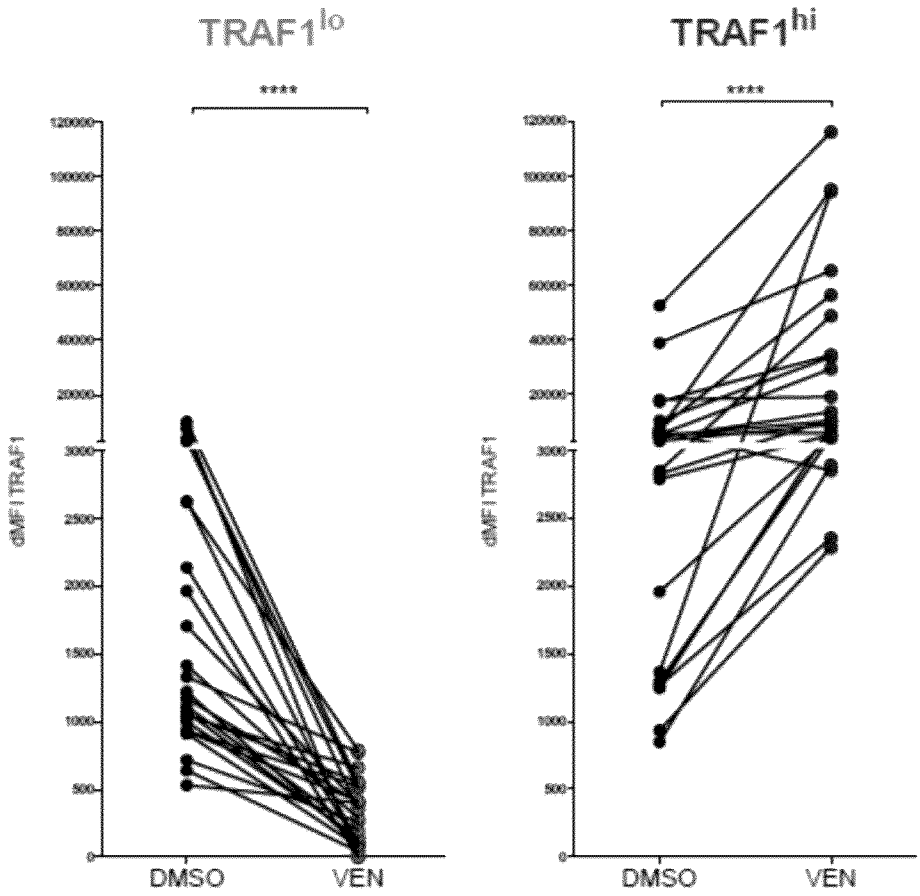
TRAF1lo
TRAF1hi
****
****
dMFI TRAF1
120000
100000
80000
60000
40000
20000
3000
2500
2000
1500
1000
500
0
DMSO
VEN
DMSO
VEN

