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(54) Title: SYNERGISTIC COMBINATIONS OF AMINO ACID DEPLETION AGENT SENSITIZERS (AADAS) AND AMINO ACID DEPLETION AGENTS (AADA), AND THERAPEUTIC METHODS OF USE THEREOF

(57) Abstract: Disclosed herein are synergistically effective combinations of Amino Acid Depletion Agents (AADA) and Amino Acid Depletion Agent Sensitizers (AADAS). Also disclosed are methods of using the disclosed combinations to treat subjects with a disease treatable by amino acid depletion-induced cell death (e.g. apoptosis). For example, the disclosed combinations are useful in the treatment or the manufacture of a medicament for use in the treatment of adult and pediatric cancers, in particular, acute lymphoblastic leukemia (ALL), as well as other conditions where amino acid depletion-induced apoptosis is expected to have a therapeutically useful effect. The synergistic combinations are also effective against solid tumors and lymphomas, including gastric cancer, pancreatic cancer, NK lymphoma, DLBCL, colorectal cancer, bladder cancer, hepatic cancer and glioblastoma.



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Synergistic Combinations of Amino Acid Depletion Agent Sensitizers (AADAS) and Amino Acid Depletion Agents (AADA), and Therapeutic Methods of Use Thereof

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to US Provisional Application No. 62/725,313, filed on 31 August 2018, and incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to the treatment of cancers using combinations of amino acid depletion agents (AADA) and amino acid depletion agent-sensitizers (AADAS), which render cancer cells more susceptible to AADA-induced cell death (e.g. apoptosis).

SUMMARY OF THE INVENTION

Asparaginase (ASNase) is a key component in the treatment of acute lymphoblastic leukemia (ALL), and is under clinical evaluation for other malignancies. A poor response to ASNase is associated with increased relapse risk. Commercially available ASNases are of bacterial origin, and their primary therapeutic effect is to deplete Asn from blood plasma. Approved versions include native *E. coli* asparaginase (ELSPAR, Lundbeck Inc.), an *E. coli*-derived peg-conjugated ASNase (e.g. ONCASPAR®, Servier) and an *E. chrysanthemi* ASNase (ERWINAZE®, EUSA Pharma). As some tumor cells, including leukemic blasts, are selectively dependent on the exogenous supply of Asn, systemic administration of ASNase leads to a cessation of growth and the induction of cell death. In ALL, the response of leukemic cells to ASNase (i.e. cellular quiescence or some degree of apoptosis) is dependent on the genetic makeup of the leukemia and the extent of Asn depletion in the cellular microenvironment.

Unfortunately, leukemic cells present in the bone marrow niche or the central nervous system (CNS) appear to be less responsive to ASNase treatment as a result of incomplete Asn depletion, enhancing the chance of relapse. Another clinical problem with the use of this protein drug is the formation of inhibitory antibodies over time that cause silent inactivation of the drug. A phenomenon associated with this immune response is the occurrence of severe allergies and related toxicities. Once patients have developed an allergic reaction to the drug,

treatment must be stopped, which enhances the chances of a relapse and reduces the chance of cure once a relapse has developed.

To address these challenges, some groups have been exploring the benefits of conjugating polymers to ASNases, to make (for example) new PEGylated and PASylated forms of ASNase. One company has addressed these challenges by encapsulating the ASNase within red blood cells (see Patents US 8,974,802 & US 8,617,840, both to Erytech Pharma SA), thereby enabling ASNase activity to be safely circulated in a patient's blood stream for extended periods of time. Another group has attempted to reduce the toxicity of ASNase by eliminating its Glutaminase (GLNase) activity (see WO 2018/050918, to Cambridge Innovations Technologies and WO 2017/151707, to the University of Illinois). And yet another group has developed endotoxin-free ASNases (WO 2018/085493 to Georgia State Research Foundation), having improved safety/toxicity profiles.

Despite these innovations, there remains a long-felt need to improve the efficacy of amino acid depletion therapies, particularly in cases where amino acid depletion alone is insufficient to completely cure or place the disease or condition into remission.

To address this problem, the Applicants performed an *in vitro* loss-of-function screen to identify novel therapeutic interventions that may synergize with ASNase to induce cell death (e.g. apoptosis) rather than mere cell quiescence. The results of this screen indicated that specifically interfering with a cell's ability to cope with amino acid starvation enhances the clinical efficacy of amino acid depletion agents (AADA).

Blocking cellular stress responses at certain kinases (e.g. the amino acid sensor GCN2, a key mediator of the amino acid stress response), sensitized cells to AADA-induced apoptosis. Unexpectedly, the Applicants observed that blocking Bruton's Tyrosine Kinase (BTK) was at least as effective in sensitizing cells to AADA-induced apoptosis. Moreover, the GCN2 kinase was effectively inhibited in response to inhibition of BTK activity, demonstrating that BTK regulates the activity of this branch of the amino acid stress response. In contrast, and rather unexpectedly, inhibition of mTOR, another important component of the amino acid stress response route, and known effector of BTK, did not affect response to AADA-induced apoptosis.

Accordingly, a first object of the disclosure is to provide therapeutically effective combinations of amino acid depletion agent sensitizers (AADAS) and amino acid depletion agents (AADA) for the treatment of diseases, including cancers. In some embodiments, the AADAS is a Bruton's Tyrosine Kinase inhibitor (BTKi) and the AADA is an ASNase. Now that the disclosure has been made, the skilled artisan will reasonably expect that a safe and effective amount of any BTK inhibitor will sensitize a variety of different tumor cells to ASNase-induced apoptosis. As further disclosed below, the efficacy of the combination of the BTKi and ASNase is greater than the additive efficacy of either component by itself. Applicants envision that other combinations of AADAS and AADA will likewise yield synergistic efficacy against cells from various cancer types.

In some embodiments, the therapeutically effective combinations provide synergistic efficacy against one or more cancers as compared with the efficacy of either active alone.

In other embodiments, the synergistic combinations are therapeutically effective against cancer types that are non-responsive to one or both of the AADA and the AADAS.

In some particular embodiments, the AADA is an ASNase and the AADAS is a BTKi, each present in subtherapeutic amounts. As used herein, a "subtherapeutic amount" means an amount of a drug or therapeutic agent that is ineffective at producing or eliciting a given therapeutic effect (e.g. a significant reduction in the size of a tumor, a significant decrease in the number of tumor cells or a significant decrease in the metastatic potential of tumor cells).

Accordingly, synergistic combinations according to this disclosure may exhibit at least the following patterns of synergistic efficacy against a given cancer indication and/or cell type:

- a) AADA Efficacy of 1 + AADAS Efficacy of 1 = Combination Efficacy of 3, 4 or greater;
- b) AADA Efficacy of 1 + AADAS Efficacy of 0 = Combination Efficacy of 2, 3, 4 or greater;
- c) AADA Efficacy of 0 + AADAS Efficacy of 1 = Combination Efficacy of 2, 3, 4 or greater;
- d) AADA Efficacy of 0 + AADAS Efficacy of 0 = Combination Efficacy of 1, 2, 3, 4 or greater;

In a second object, the disclosure provides methods of treating diseases including cancers comprising sequential or simultaneous administration of synergistically effective combinations of AADA and AADAS as disclosed herein.

In a third object, the disclosure provides kits comprising effective amounts of an AADA
5 and an AADAS, optionally including instructions for use thereof in treating cancers.

In a fourth object, the disclosure provides methods of manufacture of a medicament comprising effective amounts of an AADA and an AADAS.

In a fifth object, the disclosure provides methods and/or uses of combinations of AADA and AADAS in the treatment of cancer. In some embodiments, the use is effective in inducing
10 tumor cells that are resistant to treatment with either the AADA or the AADAS alone. In some embodiments, the use of the combination of AADA and AADAS is effective in treating a patient in whom a cancer has relapsed after a treatment with either the AADA or AADAS previously administered as a monotherapy, or in combination with an agent other than the AADA (in the case where the AADAS was previously administered) or the AADAS (in the case where the AADA
15 was previously administered).

In a sixth object, the disclosure provides methods and/or uses of combinations of AADA and AADAS in the treatment of cancer that is resistant to either or both of the AADA or the AADAS, when administered alone or with an agent other than the corresponding AADA or AADAS. In some embodiments, simultaneous or sequential administration of individually
20 subtherapeutic doses of the AADA and AADAS restores the sensitivity of the tumor cells. In some embodiments, the entire population of tumor cells is killed by a combination of the AADA and AADAS, but not either the AADA or AADAS alone.

It is a further object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the product such
25 that the Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement

requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

These and other embodiments are disclosed or are obvious from and encompassed by,
5 the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a diagram outlining the Dox inducible Cas9 Kinome gRNA library;

FIG. 1B is a WB image showing Dox regulation of Cas9 expression;

FIG. 1C is a graph showing the abundance of sgRNAs before and after treatment;

10 FIG. 1D is a graph showing the genes whose deletions correlate most significantly with resistance (right) or sensitization (left) to ASNase treatment;

FIG. 2A depicts several components of the signaling pathways modulated by treatment of cells with ASNase. KO of TRIB3 correlates with resistance to ASNase, while KO of p90RSK, EEF2K or GCN2 correlates with sensitivity to ASNase;

15 FIG. 2B is an image of a WB indicating TRIB3 levels in deletion pools of cells representing two independent gRNAs (2.1-3, 2.2-3)

FIG. 2C is an image of a WB indicting GCN2 levels in deletion pools of cells representing two independent gRNAs (1.1, 1.2)

FIG. 2D is a graph showing the growth of TRIB3 deletion cells versus control cells;

20 FIG. 2E is a graph showing deletion of TRIB3 protects cells from ASNase-induced death;

FIG. 2F is a graph showing deletion of TRIB3 reduces the percent of SubG1 cells in response to ASNase treatment;

FIG. 2G is a graph showing the relative viability among control, TRIB3del_2.1-3 and TRIB3del_2.2-3;

25 FIG. 2H is a graph showing that deletion of GCN2 increases the percent of SubG1 cells in response to ASNase treatment;

FIG. 2I is a graph showing the relative viability among control, GCN2KO-1.1 and GCN2KOdel_1.2;

FIG. 3A depicts several components signaling pathways downstream of B cell receptor signaling, including BTK;

5 FIG. 3B is a schematic representation of the in vitro ASNase treatment of nalm6 BTK KO cells vs. control nalm6 cells;

FIG. 3C is a WB image showing the relative levels of BTK in the indicated control and selected BTK KO clones, obtained after single cell cloning;

10 FIG. 3D is a WB image showing the relative levels of cleaved and uncleaved PARP in the indicated control and selected BTK KO clones;

FIG. 3E is a graph of percent dead cells in control or BTK KO clones, plus and minus 5 IU/mL ASNase;

FIG. 3F is a graph showing percent dead cells in control or GCN2KO clones, plus and minus 1 IU/mL ASNase;

15 FIG. 3G are graphs showing Hoechst staining for control and BTK KO clones, either untreated, treated with 5 IU/mL ASNase, or washed out after treatment with 5 IU/mL ASNase;

FIG. 3H is a graph of the percent of cells in SubG1 in control or BTK KO clones, either untreated, treated with 5 IU/mL ASNase (non-washout experiment);

20 FIG. 4A is a schematic representation of the in vitro ASNase + ibrutinib treatment of nalm6 cells, followed by a colony assay after washout of ASNase and Ibrutinib;

FIG. 4B is a WB showing the effective inhibition of BTK via loss of phospho-BTK with increasing concentration of ibrutinib;

FIG. 4C are graphs showing Hoechst staining for untreated cells and cells treated with 1 IU/mL ASNase, plus 0, 1 or 10 μ M ibrutinib;

25 FIG. 4D is a graph showing the percent of cells in SubG1 for Sem, Nalm6 and Reh cells treated with 0 or 1 IU/mL ASNase and 0, 1 or 10 μ M ibrutinib;

FIG. 4E is a WB image and a graph showing PARP cleavage for Nalm6, Sem and Reh cells treated with 0 or 1 IU/mL ASNase and 0 or 10 μ M ibrutinib;

FIG. 4F is a graph showing the percent SubG1 cells for Sem, Nalm6 and Reh cells treated with 0 or 1 IU/mL ASNase and 0, 1 or 10 μ M ibrutinib;

5 FIG. 4G are images of plates seeded with Nalm6 cells treated with 0 or 1 IU/mL ASNase plus 0, 1 or 10 μ M ibrutinib;

FIG. 4H are graphs presenting the colony counts for Nalm6 cells and Sem cells (below);

FIG. 4I is a schematic indicating that effective amounts of both ASNase + ibrutinib induce apoptosis, whereas these same amounts of either active alone induce quiescence;

10 FIG. 5A presents a schematic overview representing the workflow used to derive drug synergies in ALL cell lines and patient-derived xenograft (PDX) samples;

FIG. 5B is an example heat map showing the synergistic killing efficacy of the combination of ASNase and ibrutinib;

15 FIG. 5C is a schematic indicating that synergistic killing efficacy was observed in a large majority of PDX samples treated with ASNase and ibrutinib;

FIG. 5D is a graph showing the relative viability for a selection of PDX cells subjected to the indicated concentration of ASNase plus or minus the indicated concentrations of ibrutinib;

FIG. 5E is a graph showing the percent 7AAD positive cells for the indicated PDX samples treated with the indicated concentrations of ASNase and/or ibrutinib;

20 FIG. 5F is a summary table indicating the synergistic killing efficacy of the combination of ASNase and ibrutinib against patient derived xenografts (PDX);

FIG. 5G is a summary table indicating the synergistic killing efficacy of the combination of ASNase and ibrutinib (CI calculated without the 100 μ M ibrutinib group);

25 FIG. 6A are graphs showing the Hoechst staining for Nalm6 cells treated with 0 or 5 μ g/mL prednisone plus 0, 1 or 10 μ M ibrutinib;

FIG. 6B are WB images showing the cleavage of PARP Nalm6 cells treated with 0 or 5 $\mu\text{g/mL}$ prednisone plus 0, 1 or 10 μM ibrutinib;

FIG. 6C are graphs showing the percent of SubG1 cells for Nalm6, Sem and 697 cells treated with 0 or 5 $\mu\text{g/mL}$ prednisone plus 0, 1 or 10 μM ibrutinib;

5 FIG. 7A is a schematic showing the experimental design for the transplantation of patient-derived xenografts (PDX) into immunocompromised mice. Briefly, mice were injected intrafemorally with 500,000 leukemic blasts, then two weeks after transplantation, mice were administered the indicated treatment for 9 consecutive days;

FIG. 7B is a graph showing the bodyweight of the mice during and after treatment;

10 FIG. 7C is a graph showing the percent of hCD45+ cells post-transplant;

FIG. 7D is a graph showing the percent of hCD10+ cells post-transplant;

FIG. 7E is graph showing the percent hCD19+ cells as a function of treatment;

FIG. 7F is a Kaplan-Meier plot showing event-free survival. Significance was tested using a log-rank test;

15 FIG. 8A is a diagram presenting how inhibition of BTK blocks amino acid depletion agent (AADA) activation of the GCN2-ATF4 axis;

FIG. 8B is a series of WB images showing the impact on GCN2, ATF4 and ASNS protein expression in Nalm6 or Sem cells treated according to the following: untreated, ibrutinib, ASNase or combination of ibrutinib and ASNase;

20 FIG. 9 is a heat map presenting the results of the reverse phase proteomics analysis (top 50 normalized against NALM6 & SEM controls) performed on NA;

FIG. 10A is a graph showing the relative viability for HBP-ALL cells subjected to the indicated concentrations of ASNase and 0, 0.1, 1 or 10 μM ibrutinib;

25 FIG. 10B is a graph of percent dead HBP-ALL cells treated with either 0 or 0.001 IU/mL ASNase and 1 or 10 μM ibrutinib (3 day treatment);

FIG. 11A is a graph showing the relative viability for LOUCY cells subjected to the indicated concentrations of ASNase and 0, 0.1, 1 or 10 μ M Ibrutinib;

FIG. 11B is a graph of percent dead LOUCY cells treated with either 0 or 0.0001 IU/mL ASNase and 1 or 10 μ M Ibrutinib (3 day treatment);