Figure 9b

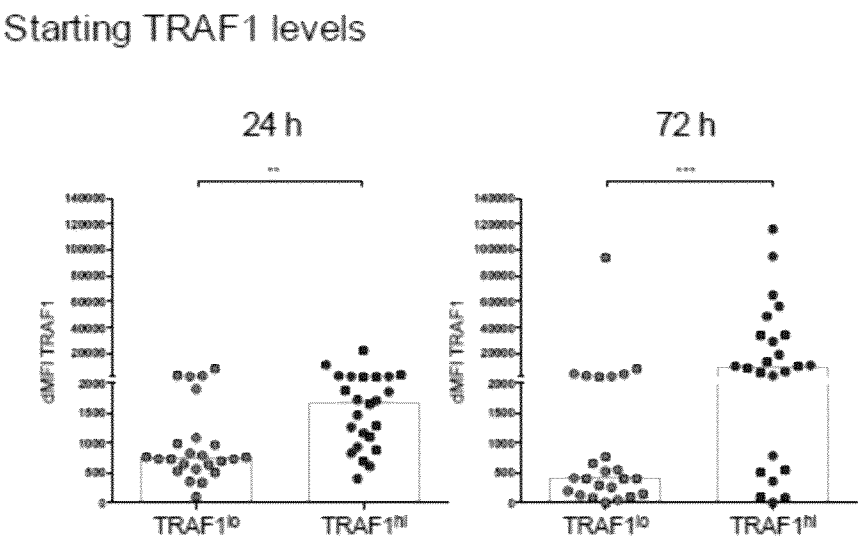

Figure 9c

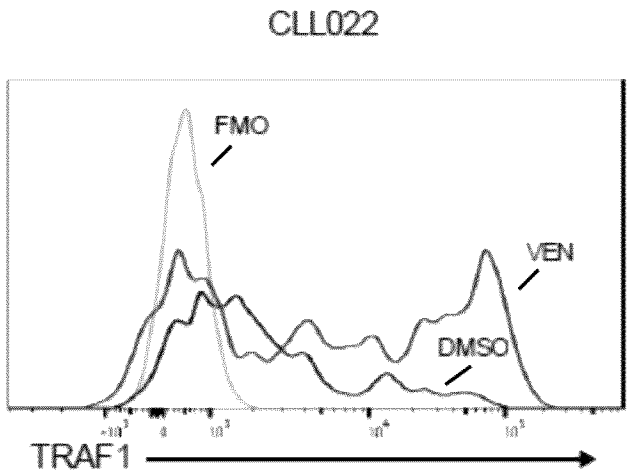

Figure 9d

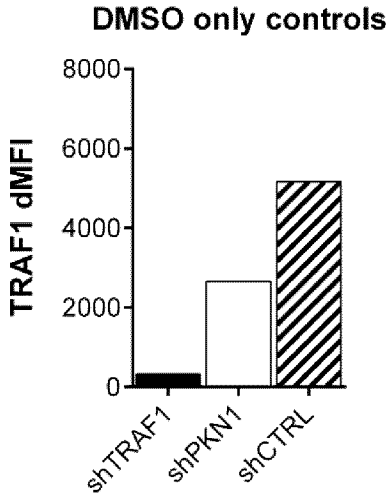

Figure 10a

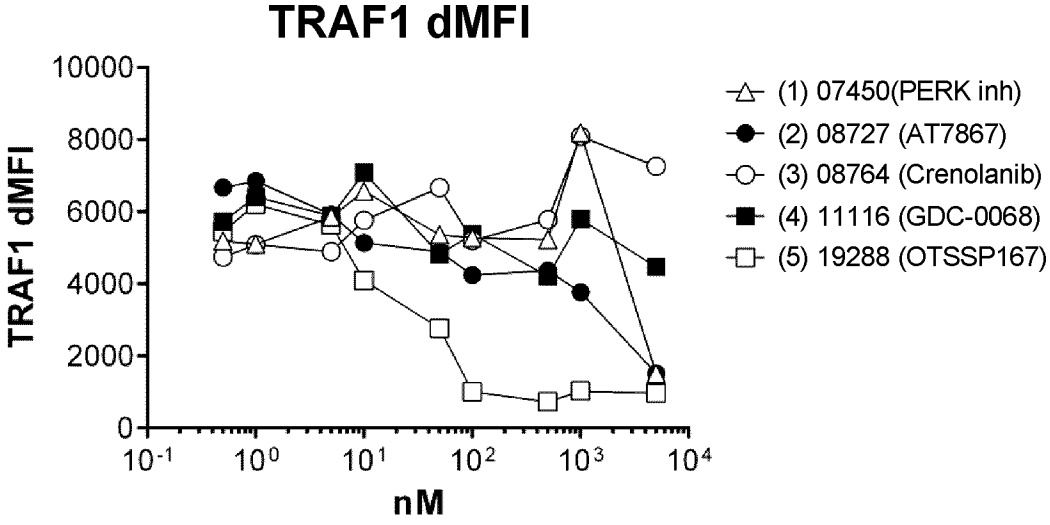

Figure 10b

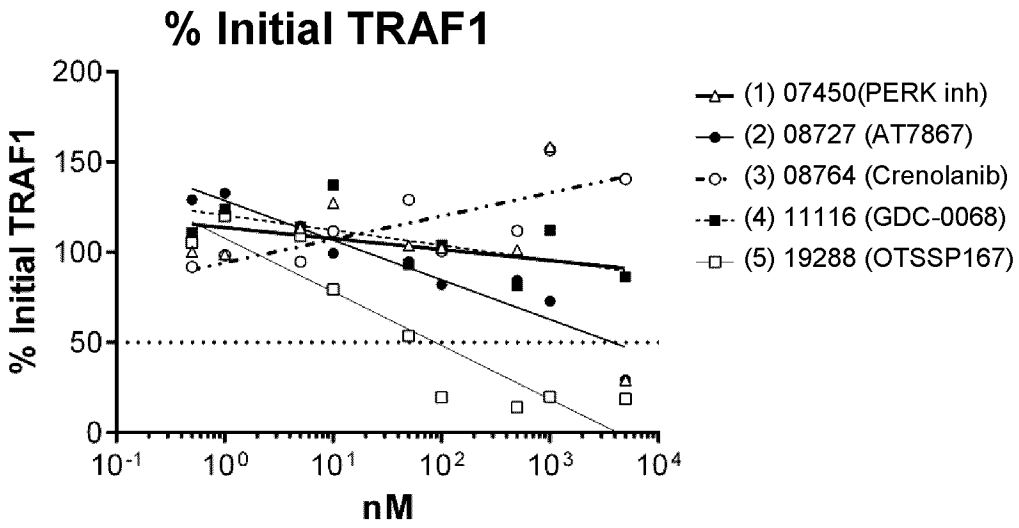
% Initial TRAF1
% Initial TRAF1
200
150
100
50
0
10-1
100
101
102
103
104
nM
(1) 07450(PERK inh)
(2) 08727 (AT7867)
(3) 08764 (Crenolanib)
(4) 11116 (GDC-0068)
(5) 19288 (OTSSP167)

Figure 10c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2020/050743

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***A61K 31/52*** (2006.01) , ***A61K 31/437*** (2006.01) , ***A61K 31/4375*** (2006.01) , ***A61K 31/454*** (2006.01) , ***A61K 31/519*** (2006.01) , ***A61P 35/00*** (2006.01) (more IPCs on the last page)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***A61K 31/52*** (2006.01) , ***A61K 31/437*** (2006.01) , ***A61K 31/4375*** (2006.01) , ***A61K 31/454*** (2006.01) , ***A61K 31/519*** (2006.01) , ***A61P 35/00*** (2006.01), ***A61P 35/02*** (2006.01) , ***C07D 401/10*** (2006.01) , ***C07D 471/04*** (2006.01) , ***C07D 473/34*** (2006.01) , ***C07D 487/04*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent database, Questel Orbit, CIPO Library Discovery Tool , Google
Search terms: protein kinase C related kinase inhibitor, PKN1 inhibitor, TRAF1, B cell related cancer, chronic lymphocytic leukemia, Bcl-2 antagonist, venetoclax, ibrutinib, nutrient stress-inducing agent, venetoclax resistance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Edilova, "TRAF1 in human health and disease", Ph. D. thesis, November 2019 (2019-11), https://tspace.library.utoronto.ca/handle/1807/97447	1-4, 9-19 and 24-27
X	WO 2015/131274 A1 (Abdul et al.) 11 September 2015 (11-09-2015) see entire document (in particular [0021], [0048], [0055], [0057]; claim 9)	1, 3 and 8
X	Edilova et al., "Control of Lymphocytic Leukemia Through Regulation of TRAF1 Protein Degradation", FOCIS 2018 Abstract Supplement W. 81, page 133, Federation of Clinical Immunology Societies, June 20-23, 2018, San Francisco, California https://www.eventscribe.com/upload/planner/links/AbstractSupplement_91.pdf see page 133, Abstract W. 81	1-4 and 8
A	Edilova et al., "TRAF1 Signaling in Human Health and Disease", Frontiers in Immunology 18 December 2018, 9:2969. doi: 10.3389/fimmu.2018.02969	

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"D"	document cited by the applicant in the international application
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 31 July 2020 (31-07-2020)	Date of mailing of the international search report 01 September 2020 (01-09-2020)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer Connie Kuang (819) 639-8664

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☑ forming part of the international application as filed:

☑ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Bishop et al., "Editorial: TRAF Proteins in Health and Disease", Frontiers in Immunology February 2019, 10:326. doi: 10.3389/fimmu.2019.00326	
A	Kato Jr et al., "Negative regulation of constitutive NF-κB and JNK signaling by PKN1-mediated phosphorylation of TRAF1", Genes to Cells 2008, 13(5), 509–520. doi: 10.1111/j.1365-2443.2008.01182.x	
A	Zapata et al., "TNF receptor-associated factor (TRAF) domain and Bcl-2 cooperate to induce small B cell lymphoma/chronic lymphocytic leukemia in transgenic mice", PNAS, 23 November 2004, 101 (47), 16600–16605.	
A	Robak, "BCL-2 inhibitors for Chronic Lymphocytic Leukemia", Journal of Leukemia 2015, 3:3, 1000e114. doi: 10.4172/2329-6917.1000e114	
A	Bose et al., "Pathways and mechanisms of venetoclax resistance", Leuk Lymphoma 2017, 58(9), pages 1-17. doi: 10.1080/10428194.2017.1283032	
A	Oppermann et al., "High-content screening identifies kinase inhibitors that overcome venetoclax resistance in activated CLL cells", Blood 2016, 128(7), pages 934-947. doi: 10.1182/blood-2015-12-687814	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2020/050743

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2015131274A1	11 September 2015 (11-09-2015)	CA2940660A1	11 September 2015 (11-09-2015)
		US2016361391A1	15 December 2016 (15-12-2016)

A61P 35/02 (2006.01) , ***C07D 401/10*** (2006.01) , ***C07D 471/04*** (2006.01) , ***C07D 473/34*** (2006.01) , ***C07D 487/04*** (2006.01)