5 FIG. 12A is a graph showing the relative viability for SupT1 cells subjected to the indicated concentrations of ASNase and 0, 0.1, 1 or 10 μ M Ibrutinib;

FIG. 12B is a graph of percent dead SupT1 cells treated with either 0 or 1 IU/mL ASNase and 1 or 10 μ M Ibrutinib (3 day treatment);

10 FIG. 12C is a graph of percent dead SupT1 cells treated with either 0 or 1 IU/mL ASNase and 1 or 10 μ M Ibrutinib (7 day treatment);

FIG. 13A is a graph showing the relative viability for SupT1 cells subjected to the indicated concentrations of ASNase and 0, 0.1, 1 or 10 μ M Ibrutinib;

FIG. 13B is a graph of percent dead SupT1 cells treated with either 0 or 1 IU/mL ASNase and 1 or 10 μ M Ibrutinib (7 day treatment);

15 FIG. 14A is a graph showing percent viability for refractory patient-derived ALL cells subjected to the indicated concentrations of ASNase and 0, 1 or 10 μ M Ibrutinib;

FIG. 14B is a graph showing the AUC for the ASNase treatment shown in FIG. 14A;

20 FIG. 15A is a graph showing % cell viability for SU-DHL-10 (DLBCL) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment;

FIG. 15B is a Combination Index (CI) plot indicating that the combination of ASNase and Ibrutinib exerted synergistic killing efficacy against SU-DHL-10 cells;

FIG. 15C is the histogram plot for the SU-DHL-10 study;

25 FIG. 16 is a graph showing % cell viability for NCI-N87 (Gastric cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 17 is a graph showing % cell viability for AsPC-1 (Pancreatic cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 18 is a graph showing % cell viability for CAPAN-1 (Pancreatic cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 19 is a graph showing % cell viability for BxPC-3 (Pancreatic cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 20 is a graph showing % cell viability for KHYG-1 (NK Lymphoma cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 21 is a graph showing % cell viability for HT-29 (Colorectal cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 22 is a graph showing % cell viability for RT4 (Bladder cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

FIG. 23 is a graph showing % cell viability for Hep3B (Hepatic cancer) cells subjected to 0.0032, 0.016, 0.08, 0.4, 2 or 10 U/mL L-ASNase and 0.0032, 0.016, 0.08, 0.4, 2 or 10 μ M Ibrutinib for 4 days of treatment. Analysis indicated synergistic efficacy;

DETAILED DESCRIPTION OF THE INVENTION

It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as “comprises”, “comprised”, “comprising” and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean “includes”, “included”, “including”, and the like; and that terms such as “consisting essentially of” and “consists essentially of” have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but

exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure
5 belongs. The singular terms “a”, “an”, and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise.

The term “about,” as used herein, means approximately, in the region of, roughly, or around. When the term “about” is used in conjunction with a numerical range, it modifies that
10 range by extending the boundaries above and below the recited values. In general, the term “about” is used herein to modify a numerical value above and below the stated value by a variance of 10%. In one aspect, the term “about” means plus or minus 20% of the numerical value of the number with which it is being used. Therefore, about 50% means in the range of 45%-55%. Numerical ranges recited herein by endpoints include all numbers and fractions
15 subsumed within that range (e.g. 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.90, 4, and 5). It is also to be understood that all numbers and fractions thereof are presumed to be modified by the term “about.”

Asparaginase (ASNase) is a key component of the multi-drug treatment that is used to treat pediatric leukemia. Upon administration, this protein converts the amino acid asparagine
20 into aspartate, effectively depleting the blood of asparagine. In contrast to most cells, lymphocytes and leukemic blasts cannot produce asparagine in sufficient amounts. The limited availability of asparagine activates the amino acid stress response pathway, which induces a state of cellular quiescence, allowing cells to overcome periods of nutrient starvation. That said, sustained amino acid starvation will eventually induce apoptosis via this same pathway.

25 However, the success of ASNase therapy is compromised by protection of tumor cells by the cellular microenvironment, causing tumor cells to go into a quiescent cell survival mode, rather than to induce apoptosis. To address this problem, Applicants performed a CRISPR/Cas9 -based *in vitro* loss-of-function screen to identify novel therapeutic interventions that may

synergize with ASNase to induce apoptosis rather than cell quiescence (see Wang *et al.* Science 2014;343 (6166):80-4). Briefly, the screen was performed using NALM6 pre-B ALL cells exposed to a IC₅₀ dose of ASNase. To facilitate rapid translation into clinical practice, the screen focused on kinases as potential targets for pharmacological intervention. After treatment, genomic DNA
5 from treated and untreated control cells was isolated, and incorporated gRNA sequences were amplified and sequenced (Illumina HiSEQ™) to identify gRNAs that were selectively enriched or depleted during treatment. The results were validated using targeted knockouts and by using small-molecule inhibitors.

As detailed in the Examples below, candidate genes were validated using ALL cell lines
10 and a co-culture of hTERT immortalized MSCs and ALL-xenografts. FIG. 2A depicts several elements of the amino acid deprivation signaling pathway, which is modulated by treatment with ASNase. FIGs. 3A & 3B summarize genes whose deletion promotes either resistance or sensitization to treatment of the cells with the AADA ASNase.

As disclosed herein, the anti-tumor effects of ASNase impinge on changes in cell
15 metabolism that occur as a result of amino acid starvation. Consistent with this notion, the loss of function screen identified genes either directly involved in the amino acid response route (TRIB3) or inhibition of protein translation in response to amino acid starvation (GCN2). Indeed, knockout of GCN2 sensitized cells to ASNase treatment whereas depletion of TRIB3 was sufficient to render these cells more resistant to the effects of ASNase on cell growth.
20 Accordingly, the Applicants have identified novel pathway interactions that can potentiate or resist the apoptosis inducing effects of the amino acid depletion agent (AADA) ASNase.

As further disclosed herein, Bruton's Tyrosine Kinase (BTK), a hematopoietic-cell specific protein kinase acting downstream of the B-cell receptor, was demonstrated to protect ALL cells from ASNase-induced apoptosis. Indeed, targeted knockout as well as inhibition by the FDA-
25 approved BTK inhibitor ibrutinib, strongly enhanced ASNase-induced apoptosis in a variety of ALL cell lines (see e.g. FIGs. 4B & 4D). Moreover, Applicants tested the effect of combinations of ASNase and ibrutinib in many different patient-derived xenograft (PDX) samples, mostly representing high risk leukemia cases and covering a wide variety of ALL subtypes. In > 80 % of

the cases, Applicants observed synergy ranging from moderate to strong with a combination index (CI) <0.8 (FIG. 5F, 5G).

Thus, it is an object of this disclosure to provide synergistic combinations of amino acid depletion agents (AADA, e.g. ASNase) and amino acid depletion agent sensitizers (AADAS, e.g. BTKi) for use in treating patients in need thereof.

It is a further object to provide methods of treating a subject or patient suffering from cancer comprising simultaneously or sequentially administering synergistically effective amounts of an AADA (e.g. ASNase) and an AADAS (e.g. BTKi). In some embodiments, the cancer may be a liquid or solid tumor. In some embodiments, the use of an AADAS may potentiate the solid tumor killing efficacy of otherwise ineffective amounts of AADA. In other embodiments, the AADAS may be combined with a better tolerated AADA, such as L-ASNase encapsulated in erythrocytes (e.g. Erytech's ERYASPASE®). Advantageous indications include, but are not limited to, solid tumor and lymphoma indications selected from Gastric cancer, Pancreatic cancer, NK Lymphoma, DLBCL, Colorectal cancer, Bladder cancer, Hepatic cancer and Glioma/Glioblastoma.

Determination of a synergistic interaction between an AADA and an AADAS may be based on the results obtained from the assays described herein. The results of these assays may be analyzed using the Chou and Talalay combination method and Dose-Effect Analysis with CalcuSyn software in order to obtain a Combination Index (Chou and Talalay, Trends Pharmacol. Sci. 4:450-454; Chou, T.C. (2006) Pharmacological Reviews 68(3):621-681; Chou and Talalay, 1984, Adv. Enzyme Regul. 22:27-55).

As further detailed in the Examples below, the synergistic AADA and AADAS combinations provided by this disclosure have been evaluated in several assay systems, and the data can be analyzed utilizing a standard program for quantifying synergism, additivism, and antagonism among anticancer agents. An exemplary program utilized is described by Chou and Talalay, in "New Avenues in Developmental Cancer Chemotherapy," Academic Press, 1987, Chapter 2. Combination Index values less than 0.8 indicates synergy, values greater than 1.2 indicate antagonism and values between 0.8 to 1.2 indicate additive effects. The combination

therapy may provide “synergy” and prove “synergistic”, i.e., the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A “synergistic effect” may be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect may be attained when the compounds are administered or delivered sequentially, e.g., by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e., serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

In some embodiments, the synergistic combination therapy of this disclosure may be effective against tumor cells having one or more of the cytogenetic profiles indicated in FIG. 5F, 5G. For example, the synergistic combination therapy may be effective against tumor cells comprising the following: t(4;11) MLL-AF4, ETV6-RUNX1 (t(12;21); EBF1del1-16,PAX5del2 8,ETV6del1-2,IL3del,CSF2RA,Ikzf1del4-7, E2A-HLF_t(17;19), E2a-PBX1; T(1;19), Ikzf1del4-7,CDKN2A~~del~~, ABL+,CDKN2A/B~~del~~,PAX5~~del~~,Ikzf1 del1-8, P53~~del~~, MLL, t(1;19), TP53 unknown (del+R248Q in relapse), TP53 del+R280T, E2a-PBX1; t(1;19), t(1;19) E2A-PBX1, t(2;12), complex.

In some embodiments, the synergistic combination therapy may be effective against solid tumor cells expressing or over-expressing a gene or mutated version thereof, whose deletion and/or inhibition sensitizes said cells to AADA-induced apoptosis. In some embodiments, the AADAS may be a BTK inhibitor and the AADA is ASNase.

In other embodiments, the synergistic combination therapy may be effective against a wide variety of solid tumor cells, including those from pancreatic cancer, breast cancer, colorectal cancer, gastric cancer, brain cancer, etc. The synergistic combination therapy may also be effective against semi-solid tumors including lymphomas. Advantageous indications include, but are not limited to, solid tumor and lymphoma indications selected from Gastric cancer, Pancreatic cancer, NK Lymphoma, DLBCL, Colorectal cancer, Bladder cancer, Hepatic cancer and Glioma/Glioblastoma.

Furthermore, the data disclosed herein indicate that the synergistic effect of AADA (ASNase) and AADAS (BTKi) is selective for ASNase-induced cell death, since no clear synergy was seen between BTK inhibition and glucocorticoid-induced apoptosis (see Example 5 and FIGs. 6A to 6C). This finding was both surprising and unexpected, since skilled artisans might have reasonably expected the AADAS to sensitize cells to the action of other inducers of apoptosis, and not only AADA-induced apoptosis. Furthermore, as indicated above, inhibition of mTOR, another important component of the amino acid stress response route and known effector of BTK, did not affect response to AADA-induced apoptosis.

Now that the invention has been disclosed, the inventors envision that other combinations of AADAS and AADA will demonstrate comparable synergistic efficacy, and include Table 1 as a non-limiting list of such combinations. When the AADA is a peptidic agent, it may be present in any pharmaceutically acceptable form, including free, pegylated, otherwise conjugated and RBC encapsulated.

Table 1. Combinations of amino acid depletion agent sensitizer (AADAS) and amino acid depletion agent (AADA)

Combination	AADAS	AADA
1	BTKi	Asparaginase (ASNase) (e.g. GRASPA®, Erytech Pharma; ONCASPAR®, Servier)
2		ASN synthetase (ASNS) inhibitor
3		Arginine deiminase (ADI) (e.g. ADI-PEG20, POLARIS)
4		Arginase (ARGase)
5		Argininosuccinate synthetase inhibitor (ASI)
6		Adenosine deaminase (ADA)
7		Methionase (METase)
8		Glutaminase (GLNase)
9		Glutamine synthetase inhibitor (GSI)
10		ASNase + METase
11		IDO (e.g. indoximod, docetaxel)
12		TDO (e.g. indole LM10)
13	p21-activated kinase inhibitor	ASNase
14		ASNS inhibitor
15		ADI
16		ARGase

17	(PAKi)	ASI
18		ADA
19		METase
20		GLNase
21		GSI
22		ASNase + METase
23		IDO
24		TDO
25	Cell division cycle 7 inhibitor (CDC7i)	ASNase
26		ASNS inhibitor
27		ADI
28		ARGase
29		ASI
30		ADA
31		METase
32		GLNase
33		GSI
34		ASNase + METase
35		IDO
36		TDO
37	TAOK2 inhibitor	ASNase
38		ASNS inhibitor
39		ADI
40		ARGase
41		ASI
42		ADA
43		METase
44		GLNase
45		GSI
46		ASNase + METase
47		IDO
48		TDO

Moreover, the combination of ASNase and ibrutinib in a mouse xenograft model produced impressive clinical responses, even after a single block of treatment (9 days). See Example 6 below. One of the surprising observations was that the effects of ibrutinib (an inhibitor of a component of the B cell receptor signaling pathway, see FIG. 3A) were also seen in leukemia subtypes that do not (yet) express a rearranged (pre) B cell receptor. Mechanistically, these data indicate that inhibition of BTK leads to inhibition of the amino acid sensor GCN2 and

the transcription factor ATF4 (see FIGs. 8A & 8B), a previously unrecognized connection between components of the B cell receptor signaling pathway and amino acid stress responses (FIG. 2A). As such, the invention also encompasses a method for overcoming ASNase resistance in patients or subjects comprising administering to patients synergistically effective amounts of a BTKi and an ASNase, such that the levels of the BTKi are sufficient to attenuate, block and/or prevent the ASNase-induced increases in ASNS expression, which would contribute to the ASNase resistance.

This unpredictable finding is further notable in that mice deficient for GCN2 show enhanced liver toxicity after exposure to ASNase, demonstrating that systemic inhibition of the amino acid stress response is not feasible (Phillipson-Weiner L *et al.* Am J Physiol Gastrointest Liver Physiol. 2016 Jun 1;310(11)) for combination with an AADA like ASNase. As such, the present disclosure demonstrates that it is possible to interfere with the GCN2-ATF4 stress response in a (tumor) cell type specific manner (i.e. because the biological target of AADAS is highly expressed in the target cells), reducing the chance of systemic toxicities.

By combining ASNase therapy with cell type specific inhibition of the GCN2-ATF4 signaling pathway, the therapeutic efficacy against ALL, other blood cancers, and solid tumors, may be enhanced while reducing side effects. In the case of ALL, this effect may be brought about by the inhibition of the hematopoietic cell specific kinase BTK, for instance by using the FDA-approved drug ibrutinib or other BTK inhibitors in combination with ASNase. Since ibrutinib is known to inhibit activation of the B cell receptor, an additional advantage could be the inhibition of antibody responses by normal B cells during treatment. Outside of ALL, the Applicants envision that sensitization of tumor cells to ASNase therapy may depend upon the expression level and/or activity of BTK (and/or related kinases of the TEC family) in such non-ALL tumor cells.

As used herein, the term “amino acid depletion agent sensitizer” (AADAS) means a drug, compound, biotherapeutic or other agent capable of sensitizing a tumor cell to treatment with an “amino acid depleting agent” (AADA).

As used herein, and “AADA” or “amino acid depleting agent” is any drug, compound, biotherapeutic or other agent capable of depleting amino acid levels to deprive one or more cell types of said amino acid. As disclosed herein, the AADAS and the AADA work together, in some embodiments synergistically, to induce apoptosis in one or more desired cell types.

5 In some embodiments, the amino acid depletion stress pathway is blocked using a Bruton’s Tyrosine Kinase inhibitor (BTKi) (the AADAS) and an ASNase (the AADA).

 In some embodiments, the ASNase is provided in the red blood cell (RBC)-encapsulated form currently produced Erytech Pharma. The RBCs are subjected to osmotic stress, which opens and reseals pores on the surface of the cells and allows the compounds to enter and be
10 trapped inside the cells. Encapsulation offers a number of benefits as compared to free-form compounds.

 The red blood cell membrane protects the therapeutic substance, which allows it to remain in the body longer and should lead to fewer administrations and a lower overall dose. The cellular membrane also protects the body against any direct toxicity of the drug substance,
15 which can result in a decreased incidence of allergic reactions and other side effects. The combination of prolonged activity and reduced toxicity is particularly beneficial for the administration of certain therapeutic enzymes, that often have short half-lives and are associated with important toxicities. Examples include enzymes such as asparaginase (ASNase) and methioninase (METase), used to starve tumors by affecting cancer metabolism.

20 In some embodiments, the synergistic combination therapy may comprise a synergistically effective amount of an RBC-encapsulated ASNase and a synergistically effective amount of a BTKi.

 In some embodiments, the synergistic combination therapy may comprise a synergistically effective amount of any ASNase, including but not limited to an Erwinase, a PEG-
25 conjugated ASNase, or an ASNase lacking glutaminase activity, and a synergistically effective amount of a BTKi.

 In some embodiments, treatment of a patient suffering from a cancer with either the synergistically effective amount of the ASNase or the synergistically effective amount of the

BTKi alone results in inhibition of tumor cell proliferation, whereas treatment with the combination of ASNase and the BTKi induces massive tumor cell apoptosis.

In other embodiments, the BTKi blocks BTK from protecting tumor cells from ASNase-induced killing.

5 Furthermore, as is well-known, there are numerous ways to activate AA depletion-induced stress, including, but not limited to the following: 1) dietary restriction (see e.g. Erytech's International Patent Application WO2017/114966, disclosing novel methods of depleting MET and ASN for treating cancer); 2) ASN depletion (including ASNase, ASNS inhibitor, etc.); 3) MET depletion (e.g. METase, MGL, MET synthesis inhibitors); 4) TYR depletion
10 (TDO, IDO inhibitors); 5) ARG depletion (ADI, ARGase...); 6) Adenosine Deaminase (ADA); and/or 7) combinations thereof. Accordingly, the AADA may encompass any one or more of the foregoing AA depletion approaches, or any other means for activating the AA depletion stress response in cancer cells.

Definitions

15 The term "acceptable" or "pharmaceutically acceptable", with respect to a formulation, composition or ingredient, as used herein, means having no persistent detrimental effect on the general health of the subject being treated or does not abrogate the biological activity or properties of the compound, and is relatively nontoxic.

"Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether
20 malignant or benign, and all pre-cancerous and cancerous cells and tissues. "Neoplastic," as used herein, refers to any form of dysregulated or unregulated cell growth, whether malignant or benign, resulting in abnormal tissue growth. Thus, "neoplastic cells" include malignant and benign cells having dysregulated or unregulated cell growth.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in
25 mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, blood cancers and solid tumors, including pancreatic ductal adenocarcinoma, colorectal cancer, breast cancer, triple negative breast cancer (TNBC), and B-cell lymphoproliferative disorders (BCLDs), such as lymphoma and leukemia. Particularly

advantageous target indications include, but are not limited to, solid tumor and lymphoma indications selected from Gastric cancer, Pancreatic cancer, NK Lymphoma, DLBCL, Colorectal cancer, Bladder cancer, Hepatic cancer and Glioma/Glioblastoma.

By “refractory” in the context of a cancer is intended the particular cancer is resistant
5 to, or non-responsive to, therapy with a particular therapeutic agent. A cancer can be refractory to therapy with a particular therapeutic agent either from the onset of treatment with the particular therapeutic agent (i.e., non-responsive to initial exposure to the therapeutic agent), or as a result of developing resistance to the therapeutic agent, either over the course of a first treatment period with the therapeutic agent or during a subsequent treatment period with the
10 therapeutic agent.

By “agonist activity” is intended that a substance functions as an agonist. By “antagonist activity” is intended that the substance functions as an antagonist. An antagonist of “Bruton’s Tyrosine Kinase” (BTK) prevents or reduces induction of any of the responses mediated by BTK.

In some embodiments, the BTK inhibitor therapeutic agent is an antagonist anti-BTK
15 antibody. Such antibodies are free of significant agonist activity as noted above when bound to a BTK antigen in a human cell.

By “BTK-mediated signaling” it is intended any of the biological activities that are dependent on, either directly or indirectly, the activity of BTK.

A BTK “signaling pathway” or “signal transduction pathway” is intended to mean at least
20 one biochemical reaction, or a group of biochemical reactions, that results from the activity of BTK, and which generates a signal that, when transmitted through the signal pathway, leads to activation of one or more downstream molecules in the signaling cascade. Signal transduction pathways involve a number of signal transduction molecules that lead to transmission of a signal from the cell-surface across the plasma membrane of a cell, and through one or more in
25 a series of signal transduction molecules, through the cytoplasm of the cell, and in some instances, into the cell’s nucleus.

As used herein, the term “agonist” refers to a compound, the presence of which results in a biological activity of a protein that is the same as the biological activity resulting from the presence of a naturally occurring ligand for the protein, such as, for example, BTK.

As used herein, the term “partial agonist” refers to a compound the presence of which
5 results in a biological activity of a protein that is of the same type as that resulting from the presence of a naturally occurring ligand for the protein, but of a lower magnitude.

As used herein, the term “antagonist” refers to a compound, the presence of which results in a decrease in the magnitude of a biological activity of a protein. In certain embodiments, the presence of an antagonist results in complete inhibition of a biological
10 activity of a protein, such as, for example, BTK. In certain embodiments, an antagonist is an inhibitor.

The term “Bruton's tyrosine kinase (BTK),” as used herein, refers to Bruton's tyrosine kinase from *Homo sapiens* (e.g. GenBank Accession No. NP—000052). The term “Bruton's tyrosine kinase homolog,” as used herein, refers to orthologs of Bruton's tyrosine kinase, e.g.,
15 the orthologs from mouse (GenBank Accession No. AAB47246), dog (GenBank Accession No. XP—549139.), rat (GenBank Accession No. NP—001007799), chicken (GenBank Accession No. NP 989564), or zebra fish (GenBank Accession No. XP 698117), and fusion proteins of any of the foregoing that exhibit kinase activity towards one or more substrates of Bruton's tyrosine kinase (e.g. a peptide substrate having the amino acid sequence “AVLESEELYSSARQ”).

20 The terms “co-administration” or “combination therapy” and the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are administered by the same or different route of administration or at the same or different time.

The term “effective amount,” as used herein, refers to a sufficient amount of an AADA
25 and an AADAS. In some embodiments, the AADAS may be a BTK inhibitory agent or a BTK inhibitor compound being administered which will result in an increase or appearance in the blood of a subpopulation of lymphocytes (e.g., pharmaceutical debulking). An appropriate

“effective amount” in any case may be determined using techniques, such as a dose escalation study.

The term “therapeutically effective amount,” as used herein, refers to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of a disease or condition. The result can be reduction and/or alleviation of the signs, symptoms, or causes of the disease or condition, or any other desired alteration of a biological system. The term “therapeutically effective amount” includes, for example, a prophylactically effective amount. An “effective amount” of a compound disclosed herein is an amount effective to achieve a desired pharmacologic effect or therapeutic improvement without undue adverse side effects. It is understood that “an effect amount” or “a therapeutically effective amount” can vary from subject to subject, due to variation in metabolism of the compound of any of Formula (A), Formula (B), Formula (C), or Formula (D), age, weight, general condition of the subject, the condition being treated, the severity of the condition being treated, and the judgment of the prescribing physician. For examples, therapeutically effective amounts may be determined by routine experimentation, including but not solely dose escalation trials.

The terms “enhance” or “enhancing” means to increase or prolong either in potency or duration a desired effect. By way of example, “enhancing” the effect of therapeutic agents refers to the ability to increase or prolong, either in potency or duration, the effect of therapeutic agents on during treatment of a disease, disorder or condition. An “enhancing-effective amount,” as used herein, refers to an amount adequate to enhance the effect of a therapeutic agent in the treatment of a disease, disorder or condition. When used in a patient, amounts effective for this use will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the physician.

The terms “inhibits”, “inhibiting”, or “inhibitor” of a kinase, as used herein, refer to inhibition of enzymatic phosphotransferase activity.

The term “irreversible inhibitor,” as used herein, refers to a compound that, upon contact with a target protein (e.g., a kinase) causes the formation of a new covalent bond with or within the protein, whereby one or more of the target protein's biological activities (e.g., phosphotransferase activity) is diminished or abolished notwithstanding the subsequent presence or absence of the irreversible inhibitor.

The term “irreversible BTK inhibitor,” as used herein, refers to an inhibitor of BTK that can form a covalent bond with an amino acid residue of BTK. In one embodiment, the irreversible inhibitor of BTK can form a covalent bond with a Cys residue of BTK; in particular embodiments, the irreversible inhibitor can form a covalent bond with a Cys 481 residue (or a homolog thereof) of BTK or a cysteine residue in the homologous corresponding position of another TK.

The term “isolated,” as used herein, refers to separating and removing a component of interest from components not of interest. Isolated substances can be in either a dry or semi-dry state, or in solution, including but not limited to an aqueous solution. The isolated component can be in a homogeneous state or the isolated component can be a part of a pharmaceutical composition that comprises additional pharmaceutically acceptable carriers and/or excipients. For example, nucleic acids or proteins are “isolated” when such nucleic acids or proteins are free of at least some of the cellular components with which it is associated in the natural state, or that the nucleic acid or protein has been concentrated to a level greater than the concentration of its in vivo or in vitro production. Also, by way of example, a gene is isolated when separated from open reading frames which flank the gene and encode a protein other than the gene of interest.

A “metabolite” of a compound disclosed herein is a derivative of that compound that is formed when the compound is metabolized. The term “active metabolite” refers to a biologically active derivative of a compound that is formed when the compound is metabolized. The term “metabolized,” as used herein, refers to the sum of the processes by which a substance is changed by an organism.

The term “modulate,” as used herein, means to interact with a target either directly or indirectly so as to alter the activity of the target, including, by way of example only, to enhance the activity of the target, to inhibit the activity of the target, to limit the activity of the target, or to extend the activity of the target.

5 As used herein, the term “modulator” refers to a compound that alters an activity of a molecule. In certain embodiments the presence of a modulator results in an activity that does not occur in the absence of the modulator.

As used herein, the term “selective binding compound” refers to a compound that selectively binds to any portion of one or more target proteins.

10 As used herein, the term “selectively binds” refers to the ability of a selective binding compound to bind to a target protein, such as, for example, BTK, with greater affinity than it binds to a non-target protein.

As used herein, the term “selective modulator” refers to a compound that selectively modulates a target activity relative to a non-target activity.

15 The term “substantially purified,” as used herein, refers to a component of interest that may be substantially or essentially free of other components which normally accompany or interact with the component of interest prior to purification. By way of example only, a component of interest may be “substantially purified” when the preparation of the component of interest contains less than about 30%, less than about 25%, less than about 20%, less than
20 about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating components. Thus, a “substantially purified” component of interest may have a purity level of about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or greater.

25 The term “subject” as used herein, refers to an animal (including a mammal, including a human), which is the object of treatment, observation or experiment.

As used herein, the term “target activity” refers to a biological activity capable of being modulated by a selective modulator. Certain exemplary target activities include, but are not limited to, binding affinity, signal transduction, enzymatic activity, tumor growth, effects on particular biomarkers related to B-cell lymphoproliferative disorder pathology.

5 As used herein, the term “target protein” refers to a molecule or a portion of a protein capable of being bound by a selective binding compound.

The terms “treat,” “treating” or “treatment”, as used herein, include alleviating, abating or ameliorating a disease or condition, or symptoms thereof; managing a disease or condition, or symptoms thereof; preventing additional symptoms; ameliorating or preventing the
10 underlying metabolic causes of symptoms; inhibiting the disease or condition, e.g., arresting the development of the disease or condition; relieving the disease or condition; causing regression of the disease or condition, relieving a condition caused by the disease or condition; or stopping the symptoms of the disease or condition. The terms “treat,” “treating” or “treatment”, include, but are not limited to, prophylactic and/or therapeutic treatments.

15 As used herein, the IC_{50} refers to an amount, concentration or dosage of a particular test compound or combination that achieves a 50% inhibition of a maximal response.

As used herein, EC_{50} refers to a dosage, concentration or amount of a particular test compound or combination that elicits a dose-dependent response at 50% of maximal expression of a particular response that is induced, provoked or potentiated by the particular
20 test compound or combination.

In some embodiments, the combinations of AADA and AADAS may be used in a method of treating a malignancy, including a hematological malignancy, in an subject in need thereof, comprising: (a) administering to the subject an amount of an AADAS (e.g. a BTKi) sufficient to sensitize a plurality of the malignant cells to treatment with an AADA (e.g. ASNase); and (b)
25 administering to the subject an amount of an AADA (e.g. ASNase) sufficient to induce apoptosis in the malignant cells sensitized thereto by the AADAS. In some embodiments, the amount of the AADA and/or the AADAS would be subtherapeutic were either to be administered as a monotherapy (e.g. without the corresponding AADA or AADAS) for the treatment of said

malignancy. As used herein, "Individually Subtherapeutic Amount" means that administration of the AADA or the AADAS absent its corresponding AADA or AADAS would be insufficient to treat a given disease or condition that could be effectively treated by a combination of the AADA and the AADAS. In some embodiments, one or both the AADA and the AADAS may be administered to a subject in individually subtherapeutic amounts. In some embodiments, both the AADA and the AADAS are administered to the subject in individually subtherapeutic amounts.

In some embodiments, the AADAS is a BTKi and the AADA is an ASNase. In such embodiments, the amount of the BTKi may be insufficient to induce death in all of the cells of the malignancy, but may be sufficient to sensitize at least a plurality of the cells to ASNase-induced apoptosis.

In some embodiments, the malignancy is a hematological malignancy including ALL, AML or CLL. In other embodiments, the malignancy is a solid tumor, including but not limited to CRC, TNBC, PANC or any other solid tumor. Advantageous target indications include solid tumor and lymphoma indications selected from Gastric cancer, Pancreatic cancer, NK Lymphoma, DLBCL, Colorectal cancer, Bladder cancer, Hepatic cancer and Glioma/Glioblastoma.

In some embodiments, the method comprises evaluating the extent to which the AADAS has sensitized the malignant cells to AADA-induced apoptosis. Evaluation may comprise measuring the level of inhibition or inactivation of a biological target of the AADAS. For example, when the biological target of the AADAS is BTK, the level of phosphorylation of BTK may be measured to determine whether a given cell has been sensitized to AADA-induced apoptosis. In some embodiments, administration of the AADA may be timed to maximize the synergistic efficacy of the combination of AADA and AADAS. For example, it may be clinically advantageous to ensure the maximum inhibition of a biological target (e.g. BTK) prior to administering the AADA (e.g. ASNase). In some embodiments, if the evaluation indicates that maximum sensitization has been achieved by the administration of the AADAS, the amount of the AADAS administered to the subject may be lowered.

As used herein, a “sensitization-effective amount” means an amount of AADAS sufficient to elicit sensitivity to AADA-induced apoptosis. Likewise, a “maximal sensitization-effective amount” means a sensitization-effective amount that is capable of eliciting the maximal sensitization effect for a given AADAS (e.g. a BTKi) to a given AADA (e.g. an ASNase).

5 In some embodiments, evaluating the extent of sensitization comprises measuring the duration of the reduction in activity of the biological target of the AADAS (e.g. phosphorylation status of BTK) as compared to before administration of the AADAS (e.g. a BTKi). In some embodiments, the method comprises administering the AADA after the activity of the biological target has remained at a predetermined reduced level for a predetermined length of time.

10 In some embodiments, the hematological malignancy is a chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), high risk CLL, or a non-CLL/SLL lymphoma. In some embodiments, the hematological malignancy is follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma, Waldenstrom's macroglobulinemia, multiple myeloma, marginal zone lymphoma, Burkitt's lymphoma, non-Burkitt high grade B cell
15 lymphoma, or extranodal marginal zone B cell lymphoma. In some embodiments, the hematological malignancy is acute or chronic myelogenous (or myeloid) leukemia, myelodysplastic syndrome, or acute lymphoblastic leukemia. In some embodiments, the hematological malignancy is relapsed or refractory diffuse large B-cell lymphoma (DLBCL), relapsed or refractory mantle cell lymphoma, relapsed or refractory follicular lymphoma,
20 relapsed or refractory CLL; relapsed or refractory SLL; relapsed or refractory multiple myeloma. In some embodiments, the hematological malignancy is a hematological malignancy that is classified as high-risk. In some embodiments, the hematological malignancy is high risk CLL or high risk SLL.

25 B-cell lymphoproliferative disorders (BCLDs) are neoplasms of the blood and encompass, *inter alia*, non-Hodgkin lymphoma, multiple myeloma, and leukemia. BCLDs can originate either in the lymphatic tissues (as in the case of lymphoma) or in the bone marrow (as in the case of leukemia and myeloma), and they all are involved with the uncontrolled growth of lymphocytes or white blood cells. There are many subtypes of BCLD, e.g., chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL). The disease course and

treatment of BCLD is dependent on the BCLD subtype; however, even within each subtype the clinical presentation, morphologic appearance, and response to therapy is heterogeneous.

Malignant lymphomas are neoplastic transformations of cells that reside predominantly within lymphoid tissues. Two groups of malignant lymphomas are Hodgkin's lymphoma and
5 non-Hodgkin's lymphoma (NHL).

In some embodiments, the combinations of AADA and AADAS may be used in a method of treating any malignancy, including a solid tumor, a hematological malignancy, a BCLD or a malignant lymphoma.

Disclosed herein, in certain embodiments, is a method for treating a DLCBL in a subject
10 in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in DLCBL cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating diffuse large B-cell lymphoma, activated B cell-like subtype (ABC-DLBCL), in a subject in need thereof, comprising
15 administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in ABC-DLBCL cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating follicular lymphoma, in a subject in need thereof, comprising administering a sensitization-effective amount of an
20 AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in follicular lymphoma cells sensitized by said AADAS. As used herein, the term "follicular lymphoma" refers to any of several types of NHL in which the lymphomatous cells are clustered into nodules or follicles.

Disclosed herein, in certain embodiments, is a method for treating a CLL or SLL in an
25 individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in CLL or SLL cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating a Mantle cell lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in Mantle cell lymphoma cells sensitized by said AADAS.

5 As used herein, the term, "Mantle cell lymphoma" (MCL) refers to a subtype of B-cell lymphoma, due to CD5 positive antigen-naïve pregerminal center B-cell within the mantle zone that surrounds normal germinal center follicles.

Disclosed herein, in certain embodiments, is a method for treating a marginal zone B-cell lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in marginal zone B-cell lymphoma cells sensitized by said AADAS.

As used herein, the term "marginal zone B-cell lymphoma" refers to a group of related B-cell neoplasms that involve the lymphoid tissues in the marginal zone, the patchy area outside the follicular mantle zone.

Disclosed herein, in certain embodiments, is a method for treating a "mucosa-associated lymphoid tissue" (MALT) lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in MALT cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating a nodal marginal zone B-cell lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in marginal zone B cell lymphoma cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating a Splenic Marginal Zone B-Cell Lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an

ASNase) sufficient to induce cell death (e.g. apoptosis) in Splenic Marginal Zone B-Cell Lymphoma cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating a Burkitt Lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in Burkitt Lymphoma cells sensitized by said AADAS.

The term "Burkitt lymphoma" refers to a type of Non-Hodgkin Lymphoma (NHL) that commonly affects children. It is a highly aggressive type of B-cell lymphoma that often starts and involves body parts other than lymph nodes. In spite of its fast-growing nature, Burkitt's lymphoma is often curable with modern intensive therapies.

Disclosed herein, in certain embodiments, is a method for treating a Waldenstrom Macroglobulinemia in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in Waldenstrom Macroglobulinemia cells sensitized by said AADAS.

The term "Waldenstrom macroglobulinemia", also known as lymphoplasmacytic lymphoma, is cancer involving a subtype of white blood cells called lymphocytes. It is characterized by an uncontrolled clonal proliferation of terminally differentiated B lymphocytes, and by the lymphoma cells making an antibody called immunoglobulin M (IgM).

Disclosed herein, in certain embodiments, is a method for treating a Multiple Myeloma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in Multiple Myeloma cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating a solid tumor or lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in solid tumor or lymphoma cells sensitized by said AADAS. The methods may be effective against any solid tumor or lymphoma, including those selected from

Gastric cancer, Pancreatic cancer, NK Lymphoma, Colorectal cancer, Bladder cancer, Hepatic cancer and Glioma/Glioblastoma.

Disclosed herein, in certain embodiments, is a method for treating a Leukemia in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in Leukemia cells sensitized by said AADAS.

Leukemia is a cancer of the blood or bone marrow characterized by an abnormal increase of blood cells, usually leukocytes (white blood cells). Leukemia is a broad term covering a spectrum of diseases. The first division is between its acute and chronic forms: (i) acute leukemia is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children; (ii) chronic leukemia is distinguished by the excessive build-up of relatively mature, but still abnormal, white blood cells. Leukemia includes, but not limited to, Acute lymphoblastic leukemia (ALL), Acute myelogenous leukemia (AML), Chronic myelogenous leukemia (CML), and Hairy cell leukemia (HCL).

Disclosed herein, in certain embodiments, is a method for treating a primary central nervous system lymphoma in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in primary central nervous system lymphoma cells sensitized by said AADAS.

Disclosed herein, in certain embodiments, is a method for treating an extranodal natural killer (NK) cell/T-cell lymphoma (ENKTL) in an individual in need thereof, comprising administering a sensitization-effective amount of an AADAS (e.g. a BTKi) and an amount of AADA (e.g. an ASNase) sufficient to induce cell death (e.g. apoptosis) in extranodal natural killer (NK) cell/T-cell lymphoma (ENKTL) cells sensitized by said AADAS.

In some embodiments, the AADAS is a BTK inhibitor selected from the group consisting of a small organic molecule, a macromolecule, a peptide or a non-peptide.

In some embodiments, the BTK inhibitor provided herein is a reversible or irreversible inhibitor. In certain embodiments, the BTK inhibitor is an irreversible inhibitor.

5 In some embodiments, the irreversible BTK inhibitor forms a covalent bond with a cysteine sidechain of a Bruton's tyrosine kinase, a Bruton's tyrosine kinase homolog, or a BTK tyrosine kinase cysteine homolog.

Irreversible BTK inhibitor compounds can use for the manufacture of a medicament for treating any of the foregoing conditions (e.g., autoimmune diseases, inflammatory diseases,
10 allergy disorders, B-cell proliferative disorders, or thromboembolic disorders).

In some embodiments, the BTK inhibitor compound used for the methods described herein inhibits BTK or a BTK homolog kinase activity with an in vitro IC₅₀ of less than about 10 μ M. (e.g., less than about 1 μ M, less than about 0.5 μ M, less than about 0.4 μ M, less than about 0.3 μ M, less than about 0.1, less than about 0.08 μ M, less than about 0.06 μ M, less than
15 about 0.05 μ M, less than about 0.04 μ M, less than about 0.03 μ M, less than about 0.02 μ M, less than about 0.01, less than about 0.008 μ M, less than about 0.006 μ M, less than about 0.005 μ M, less than about 0.004 μ M, less than about 0.003 μ M, less than about 0.002 μ M, less than about 0.001, less than about 0.00099 μ M, less than about 0.00098 μ M, less than about 0.00097 μ M, less than about 0.00096 μ M, less than about 0.00095 μ M, less than about 0.00094 μ M, less
20 than about 0.00093 μ M, less than about 0.00092, or less than about 0.00090 μ M).

In one embodiment, the BTK inhibitor compound selectively and irreversibly inhibits an activated form of its target tyrosine kinase (e.g., a phosphorylated form of the tyrosine kinase). For example, activated BTK is transphosphorylated at tyrosine 551. Thus, in these embodiments the irreversible BTK inhibitor inhibits the target kinase in cells only once the target kinase is
25 activated by the signaling events.

In some embodiments, the BTKi is selected from the following: BTKi is selected from ibrutinib (US 7,514,444 to Pharmacyclics), acalabrutinib (US 9,522,917 to Acerta), zanabrutinib (US 9,447,106 to BeiGene), tirabrutinib (US 8,557,803 to Ono), M7583 (US 9,073,947 to Merck),

vecabrutinib (US 8,785,440 to Sunesis), CT-1530 (US 9,447,106 to Centaurus), ARQ 531 (US 9,630,968 to ArQule), DTRMWXHS-12 (US 9,717,745 to Zhejiang), TG-1701 (WO 2017/118277 to TG Therapeutics), spebrutinib, CC-292 (US 7,989,465 to Celgene), LOXO-305 (US 2017/0129897 to Loxo Oncology), CG'806 (Aptose Biosciences), evorbrutinib (US 9,073,947 to Merck), RG7845, GDC-0853 (US 8,716,274 to Roche-Genentech), poseltinib, LY3337641 and HM71224 (US 8,957,065 to Lilly-Hanmi), PRN1008 (US 9,580,427 to Principia), BMS-986142 (US 8,846,673 to BMS), PRN2246 (US 9,580,427 to Principia), TAK-020 (US 9,365,566 to Takeda), AC0058 (US 9,464,089 to Acea Biosciences), BIIb-068, vecabrutinib (US 8,785,440 to Biogen) and combinations or equivalents thereof.

10 *Compositions, Formulations, and Routes of Administration*

Also disclosed is a pharmaceutical composition comprising a disclosed AADA/AADAS combination in a pharmaceutically acceptable carrier. The composition may be a kit comprising effective amounts of the AADA and AADAS, and optionally instructions for use thereof.

15 Pharmaceutical compositions containing the disclosed AADA/AADAS combination can be administered to a patient using standard techniques. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa., 1990 (herein incorporated by reference).

20 Suitable dosage forms, in part, depend upon the use or the route of entry, for example, oral, transdermal, transmucosal, or by injection (parenteral, IV, etc.). Such dosage forms should allow the therapeutic agent to reach a target cell or otherwise have the desired therapeutic effect. For example, pharmaceutical compositions injected into the blood stream preferably are soluble. The disclosed conjugates and/or pharmaceutical compositions can be formulated as pharmaceutically acceptable salts and complexes thereof.

25 Pharmaceutically acceptable salts are non-toxic salts present in the amounts and concentrations at which they are administered. The preparation of such salts can facilitate pharmaceutical use by altering the physical characteristics of the compound without preventing it from exerting its physiological effect. Useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing solubility to

facilitate administering higher concentrations of the drug. The pharmaceutically acceptable salt of an AADA (e.g. an ASNase) may be present as a complex, as those in the art will appreciate.

Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, fumarate, maleate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate, and quinate. Pharmaceutically acceptable salts can be obtained from acids, including hydrochloric acid, maleic acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, fumaric acid, and quinic acid.

Pharmaceutically acceptable salts also include basic addition salts such as those containing benzathine, chlorprocaine, choline, diethanolamine, ethylenediamine, meglumine, procaine, aluminum, calcium, lithium, magnesium, potassium, sodium, ammonium, alkylamine, and zinc, when acidic functional groups, such as carboxylic acid or phenol are present. For example, see Remington's Pharmaceutical Sciences, *supra*. Such salts can be prepared using the appropriate corresponding bases.

Pharmaceutically acceptable carriers and/or excipients can also be incorporated into a pharmaceutical composition according to the invention to facilitate administration of the particular AADA or AADAS. Examples of carriers suitable for use in the practice of the invention include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and physiologically compatible solvents. Examples of physiologically compatible solvents include sterile solutions of water for injection (WFI), saline solution and dextrose.

Pharmaceutical compositions can be administered by different routes, including intravenous, intraperitoneal, subcutaneous, intramuscular, oral, topical (transdermal), or transmucosal administration. For oral administration, for example, the compounds can be formulated into conventional oral dosage forms such as capsules, tablets, and liquid preparations such as syrups, elixirs, and concentrated drops. For injection, pharmaceutical

compositions are formulated in liquid solutions, preferably in physiologically compatible buffers or solutions, such as saline solution, Hank's solution, or Ringer's solution.

In addition, the compounds may be formulated in solid form and re-dissolved or suspended immediately prior to use. For example, lyophilized forms of the conjugate can be produced. In a specific embodiment, the conjugate is administered intramuscularly. In another specific embodiment, the conjugate is administered intravenously. In some embodiments the pharmaceutical composition is contained in a vial as a lyophilized powder to be reconstituted with a solvent.

Systemic administration can also be accomplished by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are well known in the art, and include, for example, for transmucosal administration, bile salts, and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration, for example, may be through nasal sprays, inhalers (for pulmonary delivery), rectal suppositories, or vaginal suppositories. For topical administration, compounds can be formulated into ointments, salves, gels, or creams, as is well known in the art.

The amounts of the composition to be delivered will depend on many factors, for example, the IC_{50} , EC_{50} , the biological half-life of the compound, the age, size, weight, and physical condition of the subject or patient, and the disease or disorder to be treated. The importance of these and other factors to be considered are well known to those of ordinary skill in the art. Generally, the amount of the composition (e.g. when the AADA is an enzyme like asparaginase) to be administered will range from about 10 International Units per square meter of the surface area of the patient's body (IU/m^2) to $50,000 IU/m^2$, with a dosage range of about $1,000 IU/m^2$ to about $15,000 IU/m^2$ being preferred, and a range of about $6,000 IU/m^2$ to about $15,000 IU/m^2$ being more preferred, and a range of about 10,000 to about 15,000 IU/m^2 (about 20-30 mg protein/m) being particularly preferred to treat a malignant hematologic disease, e.g., leukemia. Such dosages may be administered via intramuscular or intravenous injection at an interval of about 3 times weekly to about once per month, or once per week or once every

other week during the course of therapy. Of course, other dosages and/or treatment regimens may be employed, as determined by the attending physician.

Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Example 1 - CRISPR/Cas9 Based Kinome Screen Identifies BTK as an Important Determinant of Asparaginase Treatment Response in Acute Lymphoblastic Leukemia (ALL)

Unless otherwise specified below, the *in vitro* studies were conducted using native *E. coli* ASNase (NCBI WP_000394140.1), and *in vivo* studies were conducted using ONCASPAR®. Moreover, all references to commercial product specifications should be interpreted to mean as understood by the skilled artisan as of the time of the filing of this application.

Briefly, a CRISPR/Cas9-based kinome screen (outlined in FIG. 1A) was conducted to identify genes whose deletion would have an impact on the ALL cells' sensitivity to amino acid depletion by ASNase. Nalm6 pre-B ALL cells were transduced with a lentivirus encoding a doxycycline inducible Cas9. Subsequently, a gRNA library targeting 506 kinases was introduced by lentiviral transduction with an average of one gRNA per cell. Each individual kinase in the library was represented by 10 gRNAs. After selection of transduced cells, doxycycline was added for one week to allow Cas9 expression to induce mutations. Thereafter, expression of Cas9 in response to doxycycline exposure was verified by Western Blotting (FIG. 1B). After verification of library complexity, cells were split for ASNase or mock treatment. Following treatment, DNA was harvested and subjected to NGS based sequencing to determine relative frequencies of each individual gRNA in the ASNase and control treated cells. and the distribution of gRNAs in the pool of Nalm6 cells before and after treatment with Asparaginase is shown in FIG. 1C. A deviation from the diagonal indicates enrichment (left) or depletion (right) of a specific gRNA during treatment. Finally, FIG. 1D indicates genes significantly enriched/depleted during asparaginase treatment.

Conclusion: These results show that the CRISPR-Cas9 based reverse genetics screen successfully identifies gene products that modulate the sensitivity to asparaginase treatment.

Example 2 – ASNase response is affected by kinases regulating the ER stress response pathway

A series of experiments was conducted to begin to understand the mechanisms involved in the observed gene-deletion-mediated sensitization or resistance to amino acid starvation stress induced by ASNase.

FIG. 2A presents a schematic overview of the amino acid response pathway. Kinases representing gRNAs enriched in the screen are shown in blue, and kinases representing gRNAs that are selectively lost (dropouts) are shown in purple. Targeted knockout experiments were used to validate these results in independent experiments. As indicated by the Western Blot data shown in FIG. 2B, the TRIB3 gene was effectively disrupted by CRISPR/Cas9 mediated introduction of frame shift mutations/deletions in a pool of Nalm6 cells. In another pool of Nalm6 cells, the GCN2 gene was similarly disrupted by CRISPR/Cas9 mediated introduction of frame shift mutations/deletions (FIG. 2C). Nalm6 wt and TRIB3 knockout cells (pool) were then left untreated or treated with ASNase and their proliferation was determined by measuring cell numbers over time (FIGs. 2D & 2E), the SubG1 (apoptotic) fraction was determined by Hoechst staining (FIG. 2F). Additionally, viability was measured using a MTT based viability assay (FIG. 2G). Nalm6 control and GCN2 deleted cells (pools) were either left untreated or treated with ASNase and the SubG1 (apoptotic) fraction was determined by Hoechst staining (FIG. 2H). Asterisks indicates a significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Viability was measured using a MTT based viability assay (FIG. 2I).

Conclusions: These results indicate that two candidate genes that were identified with the reverse genetic screen, TRIB3 and GCN2, previously implicated in the amino acid stress response pathway, are indeed valid modifiers of the response to asparaginase.

Example 3 – Targeted deletion or pharmacological inhibition of BTK sensitizes Nalm6 cells to ASNase treatment

A series of experiments was conducted to study the impact of deletion or pharmacological inhibition of Bruton's Tyrosine Kinase (BTK) on the response of cells to ASNase treatment.

BTK Deletion. FIG. 3A presents a schematic overview depicting the (pre-) B cell receptor pathway. Initially, the BTK gene was disrupted by CRISPR/Cas9 mediated introduction of frame shift mutations/deletions in Nalm6 cells and single cell clones were evaluated for BTK expression using Western blot (FIG. 3C). Nalm6 wt Clones and BTK knockout clones were then either treated with 1 or 5 IU/ml ASNase or left untreated for 1 week before the SubG1 (apoptotic) fraction was measured by Flow cytometry. (FIGs. 3D & 3E) The same cells were also analyzed for the presence of apoptotic cells by Hoechst staining (FIGs. 3G & 3H). Asterisks indicate a significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

BTK inhibition. Wildtype Nalm6, Sem and Reh cells were cultured for 7 days in the presence or absence of 1IU/ml ASNase and increasing concentrations of the BTK inhibitor ibrutinib. After 7 days, drugs were washed out and remaining cells reseeded in soft agarose containing medium (scheme presented in FIG. 4A). Cell viability was then measured by cell cycle distribution, (FIGs. 4C & 4D), and by blotting for the apoptosis marker PARP and calculating the ratio between cleaved and uncleaved PARP protein or (FIG. 4E) by amine staining (FIG. 4F). After a washout of ibrutinib and ASNase, viable cells were maintained in semi soft agar for three days and colonies were stained with Cristal violet and quantified by cell counting (FIGs. 4G & 4H). Finally, FIG. 4I is a model explaining how the asparaginase / ibrutinib combination therapy shifts cells from a survival mode into an apoptosis mode.

Conclusions. The ability of the combination of ibrutinib and ASNase to elicit such a significant apoptotic killing efficacy was surprising and unexpected, particularly in view of the relatively small effect that ibrutinib and ASNase exerted against cells when used alone (FIGs. 4D, 4E, 4F & 4H).

Example 4 – ASNase and ibrutinib exhibit synergistic efficacy in ALL cell lines and ALL xenografts

A series of experiments was conducted to test the extent of synergistic efficacy between ASNase and ibrutinib against patient-derived xenografts (PDX). The viability and proliferative

capacity of primary human ALL cells outside of the body is extremely poor. In order to expand the number of tumor cells, human ALL cells are injected into the bone marrow of immunocompromised mice. After 2-4 months these mice developed full blown leukemia with an immunophenotype and genetic composition that in the large majority of cases is identical to the original material at the time of injection. These cells can then be isolated and used immediately or viably frozen for future use in in vitro and in vivo experiments. These PDX cells were used to test for synergistic effects between Ibrutinib and asparaginase of cell viability (FIGs. 5A-5G).

FIG. 5A presents a schematic overview representing the workflow used to derive drug synergies in ALL cell lines and PDX samples. Briefly, hTERT immortalized MSCs were seeded in either a 384 wells format (microscopy) or a 96 wells format (7AAD staining) and allowed to settle for 24 hours prior to the addition of ALL xenografts. Cells were again allowed to settle for 24 hours before ASNase and ibrutinib were added as serial dilutions. After 3 and 7 days of incubation, cell death was analyzed using microscopy using live cells staining or flowcytometry using 7AAD to discriminate between live or dead cells. Quantification of the microscopy images was done using matlab. In a second step the data were analyzed using R-software to calculate a Combination Index (CI). For 7 day studies, cells were refed midway with ASN-depleted media.

Using this workflow, 73 experiments were performed, using 35 unique PDX samples. A total of 59 experiments passed quality control and were submitted for analysis. A strong synergy between asparaginase and Ibrutinib was found in over 70% of experiments ($CI \leq 0.8$) (FIG. 5C). FIG. 5D shows representative examples of synergistic, additive and antagonistic effects of the combination treatment obtained using the automated microscopy. Of note, antagonistic effects were only observed in those cases that already showed a strong response to asparaginase alone. These results were validated in an independent experiment using flowcytometry based evaluation of cell viability after treatment. FIG. 5E shows the response of 10 different PDX samples to combination treatment. FIG. 5F is a summary table indicating the synergistic killing efficacy of the combination against PDX; and FIG. 5G is a summary table showing the synergistic killing efficacy, but this time, with the 100 μ M ibrutinib group excluded from the CI calculation.

Conclusion: A strong synergy between asparaginase and Ibrutinib in (ex vivo) tumor cell killing was observed in over 70% of ALL cases (PDX).

For the following Tables: A=ASNase; A0=0; A1=0.01; A2=0.03; A3=0.1; A4=0.3; A5=1; A6=3; A7=10; A8=32; A=100; B=ibrutinib; B0=0; B1=0.001; B2=0.004; B3=0.02; B4=0.1; B5=0.3; B6=1; B7=6; B8=24; B9=100.

Table 2. m3810 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	49	49	50	32	32	38	67	36	7	3
B8	67	69	53	64	55	50	58	70	32	2
B7	78	87	67	80	50	66	91	49	41	1
B6	85	105	77	121	87	102	108	122	42	2
B5	163	151	153	116	115	144	91	81	27	2
B4	373	286	334	301	220	243	198	90	44	3
B3	529	383	494	411	369	329	274	126	55	4
B2	564	429	500	442	376	304	261	138	49	3
B1	563	492	505	418	476	379	302	100	63	4
B0	2527	1776	1752	1563	1519	1136	1251	686	87	4

Table 3. m764 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	24	16	21	32	41	32	17	18	18	7
B8	52	81	53	82	66	98	55	63	57	4
B7	76	92	82	109	105	124	67	110	79	4
B6	74	124	88	87	103	78	93	59	46	8
B5	111	107	167	113	104	128	117	82	146	5
B4	123	113	114	119	102	101	152	73	85	5
B3	111	111	103	142	118	129	136	99	75	10
B2	105	114	124	113	120	92	138	92	145	3
B1	112	92	116	104	85	114	139	94	73	7
B0	697	546	1126	109	250	915	887	594	157	2

Table 4. m838 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	249	430	397	508	641	254	245	50	40	16
B8	628	1082	924	986	1084	997	476	372	18	14
B7	1014	992	1024	1214	1215	1227	798	532	47	14
B6	1169	749	1233	1225	1192	716	1101	747	61	13

B5	1019	1074	1148	1361	1334	1369	1143	527	67	10
B4	1638	938	1479	1605	1643	925	1264	706	72	11
B3	1765	1476	1447	1589	1294	1385	1241	949	83	12
B2	1646	1465	1759	1002	993	1424	918	876	99	7
B1	1577	911	1662	1755	1125	789	1437	678	173	15
B0	3112	2793	3003	3109	2839	2454	3049	2431	175	18

Table 5. m1095 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	420	408	342	333	335	289	251	224	40	12
B8	813	860	826	708	763	777	806	518	56	19
B7	773	887	747	703	611	770	689	620	174	13
B6	831	919	902	683	779	906	751	584	153	23
B5	858	695	739	686	878	726	865	606	136	17
B4	853	834	914	812	716	660	805	494	198	9
B3	745	787	754	734	814	765	701	534	144	13
B2	758	753	723	785	721	616	481	352	174	18
B1	747	749	724	658	551	603	557	320	312	16
B0	3148	2935	2914	3089	3205	2579	549	2033	238	30

Table 6. m4136 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	100	122	85	165	122	128	92	64	27	13
B8	132	94	95	141	105	130	114	177	79	17
B7	158	126	95	164	113	116	166	180	130	24
B6	125	132	129	132	163	149	120	149	152	16
B5	184	152	158	127	160	204	157	211	164	36
B4	165	161	171	213	168	224	215	320	324	19
B3	215	185	175	195	205	251	236	381	489	13
B2	366	202	229	195	192	218	272	293	522	21
B1	293	200	289	207	193	205	314	408	254	15
B0	2590	1992	1998	1980	1892	2157	2652	2136	2172	39

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Table 7. m1600 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	903	752	962	655	916	1025	362	84	11	0
B8	1378	681	1068	864	860	1201	710	506	1	3
B7	1293	914	843	1125	949	1010	788	603	4	1
B6	2324	1520	1322	1461	1377	1189	1084	776	16	1
B5	3669	2665	2421	2385	2306	1815	1758	1316	39	0

B4	4993	3411	3113	2983	3394	2569	2209	1469	155	2
B3	5554	3343	3485	3461	3097	3047	3026	2491	149	1
B2	3632	3511	3292	2799	3030	2658	3402	2449	348	1
B1	4128	3619	3680	3292	2673	3558	3084	2515	279	1
B0	4508	4146	4603	3714	3134	4045	4366	3730	487	4

Table 8. m877 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1493	1001	1072	939	1022	602	545	115	33	1
B8	3082	2044	2043	1723	1443	1300	1062	887	120	1
B7	3178	2006	2363	2011	1958	1806	1434	846	30	3
B6	4243	3191	3536	3418	2958	2503	2316	1535	124	1
B5	5835	4602	4504	4313	4520	4483	3710	1803	164	2
B4	5764	5424	5650	5427	4935	4678	4574	2984	409	3
B3	6331	5504	5305	5445	5331	5070	5369	3823	388	6
B2	5629	5066	5289	5210	4956	5140	5345	4548	739	0
B1	4914	4588	4458	4325	4972	5022	5021	4239	600	4
B0	5076	5090	4877	4455	4789	5008	5125	4350	1205	2

Table 9. m3657 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	40	28	50	54	31	26	22	37	20	18
B8	158	178	171	189	197	143	72	34	38	19
B7	229	210	214	239	223	185	112	39	37	3
B6	293	298	295	347	322	250	118	53	39	13
B5	433	385	524	439	437	374	211	86	37	16
B4	1355	1081	1107	1194	1370	1056	882	199	48	15
B3	1167	1389	1338	1314	1287	1074	800	338	73	14
B2	1436	1334	1375	1157	1467	1102	1074	395	83	26
B1	1326	1487	1245	1116	1115	1277	913	385	36	10
B0	3780	3562	4473	4173	1159	3935	3317	4220	723	17

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Table 10. m1338 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	416	154	93	114	91	35	22	1	0	1
B8	719	506	566	475	370	343	219	21	0	0
B7	1374	825	640	590	674	594	292	57	0	0
B6	1658	1073	825	883	781	724	431	114	3	0
B5	1856	1407	1504	1426	1482	961	907	211	7	1
B4	2628	2437	2519	2250	2363	2096	1537	668	27	0

B3	3547	2493	2245	2641	2576	1893	2322	1565	85	0
B2	2770	2366	2360	2735	2568	2327	2108	1704	222	0
B1	3024	2426	2405	2281	2185	2636	2264	1410	171	1
B0	3373	2207	3174	2916	2372	2788	2166	1468	292	1

Table 11. m4206 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1021	977	917	832	852	708	643	314	289	35
B8	695	717	563	724	607	471	504	361	53	68
B7	455	485	529	546	426	386	293	352	86	136
B6	387	855	419	544	501	325	442	453	76	110
B5	936	795	787	807	776	671	806	592	103	158
B4	1460	1254	1439	1015	1115	1284	1069	866	108	157
B3	1522	1967	1303	1395	1194	1189	1204	1066	81	223
B2	1693	1681	1288	1313	1405	1141	1221	847	109	165
B1	1859	1428	1318	1175	1552	996	1415	967	83	84
B0	3409	3112	2484	3106	2323	2141	2304	1979	301	69

Table 12. m1717 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	930	769	609	614	528	422	123	30	21	5
B8	1991	1202	1127	1053	1309	1141	604	136	44	4
B7	2044	1504	1314	1249	1388	1502	836	305	43	18
B6	2596	1979	1651	1683	1793	1864	1330	716	28	10
B5	3758	2853	2406	2418	2803	2826	2797	1036	28	6
B4	6464	4162	3717	4089	4206	4587	3992	1813	104	15
B3	6851	5534	4497	4211	4632	4665	4904	2817	146	3
B2	7148	5854	5011	4310	5056	5180	4562	3396	309	17
B1	6284	5714	4884	5097	4946	5285	5007	3081	278	13
B0	6722	5977	6674	5155	6700	6589	5782	3762	571	24

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Table 13. m3810 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	74	106	74	101	79	113	77	30	7	10
B8	113	164	141	163	144	184	167	102	16	11
B7	130	139	143	178	136	144	139	134	14	10
B6	111	185	158	199	157	185	165	175	23	17
B5	265	295	302	287	295	262	268	235	60	7
B4	613	757	612	596	530	470	404	358	87	11
B3	597	837	764	780	627	566	486	405	85	6

B2	727	763	701	676	647	478	482	385	139	15
B1	582	530	552	556	613	663	512	498	104	16
B0	2123	1232	2209	1405	1982	1472	1497	1054	268	8

Table 14. m4206 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	60	83	120	121	214	508	1228	1264	1368	4881
B8	91	65	108	92	156	663	1243	1047	1229	3666
B7	84	93	70	103	204	602	1188	1369	1355	4685
B6	70	60	82	118	111	728	1150	1110	923	3684
B5	71	53	108	76	196	429	1285	1421	1180	3902
B4	58	80	91	112	137	501	927	1090	942	3737
B3	58	73	111	126	91	295	720	844	688	3282
B2	147	129	98	115	164	131	306	309	245	1532
B1	161	74	181	86	222	220	308	258	241	143
B0	32	56	52	86	37	83	87	68	24	35

Table 15. X-SK-5864D (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	51	99	75	55	75	47	43	37	24	19
B8	65	114	137	138	122	94	87	60	41	7
B7	158	177	148	152	196	117	93	88	49	37
B6	189	156	250	273	158	159	129	122	47	93
B5	402	398	390	410	338	332	343	184	36	114
B4	2636	1761	1791	1219	1433	1417	1291	591	86	114
B3	3379	4002	3867	3538	3231	3073	3105	2044	176	103
B2	4746	3760	3869	3618	3415	3979	1782	2008	267	72
B1	4600	4300	4111	3727	4171	3638	2151	1541	251	51
B0	8994	8655	8407	8740	9490	9099	8295	6625	1834	39

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Table 16. m1560 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	119	143	155	113	150	141	154	85	85	11
B8	978	940	1061	886	776	753	679	340	99	18
B7	1615	1448	1443	1600	1556	1230	1033	549	102	21
B6	2147	1676	2022	2336	1966	1799	1513	933	210	8
B5	3294	3216	3318	3021	3440	3225	2479	1175	233	18
B4	3833	4207	3976	3657	3629	3192	2661	1564	848	7
B3	4034	3754	4065	4060	4553	3549	3858	3156	912	9
B2	4644	3954	4070	4143	4767	3794	3995	3859	1144	19

B1	4009	3477	3926	3794	4154	3510	4199	3495	979	12
B0	3868	3831	4353	3990	4102	4790	4389	4671	1355	11

Table 17. XX-SK-15723 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	127	164	148	185	154	146	129	68	116	251
B8	219	252	148	316	357	185	181	119	101	353
B7	358	290	248	331	325	281	243	174	105	455
B6	440	391	353	309	440	298	275	219	123	571
B5	574	500	642	561	786	533	618	367	189	552
B4	807	845	688	888	834	747	822	521	151	658
B3	882	944	983	973	951	1114	961	718	181	421
B2	847	863	821	748	900	1008	771	632	165	503
B1	875	894	915	723	811	808	909	766	155	548
B0	1151	1111	1324	1511	1030	1379	1698	1672	381	465

Table 18. X-SK-14316 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	102	148	78	65	69	58	67	17	21	7
B8	191	116	100	110	73	50	49	14	11	5
B7	140	171	164	147	156	139	70	15	20	2
B6	279	230	229	202	181	235	135	69	14	5
B5	311	292	338	318	375	284	299	218	33	7
B4	603	373	415	426	436	416	463	337	131	13
B3	516	453	457	596	595	560	612	460	215	4
B2	617	423	515	432	459	410	522	467	278	1
B1	684	570	488	502	561	463	503	460	198	1
B0	1067	825	708	984	778	710	727	584	345	2

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Table 19. m3596 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	658	682	757	545	767	658	278	82	34	10
B8	1101	1035	1472	917	1258	743	926	448	131	39
B7	1079	1248	1240	1111	1215	1313	924	622	111	25
B6	1344	1196	1412	1309	1014	1161	1149	648	64	11
B5	1853	1900	2016	1756	1947	1385	1129	658	67	16
B4	3093	3011	3067	2970	2953	2790	2388	1594	136	16
B3	3031	2989	2977	3050	2966	2368	2683	1829	165	25
B2	3392	3588	2861	3154	3237	2842	2462	1768	103	21
B1	3162	3260	3091	3260	2900	2698	2869	1414	137	22

B0	5541	5112	5583	5099	4098	5124	4525	3446	178	14
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Table 20. XX-IV-182 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	999	897	955	924	876	851	1022	480	27	14
B8	1497	1680	1723	1492	1717	1430	1533	966	93	9
B7	2067	1897	1727	1697	1861	1679	1579	1069	65	16
B6	2253	2501	1925	1545	1702	1701	1818	1152	203	19
B5	2324	2869	2703	2308	2611	2573	2328	1139	248	2
B4	3138	3353	3121	3150	3167	2990	2731	2323	649	3
B3	2857	2994	3325	3426	3359	3282	3013	2810	1228	19
B2	3257	3303	3281	3127	3098	3480	2446	2845	1612	18
B1	3169	3025	3402	3353	2759	2989	2927	2748	1302	10
B0	3226	3476	3328	3487	3721	3248	3111	2076	1869	0

Table 21. m1594 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	865	442	451	383	512	391	139	88	17	17
B8	1142	576	462	534	691	524	324	165	136	5
B7	1253	672	763	649	641	671	453	196	124	16
B6	1692	1363	1093	1111	995	1015	790	333	103	11
B5	2745	2640	2671	2444	2012	2458	1929	929	249	23
B4	4818	3651	4455	4229	3751	3578	4408	2798	230	29
B3	4966	5215	4568	3850	4854	5223	4688	3445	492	41
B2	5720	4808	1079	4927	4907	4909	4890	3847	697	70
B1	5417	4645	4754	5119	4584	4351	5212	3919	533	24
B0	7645	6401	5827	5690	5212	5817	6001	5577	1349	21

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Table 22. XX-SK-15723 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	127	164	148	185	154	146	129	68	116	251
B8	219	252	148	316	357	185	181	119	101	353
B7	358	290	248	331	325	281	243	174	105	455
B6	440	391	353	309	440	298	275	219	123	571
B5	574	500	642	561	786	533	618	367	189	552
B4	807	845	688	888	834	747	822	521	151	658
B3	882	944	983	973	951	1114	961	718	181	421
B2	847	863	821	748	900	1008	771	632	165	503
B1	875	894	915	723	811	808	909	766	155	548
B0	1151	1111	1324	1511	1030	1379	1698	1672	381	465

Table 23. X-SK-6731 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	133	47	48	44	87	35	47	1	0	0
B8	335	200	164	122	170	156	174	50	0	0
B7	362	175	151	134	193	149	165	80	0	0
B6	549	385	235	258	310	273	262	110	4	0
B5	1553	826	723	672	790	721	650	160	0	0
B4	3585	2061	1645	1597	1448	1532	1388	554	23	0
B3	3856	2579	2112	2186	1870	1821	1427	652	16	0
B2	3698	2289	2404	1793	1991	1715	1767	818	76	0
B1	3499	2563	2168	2073	2006	1830	1827	858	58	0
B0	4324	3005	2586	2601	2507	2249	2333	1142	160	0

Table 24. m3596 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	135	75	124	85	60	41	50	15	3	0
B8	265	289	115	324	221	383	355	100	3	1
B7	273	304	172	186	346	297	173	165	2	4
B6	325	345	297	379	340	383	387	117	21	0
B5	125	369	300	365	541	514	511	241	8	2
B4	933	1003	950	707	917	1034	1075	645	38	0
B3	1423	1544	1380	1861	1551	1880	1018	864	58	0
B2	1727	1460	1675	1459	1476	1610	1647	943	45	0
B1	1727	1492	1496	1719	1652	1240	1577	1046	33	0
B0	3310	3788	4101	3585	3908	3717	3560	2162	94	1

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Table 25. m3657 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	181	134	192	218	160	127	70	12	8	3
B8	420	388	377	369	394	214	166	47	11	0
B7	423	403	515	478	456	639	207	103	35	2
B6	568	476	581	603	506	486	275	113	47	1
B5	681	790	726	661	937	527	488	183	79	3
B4	1208	1076	1353	1081	1570	1326	926	558	161	2
B3	1194	1328	1385	1408	1317	1108	1337	729	214	0
B2	1311	996	1404	1339	1498	1325	1385	919	223	3
B1	1345	1538	1260	1552	1246	1210	1436	749	228	2
B0	1632	1929	2171	2106	2057	2138	2291	1780	1080	2

Table 26. m1307 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	388	386	0	342	321	239	120	46	6	9
B8	534	638	0	549	505	434	241	41	106	0
B7	960	997	965	1063	900	695	385	101	209	2
B6	1452	1491	1437	1433	1302	1090	528	74	367	4
B5	3518	3168	3282	3438	3088	1227	2237	417	506	7
B4	5749	5024	6133	5253	5504	5263	4846	2818	697	6
B3	4119	5503	6015	5491	5466	5547	5522	3270	1570	1
B2	5036	5245	5008	5320	5112	4896	4325	3420	1513	5
B1	5093	5163	1240	5415	5186	5088	5274	3445	1610	5
B0	5757	6152	6130	6246	5821	5979	6580	6173	3087	3

Table 27. m1307 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	31	25	50	38	45	44	26	11	0	1
B8	105	167	99	130	167	83	55	40	41	0
B7	295	224	225	359	264	149	310	35	58	0
B6	409	406	459	476	441	26	83	70	55	1
B5	729	1441	1401	1564	1537	614	573	140	99	0
B4	3714	3643	3874	2696	4135	3031	2413	846	112	0
B3	4767	5023	5325	4275	4510	4317	3435	1691	187	0
B2	3973	4581	5143	4683	4904	4696	3887	2438	411	1
B1	4509	4912	4491	4869	4162	4063	3458	2487	293	0
B0	6796	7142	7897	6024	7716	7448	6827	6821	3341	0

5 Table 28. m4138 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	56	65	51	72	54	45	41	91	121	144
B8	63	54	74	76	67	50	47	99	109	161
B7	90	58	91	42	101	53	36	64	92	210
B6	69	110	53	95	77	94	74	54	40	168
B5	174	111	150	134	101	98	107	66	63	137
B4	467	230	397	365	332	271	116	104	28	114
B3	1115	386	1185	1033	975	1015	929	108	110	172
B2	1232	1233	1406	1414	479	1319	632	294	55	135
B1	804	1419	1197	1277	1425	677	1114	114	86	69
B0	1058	1607	2092	2136	1740	664	1893	865	128	97

Table 29. m1106 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	650	646	546	741	959	1193	2189	1863	3	12
B8	1731	1724	1447	1244	1545	1473	1887	1369	793	31
B7	2418	2400	2252	2591	2682	2315	2209	1392	623	48
B6	2654	2651	2553	2732	2531	2543	2651	1944	562	47
B5	3473	3349	3123	2866	3057	2720	3629	2572	481	49
B4	3863	3955	3831	3951	4091	3768	4178	2661	519	47
B3	4387	4626	4713	4504	4405	4092	3979	3063	562	47
B2	4081	4611	4537	4426	4477	4039	4568	2968	1117	34
B1	4532	4585	4829	4710	4765	4348	4486	2908	626	49
B0	5947	7059	6717	7505	7175	6517	6405	4699	1192	40

Table 30. m1371 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	194	234	219	184	226	177	105	55	10	10
B8	384	371	372	341	333	353	347	258	24	11
B7	441	465	346	431	417	421	429	312	28	16
B6	571	515	564	589	531	582	539	424	40	7
B5	778	928	772	745	810	725	820	637	71	9
B4	1139	1278	1054	979	1018	1109	1308	996	73	7
B3	1046	1071	1074	1160	1251	722	1184	1046	461	2
B2	990	1044	1094	1133	1361	1049	1501	1065	169	4
B1	1099	1093	121	1071	1189	1223	1438	897	113	3
B0	1382	1469	1056	1428	1411	1380	1399	1172	205	9

Table 31. m4138 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	176	272	176	228	224	212	187	63	76	5
B8	193	214	220	231	203	215	173	111	36	10
B7	266	251	275	236	219	219	247	105	71	5
B6	279	239	298	276	318	304	277	129	64	10
B5	605	683	625	596	699	673	501	135	74	7
B4	1497	1661	1509	1499	1533	750	1257	577	84	5
B3	1881	2305	2198	1181	2342	2103	1544	551	45	8
B2	2177	1676	1440	2557	2302	2100	756	601	66	17
B1	2188	2236	2127	1913	2477	1903	1610	542	68	7
B0	3174	3250	3053	3433	3000	3209	2230	1278	108	7

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Table 32. m3320 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
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B9	15	16	19	27	20	24	14	9	5	3
B8	24	26	17	33	23	33	20	8	1	0
B7	30	37	32	46	37	14	25	12	3	0
B6	63	52	48	81	78	54	40	13	9	1
B5	515	624	511	431	493	575	451	90	12	2
B4	3239	2531	2320	2283	2228	2322	1999	1351	73	1
B3	3272	2712	2588	2856	2542	2648	3112	2249	90	0
B2	3622	2560	2755	2800	2646	2769	3104	2355	150	2
B1	3835	3190	2471	2690	2573	2501	3137	2542	175	0
B0	11445	8255	8872	9248	9768	9770	10719	4017	268	1

Table 33. XX-IV-113 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	17	18	10	14	9	19	12	21	14	12
B8	47	36	52	30	36	15	26	22	5	16
B7	151	105	101	91	109	87	57	29	29	0
B6	118	119	137	109	99	70	66	62	46	24
B5	289	113	207	146	117	177	142	121	34	62
B4	11422	1623	1975	12276	12872	4413	11328	1129	870	67
B3	12123	11710	8351	12522	12973	13308	12790	10315	1345	53
B2	12532	9461	12821	14151	13255	12266	12504	10300	1807	31
B1	13471	10837	12433	3253	11829	13564	13778	9590	1622	63
B0	11196	13403	13329	13281	12393	13143	13327	9922	2073	83

Table 34. m3794 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	212	216	162	131	132	149	51	37	93	28
B8	402	420	271	295	408	334	197	88	52	42
B7	364	341	334	401	319	291	185	64	49	47
B6	482	507	560	326	504	458	262	98	76	23
B5	923	1120	1013	1391	1093	982	670	175	52	50
B4	2005	2608	2312	2000	1860	2214	1942	977	84	16
B3	1841	2123	2555	2387	2294	2372	2374	1700	99	30
B2	2257	2301	2703	2356	2362	2093	2395	1637	165	20
B1	2665	2690	2752	2270	2289	2193	2524	1492	148	37
B0	2813	2473	2024	2610	2444	2859	2760	2195	254	28

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Table 35. m3147 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	34	53	21	30	55	18	18	8	3	10
B8	247	234	229	304	195	267	178	103	29	4

B7	294	456	209	406	408	303	274	161	32	0
B6	548	386	440	371	444	514	409	266	27	1
B5	836	803	784	730	975	829	748	456	74	3
B4	1534	1494	1492	1511	1072	1199	1283	1008	289	4
B3	2244	1912	1979	1913	2093	1971	2063	1290	647	3
B2	1812	1663	1983	1883	2046	1993	1785	1590	745	4
B1	1731	1808	1968	1737	2138	1467	2102	1984	741	2
B0	2298	2778	3241	2785	2761	2128	2825	2125	1167	4

Table 36. m1594 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	25	21	10	18	25	21	40	20	5	1
B8	338	54	25	31	28	24	24	19	49	4
B7	550	92	49	34	47	22	58	20	8	1
B6	742	192	128	77	69	74	38	33	10	4
B5	2020	588	454	321	277	209	241	119	25	29
B4	3788	1571	1660	1212	1270	1080	1147	420	45	33
B3	4466	2702	2772	2471	2244	2276	1863	1178	99	15
B2	4888	3427	3001	2499	2276	1849	2480	1431	168	46
B1	5789	2679	2665	2247	2219	2406	2568	1518	151	9
B0	8723	5777	4973	4136	3129	4102	3755	3900	725	20

Table 37. m3794 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	9	10	14	2	5	7	6	4	8	7
B8	36	37	41	37	64	37	12	8	5	8
B7	88	68	43	79	45	51	51	8	2	35
B6	126	143	138	137	124	119	62	15	9	17
B5	467	499	523	398	496	391	200	41	12	35
B4	1208	1507	1452	1448	1162	1352	1108	272	13	43
B3	1801	2029	1852	2211	2178	2226	1646	819	15	13
B2	2239	2274	2070	2253	1826	2104	2044	939	18	12
B1	1864	2471	2097	2481	2411	2192	1655	905	16	8
B0	4936	3956	4074	4998	3613	4348	3709	2630	271	26

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Table 38. m3153 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	46	21	10	17	10	28	38	15	13	13
B8	132	107	85	122	84	124	116	61	15	28
B7	178	212	202	241	237	270	171	133	15	28
B6	334	318	290	231	323	358	378	181	24	32

B5	550	464	450	296	499	507	632	336	27	18
B4	1178	1208	1194	1094	1311	1255	1257	976	73	19
B3	1296	1262	1292	1430	1444	1466	1488	1208	101	16
B2	1202	1170	1303	1440	1349	1395	1403	1176	183	17
B1	1122	1272	1413	1242	1393	1412	1483	1096	115	31
B0	2081	2288	2291	2505	2446	1542	2303	2087	545	73

Table 39. m1560 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	10	6	8	13	16	9	6	9	18	3
B8	128	135	137	113	126	64	45	16	32	2
B7	370	334	306	288	280	218	222	72	30	0
B6	342	669	479	664	657	628	447	171	42	29
B5	1595	1420	1542	1620	1729	1781	1241	435	101	7
B4	3358	3091	3094	3145	2911	3093	2637	1299	154	0
B3	3970	3673	3758	3461	4196	3484	3652	2574	276	2
B2	4127	3975	3786	3621	3415	3068	3797	2654	650	1
B1	3953	4213	3135	4059	4085	3554	3619	2956	755	4
B0	4122	5402	5149	5598	4723	4546	4296	3475	1078	1

Table 40. XX-IV-182 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	194	88	191	136	175	201	203	52	18	3
B8	716	655	709	543	534	447	316	192	10	2
B7	1068	983	962	844	938	745	605	220	14	3
B6	1741	1378	1362	1249	1284	972	977	379	25	8
B5	2109	1839	1815	1749	1644	1620	1326	806	24	3
B4	3097	2977	2947	3070	3010	2719	2267	1220	399	11
B3	2995	4635	4202	4028	3463	3656	2838	1757	482	5
B2	3863	3654	4246	4004	3429	3076	3078	1973	759	14
B1	3806	4090	3935	3993	3759	3804	3047	1835	798	3
B0	2952	4034	4692	4103	4991	4074	2926	2085	1052	7

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Table 41. m1600 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	143	112	120	73	89	76	76	119	43	12
B8	693	457	398	370	394	307	328	234	226	12
B7	812	643	649	564	614	580	374	255	342	14
B6	1500	967	990	736	928	667	589	433	315	16
B5	2919	2113	2163	2060	1571	1477	968	550	98	13
B4	5606	4696	3726	3800	2287	2862	1955	1141	200	12

B3	2457	5339	4467	4524	3406	2993	2907	1302	347	11
B2	4478	4668	4263	4253	3898	3420	2831	1591	314	11
B1	2362	4454	4457	3993	3889	3143	2348	1502	369	19
B0	471	1893	2130	3491	5488	5243	4346	2600	315	14

Table 42. XX-IV-153 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	265	234	255	252	301	249	162	34	20	12
B8	625	479	526	528	425	475	406	235	96	7
B7	821	828	716	711	771	817	614	355	147	10
B6	893	938	841	878	885	649	699	505	214	12
B5	1454	1325	1153	1317	1327	1471	1191	944	244	11
B4	1963	1900	1823	1695	1732	1847	1809	1544	332	3
B3	2073	1772	1998	1991	2153	1338	1849	1413	317	4
B2	1018	1866	1864	2081	1954	1729	1602	1226	389	15
B1	1442	702	1349	1429	1554	1628	1642	1201	302	4
B0	2449	2404	3545	3359	2839	3303	2330	2729	1186	18

Table 43. X-SK-8266 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	260	213	183	223	217	191	96	64	47	3
B8	322	283	217	218	241	231	230	107	34	11
B7	325	284	264	257	314	327	236	130	61	21
B6	432	416	287	402	436	435	358	217	104	22
B5	707	654	610	675	664	625	566	324	74	30
B4	1171	810	891	502	785	731	784	508	166	18
B3	1598	1072	827	741	1007	789	739	488	90	30
B2	1534	952	912	875	912	747	728	625	117	57
B1	1476	947	713	780	718	818	848	663	152	19
B0	1717	985	782	766	866	856	619	558	161	25

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Table 44. X-SK-8266 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	72	99	61	67	24	24	30	20	25	8
B8	110	117	90	114	61	95	68	35	35	16
B7	99	119	110	96	121	94	55	55	42	14
B6	224	115	185	167	176	113	58	105	8	34
B5	514	319	379	314	369	307	324	136	60	12
B4	2316	1026	952	621	617	846	944	320	20	13
B3	2548	1452	1133	1027	930	863	798	279	37	27

B2	3359	1655	1236	989	992	821	632	211	58	38
B1	2523	1255	1121	1083	852	815	604	396	118	16
B0	1679	1643	1100	1193	998	706	868	371	99	40

Table 45. m1730 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	5	5	5	8	8	6	5	22	27	16
B8	3	4	4	5	3	2	8	15	42	8
B7	5	4	2	0	2	8	8	13	36	17
B6	3	7	4	6	6	6	9	9	45	17
B5	52	82	63	43	41	24	14	15	44	10
B4	599	1720	1785	1388	1463	773	174	13	43	7
B3	2390	2886	3265	3212	2483	2057	894	37	28	9
B2	2463	3560	3295	2959	2768	2029	749	40	25	12
B1	3052	3399	3610	3583	3250	2337	1141	48	29	6
B0	3463	4260	5214	5497	5598	4127	2393	258	19	5

Table 46. X-R-IV-109 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	37	25	19	32	33	22	36	27	16	33
B8	188	175	175	178	209	233	300	392	118	28
B7	430	341	390	287	271	472	546	642	164	29
B6	483	287	439	502	399	766	727	1143	333	135
B5	949	774	801	652	258	1052	1288	1490	598	56
B4	3695	2466	2177	2065	932	2208	2880	2431	795	98
B3	2605	3405	3511	4065	3407	3186	4361	3562	1199	57
B2	3096	4304	3628	3196	3584	3770	4295	3860	1410	22
B1	3139	3299	2951	2903	4018	4071	4909	3925	1425	174
B0	5287	7093	3309	3369	8255	7783	7909	5856	2103	61

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Table 47. XX-IV-153 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	28	49	41	38	39	47	53	48	20	13
B8	28	48	48	45	50	53	59	50	40	4
B7	90	72	40	61	58	77	66	66	59	8
B6	114	61	104	92	95	148	86	80	51	13
B5	138	119	97	203	147	245	173	136	98	17
B4	8572	8348	3056	7879	7765	8096	8512	5342	529	11
B3	9108	8837	8710	9755	8690	2713	7598	5710	225	9
B2	7675	8636	8808	8558	7832	9902	8177	3042	847	11

B1	8286	8813	8992	9256	8050	8196	8044	5960	454	19
B0	7758	6964	8639	9007	8949	8707	6201	5199	1095	13

Table 48. X-SK-5486-R (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	53	61	48	47	39	49	42	22	11	10
B8	105	102	128	132	153	146	80	88	54	6
B7	115	176	188	191	202	187	93	90	34	22
B6	215	226	247	203	302	214	271	201	52	33
B5	450	469	537	592	663	518	398	245	60	40
B4	3914	4752	4394	4146	4090	3409	3295	1313	81	30
B3	9069	8915	9771	9515	8986	8612	7187	4863	152	37
B2	9257	10025	10404	10038	10548	8922	8595	4929	337	48
B1	9754	9906	10564	10158	9855	9274	8138	4818	324	36
B0	3970	9051	5484	10118	9117	9577	8739	3575	1008	23

Table 49. X-SK-6731 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1	3	1	1	4	6	4	0	2	1
B8	42	33	28	20	20	14	20	2	1	1
B7	31	40	27	38	24	34	23	9	3	0
B6	66	39	51	55	51	68	33	23	4	0
B5	604	354	377	240	230	217	153	50	11	0
B4	4670	2566	2609	1976	1659	1435	953	172	19	0
B3	8277	4954	3685	3355	2652	2600	1774	391	6	0
B2	7321	5165	4287	3751	2679	2525	1628	488	5	0
B1	7019	4807	4075	3273	3031	2598	1564	513	2	0
B0	9279	6018	4739	3509	3668	2826	1860	834	11	2

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Table 50. m1371 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	113	138	97	121	107	109	85	36	27	31
B8	273	308	242	319	285	237	244	252	35	0
B7	322	324	273	326	313	351	325	207	12	0
B6	346	449	427	373	411	422	438	203	39	2
B5	576	543	552	571	537	632	522	377	42	12
B4	623	762	656	784	642	710	956	705	54	4
B3	629	635	686	663	775	753	1047	728	55	23
B2	713	694	703	730	784	728	893	549	42	25
B1	512	754	583	808	880	718	783	517	54	13

B0	700	771	740	857	805	1006	836	688	61	4
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Table 51. m1730 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	11	10	4	2	8	5	1	31	14	0
B8	39	32	26	12	11	20	6	11	10	5
B7	51	44	26	42	30	15	14	14	22	9
B6	151	127	96	88	59	64	24	7	31	2
B5	669	750	840	689	760	525	179	28	23	14
B4	2329	2925	2886	2149	2409	2286	1429	149	9	12
B3	2830	3105	3486	3022	3175	2685	1918	310	38	8
B2	2821	3144	3435	3228	3224	2746	2111	415	43	15
B1	3513	3268	3661	3401	3242	2971	2505	322	22	6
B0	4928	4546	3818	4179	3944	4072	3653	1224	19	23

Table 52. m877 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	185	20	22	5	6	12	5	2	0	1
B8	814	223	236	105	83	69	27	19	9	1
B7	1388	462	383	290	199	139	63	24	19	1
B6	1908	961	680	583	381	281	137	19	24	0
B5	4383	1789	1371	1265	861	843	295	48	32	1
B4	5862	3183	2517	2529	1806	1327	541	39	13	1
B3	7279	3759	3270	3124	2663	2067	1468	332	9	1
B2	5532	4103	3815	2733	2960	2362	1860	556	31	0
B1	4746	3688	3290	2657	2573	2177	1639	781	20	2
B0	6011	3350	3024	2636	2367	2008	1592	917	32	0

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Table 53. m3320 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1	12	7	10	44	4	11	10	3	7
B8	29	30	12	17	20	37	22	18	9	1
B7	26	27	22	30	31	28	37	19	9	3
B6	38	40	26	44	52	42	33	21	10	7
B5	235	200	203	126	212	197	162	32	10	5
B4	4084	2863	2869	2824	2519	2939	2645	1498	17	0
B3	16626	10918	10124	8541	9882	8078	8799	4974	30	4
B2	16930	12456	12090	9527	10090	9997	11230	4831	49	4
B1	17469	11471	11449	9934	10910	11491	11146	6334	53	3
B0	22081	14399	13135	15293	8420	5988	940	450	1820	1

Table 54. X-R-IV-109 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1471	1466	1501	1602	1676	1794	1398	1515	68	16
B8	1659	1706	1839	1630	1825	1954	1968	1901	812	6
B7	1533	2270	2237	2166	2224	2087	2273	2284	714	7
B6	2758	2518	2926	2685	2544	2379	2678	2985	1126	6
B5	2626	3687	3533	3200	3527	3723	3654	2287	1870	1
B4	4139	4184	4454	4353	4755	5017	4927	4752	2255	6
B3	4480	4491	4588	4587	4602	5014	4953	4743	2461	3
B2	4024	4404	4609	4414	4709	4840	4834	4639	2587	3
B1	4195	4612	4542	4638	4777	5116	4860	4664	2590	6
B0	4941	5128	5836	5521	4885	6309	6138	5751	2981	7

Table 55. m1339 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	1223	0	1388	1583	1364	1363	788	0	3	1
B8	1626	1144	1102	947	1188	1036	882	136	3	1
B7	1335	1431	995	1154	1443	1523	877	285	8	1
B6	2353	2049	1565	1515	2108	1436	1066	387	8	0
B5	2809	2337	2513	3019	2221	2553	1983	731	17	0
B4	3264	2809	3051	3208	3243	3182	3254	1522	60	2
B3	3585	3150	3079	3499	3127	2871	3718	2054	110	1
B2	2857	2713	3164	2818	3359	2725	4098	2745	272	1
B1	3028	3038	3297	3158	3380	3308	3549	2517	189	0
B0	4770	3945	4050	3763	4321	4701	4063	3346	731	0

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Table 56. m3285 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	103	166	223	359	231	425	452	585	381	665
B8	149	142	203	228	349	638	873	643	646	952
B7	96	120	274	316	428	601	828	591	812	1173
B6	64	151	242	217	314	784	854	542	640	988
B5	81	126	196	236	410	801	849	685	650	651
B4	104	102	183	181	285	789	1003	773	942	1230
B3	58	64	90	94	227	577	617	593	829	1162
B2	63	103	57	70	133	190	329	455	265	996
B1	18	13	54	53	62	84	125	162	297	916
B0	15	5	4	12	10	10	24	15	14	11

Table 57. m1675 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	44	26	38	34	54	32	35	7	27	5
B8	94	102	101	81	152	136	144	72	11	3
B7	103	131	118	138	192	184	227	137	3	9
B6	93	105	109	101	154	165	199	216	3	4
B5	131	126	143	163	220	190	248	181	7	3
B4	149	155	214	145	246	274	314	204	7	7
B3	138	201	196	194	260	263	325	289	9	12
B2	181	173	211	317	266	267	310	254	23	5
B1	139	179	182	158	333	199	306	218	9	5
B0	504	612	661	766	762	420	817	846	73	9

Table 58. XX-IV-113 (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	600	637	800	832	660	346	300	105	16	7
B8	1637	1643	1541	1555	1680	1250	953	665	148	9
B7	1514	1903	1845	1896	1923	1650	1340	735	265	8
B6	2012	1955	1897	1729	1955	1529	1413	983	414	8
B5	1730	2067	2187	1973	2225	2040	1761	1273	447	4
B4	1920	1610	2582	1751	2412	2031	2749	1436	485	11
B3	1909	2167	2780	2668	2333	2723	2551	1777	553	13
B2	2026	2392	2425	2516	2652	2387	1611	1725	563	6
B1	2186	1655	1881	1876	2421	2759	2425	1699	605	2
B0	4890	4668	4570	3075	5106	5496	6086	5260	2907	9

5 Table 59. m1717 (7d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	53	60	48	49	32	28	7	3	5	1
B8	361	444	472	405	361	364	133	9	17	0
B7	538	585	476	723	628	397	291	43	25	4
B6	1033	995	942	930	919	679	428	107	9	1
B5	1810	1397	1864	1309	1554	1321	737	69	4	2
B4	2377	6494	5263	4346	4291	4008	2808	439	12	4
B3	2172	6475	6506	6141	5928	4514	2145	1367	13	1
B2	2149	6013	7131	6203	5290	5987	4742	2024	25	1
B1	2521	6432	6411	6085	6481	6272	4572	2069	26	1
B0	1166	3890	5665	5980	6794	5897	4734	2420	147	0

Table 60. X-SK-5486-R (3d)

	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9
B9	807	880	856	753	803	797	571	254	41	13
B8	1403	1402	1660	1523	1468	1518	1328	954	223	41
B7	1440	1413	1485	1543	1446	1414	1483	1099	299	74
B6	1590	1687	1788	1712	1649	1606	1611	1309	211	35
B5	2541	2551	2643	2940	2641	2574	2591	1987	378	89
B4	3959	3996	3884	3497	3438	3869	4035	2935	488	34
B3	4572	4362	4432	4451	4378	4489	4434	3429	939	57
B2	4412	4798	4155	5023	4747	4682	5086	3938	1365	86
B1	4810	4630	4520	4525	4447	3983	4676	3699	980	53
B0	5290	5690	5181	6450	5880	5687	5636	5695	2554	34

Conclusion: A strong synergy between ASNase and ibrutinib was found in 72% of these PDXs at concentrations between about 0.01 μ M and about 10 μ M ibrutinib.

Example 5 – Prednisone and ibrutinib effect on Nalm6, Sem and 697 cells

5 A series of experiments was conducted to test for the specificity of the synergistic interaction between BTK inhibitor ibrutinib and asparaginase. Synthetic glucocorticoids (GCs, e.g. prednisone, dexamethasone) are essential drugs for the treatment of ALL. To test whether BTK inhibition could modulate the response to GCs, the effect of combination treatment was tested. First, Nalm6 cells were exposed to 1 μ l of prednisone in the absence or presence of
10 Ibrutinib for 7 days. Next, cell viability was assayed by testing for membrane integrity using a amine-dye followed by flowcytometry. This showed that although combining prednisone treatment with the highest teste dose of 10 μ M Ibrutinib resulted in enhanced cell death, this is at best a modest effect when compared to the synergy between asparaginase and Ibrutinib. Similarly, in alternative assays, measuring cell death by testing for PARP cleavage using western
15 blot or DNA fragmentation using Hoechst staining followed by flow cytometry, only a small effect of combination treatment was observed. See FIGs. 6A to 6C.

Example 6 – ASNase and ibrutinib Combination Therapy to Treat Human Leukemia in an Immune Compromised Mouse Model

Objective. To study the safety and efficacy of the ASNase + ibrutinib combination
20 treatment. *Compounds:* ibrutinib (Sellekchem), 25mg/kg, dissolved in 5% DMSO + 30% PEG 300 + 5% Tween 80 + ddH₂O; PEG-Asparaginase (ONCASPAR®), 300 IU/kg in phosphate buffered

saline solution. *Power calculation*: Using a quite modest expected minimal effect size of 20% (a relevant effect in median survival time between the groups), a standard deviation <10%, an alpha-value of 0.05 and a power of 80%, this results in 7 mice per group.

Procedure. The experimental design is schematized in FIG. 7A. Briefly, thirty-two (32) NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ ("NSG") mice were injected intrafemorally with 500,000 leukemic blasts (2 injections / mouse) (Human Acute Lymphoblastic Leukemia). Two weeks after transplantation, mice received treatment for 9 consecutive days: 1) daily (9 days) oral administration of ibrutinib (groups 2 and 4) or vehicle (groups 1 and 3) via gavage; 2) IV administration of ONCASPAR® (groups 3 and 4) or vehicle (groups 1 and 2) on days 1, 4 and 7. During and after treatment, mice were weighed weekly, and their welfare was evaluated daily. Blood samples were taken weekly for assessment of the leukemia burden. Termination was predefined with a leukemia burden reaching 50% leukemic blasts in peripheral blood as measured by flow cytometry or euthanasia because of overt signs of discomfort due to disease.

The primary outcome of this experiment was event-free survival, where event was defined as early termination because of other causes will lead to censoring or exclusion from the experiment. Differences in event-free survival were analyzed using a Kaplan-Meier plot and significance was tested using the log-rank test.

Results. Thirty-one (31) of the thirty-two (32) mice were successfully engrafted with human leukemia. None of the mice showed treatment related signs of discomfort other than weight loss. In line with previous experiments, mice treated with ASNase experienced a strong but transient weight loss (FIG. 7B). ibrutinib treatment resulted in a mild and transient drop in bodyweight. Combination treated mice did not show any signs of additional discomfort in comparison to the PEG-Asparaginase treatment arm.

Weekly analysis of blood by flow cytometry showed that 5 weeks after transplantation, human leukemic blasts markers were detectable in control treated mice as identified by expression of human CD45 (FIG. 7C), CD10 (FIG. 7D) and CD19 (FIG. 7E). These numbers increased in time up to >50% which was defined as termination point. For 6 out of 8 control treated mice, this point was reached within 10 weeks after transplantation. Although ibrutinib

treatment initially gave a growth retardation, 4/7 mice reached the termination point after 10 weeks and the remaining 3 mice were terminated 1 week later. Asparaginase treatment resulted in a stronger delay in leukemia development, with mice reaching the termination point between 10 and 13 weeks. Combination treatments significantly decreased the % of hCD19+ cells (FIG. 7E), and significantly prolonged survival compared to the ASNase treatment, with mice reaching termination point between 10 and 15 weeks after transplantation (FIG. 7F).

Accordingly, combination treatment of ASNase and ibrutinib significantly delays leukemia development in mice transplanted with human acute lymphoblastic leukemia relative to control mice or mice treated with either compound as a single agent. No overt signs of toxicities as a result of combination treatment were observed, other than the transient weight loss that was also observed in mice treated with ASNase alone.

Overall Conclusions

The anti-tumor effects of ASNase impinge on changes in cell metabolism that occur as a result of amino acid regulation. Consistent with this notion, we identified genes either directly involved in the amino acid response route (TRIB3) or inhibition of protein translation in response to amino acid starvation (GCN2). Indeed, knockout of GCN2 sensitized cells to ASNase treatment whereas depletion of TRIB3 was sufficient to render these cells more resistant to the effects of ASNase on cell growth.

In addition, we found that Bruton Tyrosine Kinase (BTK), a hematopoietic-cell specific protein kinase acting downstream of the B-cell receptor, protects ALL cells from ASNase-induced apoptosis. Indeed, targeted knockout as well as inhibition by the FDA-approved BTK inhibitor ibrutinib, strongly enhanced ASNase-induced apoptosis in a variety of ALL cell lines (Figure 1). Moreover, we tested the effect of combination treatment in 35 different patient-derived xenografts samples, mostly representing high risk leukemia cases and covering a wide variety of ALL subtypes. In more than 75 % of the cases we observed synergy ranging from moderate to strong with a combination index (CI) <0.8.

In summary, the nutrient stress response pathway is a key modulator of ASNase-induced cell death; BTK provides a strong survival signal under (ASNase-induced) nutrient stress; and the BTK inhibitor ibrutinib potentiated ASNase therapy response in vivo.

Example 7 – AADAS and AADA combination therapy to kill solid tumor cells in an in vitro model

Although AADA asparaginase is primarily used in the treatment of leukemia, the responses in various solid tumor types suggest therapeutic efficacy beyond leukemia. Cell line and organoid models of different tumor origin (including, but not limited to pancreatic cancer, colon cancer, ovary cancer, brain tumors, breast cancer and lymphoma) are tested for sensitivity to AADA therapy (selected from an asparaginase (ASNase), an arginase (ARGase), an arginine deiminase (ADI), a methioninase (METase), an adenosine deaminase (ADA), an IDO, a TDO, fumagillin) in combination with an AADAS (ibrutinib and other AADAS) using a matrix of concentrations. Cells are seeded in 96 well or 384 well and incubated with the combination of AADA and AADAS at concentrations ranging from 0 to a 10-fold excess of the maximum tolerated dose in vivo. For asparaginase as AADA, these concentrations range from 0 to 100 IU/ml. For ibrutinib as AADAS, concentrations range from 0 to 100µM. After incubation for 3-7 days, cell viability is determined using automated microscopy using live cells staining or flowcytometry using 7AAD or amine staining to discriminate between live or dead cells. Quantification of the microscopy images was done using matlab. In a second step the data is analyzed using R-software to calculate a Combination Index (CI). These results demonstrate that AADA and AADAS synergize in killing solid tumor cells at clinically relevant concentrations. Again, for 7 day studies, cells are refed with [particular amino acid]-depleted media at the midway point.

Example 8 - AADAS and AADA combination therapy to kill solid tumor cells in an in vivo model

The efficacy of combination therapy of AADA and AADAS depends on the pharmacokinetics and dynamics of the drugs within a tumor type. *In vivo* models are used to demonstrate synergy at clinically relevant concentrations. Solid tumor models (cell lines and

primary patient cells) are either grown in spheroids, organoids or in a growth supporting matrix (matrigel, growth factor containing scaffolds). Next, tumor cells grown as described are transplanted into a host (in most cases an immunocompromised mouse, commonly known as NSG-mice) and allow to grow and to be innervated by the host's blood vessels. When a tumor is established, the host will be treated with control compounds (solvents), AADA, AADAS and a combination thereof in concentrations either used in clinical practice, commonly used in experimental *in vivo* models or at the maximal tolerable dose using the administration route that is common practice for the compounds. The duration and frequency of administration are adapted to the compound based on pharmacokinetics and dynamics.

In the case where the AADA is PEG-asparaginase, the dose may be about 300 IU/kg, administered IV once every 3 days. In case the AADAS is ibrutinib, the host may be treated with about 25mg/kg, administered orally once every day. The effect of tumor growth is monitored by *in vivo* imaging (in case the tumor cells are labelled with a fluorescent or bioluminescent marker), SPECT/CT and in case the tumor is transplanted subcutaneous, by directly measuring tumor size. The effect of control treatment, single treatment with either AADA or AADAS and the combination thereof on tumor development and/or the formation of metastasis is compared using predefined end-points such as tumor size, morbidity or mortality of the host. The results indicate that AADA and AADAS synergize in the treatment of solid tumor cells in *in vivo* models at clinically relevant concentrations.

Example 9 – Efficacy of AADAS + AADA against HB-ALL, LOUCY, SupT1 and JURKAT cells

T-ALL cell lines were tested as described in the above examples, and sensitivity to Asparaginase (SPECTRILA®, the recombinantly produced *E. coli* ASNase) and Ibrutinib (described above) were tested as detailed in Table 61. HBP-ALL (ThermoFisher); Loucy cells (ATCC® CRL-2629™); and SubT1 (ATCC® CRL-1942™); Jurkat (ATCC® Clone E61).

Table 61. Treatment details. Cell were incubated as indicated and tested at either day 3 or 7 (FIGs. 10 to 13 summarize the results)

ALL line	[ASNase] tested	[Ibrutinib] tested	Assay	Assay Day
HBP-ALL	Range across 6.5 logs	0, 1, 10 µM	MTT	
HBP-ALL	0.001 IU/mL	0, 1, 10 µM	Cell Death	3

LOUCY	Range across 6.5 logs	0, 1, 10 μ M	MTT	
LOUCY	0.0001 IU/mL	0, 1, 10 μ M	Cell Death	3
SubT1	Range across 8.5 logs	0, 0.1, 1, 10 μ M	MTT	
SubT1	1 IU/mL	0, 1, 10 μ M	Cell Death	3
SubT1	1 IU/mL	0, 1, 10 μ M	Cell Death	7
JURKAT	Range across 8.5 logs	0, 0.1, 1, 10 μ M	MTT	
JURKAT	1 IU/mL	0, 1, 10 μ M	Cell Death	7

Results. As indicated in FIGs. 10 to 13, cell death (after either 3 or 7 days) was observed as determined by amine exposure. The first two cell lines (HBP-ALL, Loucy) appeared to be extremely sensitive to ASNase as a single agent. Even so, the addition of ibrutinib appeared to produce some synergistic efficacy (FIG. 11A-B). Further, the SupT1 line appeared to be largely unresponsive to any of the treatments (FIGs. 12A-C), while the Jurkat cells exhibited the most prominent “synergistic sensitivity” to the combination (FIGs. 13A-B). And while these results highlight the unpredictable nature of the response/sensitivity of cancer cells to the combined efficacy of AADAS + AADA, the skilled artisan reading this application will now be able to conduct routine tests to evaluate the synergistic—or other *supra-additive*—efficacy of the combination against any other cell/cell type.

Furthermore, an ongoing study (performed substantially as described in Examples 4 & 6) indicates that the disclosed combinations may be synergistically effective against PDX cells taken from an *extremely ASNase-refractory* ALL patient. This particular patient’s leukemia cells exhibited both IKZF1 deletion and PAX5 translocation, and, as indicated in FIGs. 14A-B, were significantly sensitive to the combination of native *E. coli* ASNase + Ibrutinib. These cells are currently amplifying in a mouse for subsequent analysis as detailed in Example 6.

Taken together, these data suggested to Applicants that it would be therapeutically useful to begin the combination treatment *before* the emergence of ASNase resistance. As such, in some embodiments, the invention encompasses a method for preventing ASNase resistance comprising the following steps: 1) determining the emergence of a predetermined level of ASNase blocking and/or inhibitory antibodies in a patient who is receiving ASNase therapy; 2) administering to the patient an effective amount of a BTKi to reduce and/or prevent the resistance; 3) continuing the ASNase therapy in combination with the BTKi therapy; 4)

optionally discontinuing the BTKi therapy after the anti-ASNase antibodies reduced to some predetermined level; 5) optionally periodically monitoring the patient for the re-emergence of anti-ASNase antibodies; and 6) in the event of re-emergence of anti-ASNase antibodies at the predetermined level, repeat steps 2 to 5 as needed, for example, until the ALL has been eliminated.

Employing such a method would allow the clinician to selectively apply the disclosed combination for ALL patients that would most benefit from the combination. In some embodiments, the method may be useful in cases where ALL patients relapse and are also intolerant to ASNase therapy. In such embodiments, the addition of the BTKi may allow for the re-sensitization of the relapsed patient's ALL cells to ASNase. In still further embodiments, these same methods and experiments may be extended to cancers outside of ALL, including to solid tumors and lymphomas.

Example 10 – Efficacy of AADAS + AADA against Solid and Lymphoma cancer cells

To begin to determine the range of cancer types that are sensitive to the combination of AADAS + AADA, the components and their combinations were tested against 30 cell lines covering a variety of cancer cell types (Table 62). Two incubation times were tested. First, an overnight incubation detected early pro-apoptotic activity induced by the active ingredients (i.e. the AADAS and the AADA) via the measurement of caspase 3/7. Second, a longer incubation time (3-4 days) was performed to look simultaneously at viability, toxicity and apoptosis. For the experiments of this example, the AADA was the asparaginase known as SPECTRILA®, which is recombinantly produced *E. coli* ASNase having the amino acid sequence as set forth in NCBI Ref Seq WP_000394140.1, and having the ATC Code L01XX02. Ibrutinib was the AADAS as detailed above.

Table 62. Cancer cell lines

Cell line	Tissue / Type	Cell line	Tissue / Type	Cell line	Tissue / Type
Capan-1	Pancreas	SU-DHL-8	DLBCL	HepG2	Liver
AsPC-1	Pancreas	SU-DHL-10	DLBCL	MDA-MB-436	Breast
PANC-1	Pancreas	Toledo	DLBCL	HCT-116	Colon
Mia PaCa-2	Pancreas	NK-92	NK Lymphoma	HT29	Colon
BxPC-3	Pancreas	KHYG-1	NK Lymphoma	SW620	Colon

SW1990	Pancreas	LN-229	Glioblastoma	UM-UC-3	Bladder
AGS	Stomach	U87 MG	Glioblastoma	RT-4	Bladder
NCI-N87	Stomach	PC-3	Prostate	RS4;11	ALL
NIH: OVCAR-3	Ovary	SK-HEP-1	Liver	NALM-6	ALL
SK-OV-3	Ovary	Hep3B	Liver	SEM	ALL

Cell culture conditions. Adherent tumor cells were grown as monolayer at 37°C in a humidified atmosphere (5% CO₂, 95% air). For experimental use, tumor cells were rinsed twice with versene (ref.: 15040033, Thermofisher), detached from the culture flask by a 5-minute treatment with trypsin (ref.: 25300054, Thermofisher) and neutralized by addition of complete culture medium. The cells were counted and their viability assessed by 0.25% trypan blue exclusion assay.

Table 63. Culture conditions

Cell line	Culture Medium	Growth
Capan-1	IMDM + 20% FBS	Adherent
AsPC-1	RPMI + 10% FBS + 1% GlutaMax + 1% PS	Adherent
PANC-1	DMEM + 10% FBS + 1% PS + 1% Na Pyr	Adherent
Mia PaCa-2	DMEM + 2.5% Horse serum + 10% FBS + 1% PS + 1% Na Pyr	Adherent
BxPC-3	RPMI + 10% FBS + 1% GlutaMax + 1% PS	Adherent
SW1990	Leibovitz's + 10% FBS + 1% PS	Adherent
AGS	RPMI +10% FBS + 1% GlutaMax	Adherent
NCI-N87	RPMI +10% FBS + 1% GlutaMax	Adherent
NIH: OVCAR-3	RPMI + 10mM Hepes + 1mM Na Pyr + 10% FBS + 2.5 g/l glucose + 0.01 mg/ml bovine insulin	Adherent
SK-OV-3	RPMI +10% FBS + 1% GlutaMax	Adherent
SU-DHL-8	RPMI 1640 + 10% FBS + 1% GlutaMax + 1% PS	Suspension
SU-DHL-10	RPMI 1640 + 10% FBS + 1% GlutaMax + 1% PS	Suspension
Toledo	RPMI + 10mM Hepes + 1mM Na Pyr + 15% FBS + 2.5 g/l glucose	Suspension
NK-92	AlphaMEM + 10%FBS + 10% Horse serum + 1% PS + 100U/ml IL-2 +0.2mM Myoinositol +0.1mM mercaptoethanol + 0.02mM folic acid	Suspension
KHYG-1	RPMI 1640 + 10% FBS + 1% GlutaMax + 1% PS + 100 U/ml IL2	Suspension
LN-229	DMEM + 5% FBS+1% Na Pyr	Adherent
U87 MG	EMEM + 10% FBS	Adherent
PC-3	RPMI +10% FBS + 1% GlutaMax	Adherent
SK-HEP-1	RPMI 1640 + 10% FBS + 1% GlutaMax	Adherent
Hep3B2.1-7	RPMI 1640 + 10% FBS + 1% GlutaMax	Adherent
HepG2	EMEM + 10% FBS + 1mM NaPyr + 0.1mM NEAA	Adherent
MDA-MB-436	RPMI 1640 + 10% FBS + 1% GlutaMax	Adherent
HCT-116	McCoy's 5A + 10% FBS	Adherent

HT29	RPMI 1640 + 10% FBS + 1% GlutaMax	Adherent
SW620	RPMI 1640 + 10% FBS + 1% GlutaMax	Adherent
UM-UC-3	EMEM + 10% FBS + 1mM NaPyr + 0.1mM NEAA	Adherent
RT-4	McCoy's 5A + 10% FBS	Adherent
RS4;11	RPMI + 10% FBS + 1% PS	Suspension
NALM-6	RPMI + 10% FBS + 1% PS	Suspension
SEM	RPMI + 10% FBS + 1% PS	Suspension

Cell plating for 3 to 4 day incubation. The tumor cells were seeded at optimal density (Table 64) in 96-well flat-bottom microtitration plates. Cells were seeded in 90 μ L of drug free minimal essential medium and incubated at 37°C under 5% CO₂. Adherent cells were seeded overnight before treatment whereas cells in suspension were seeded on the same day as treatment.

Table 64. Seeding conditions

Cell line	Cells/Well	Cell line	Tissue / Type	Cell line	Tissue / Type
Capan-1	5000	SU-DHL-8	10000	HepG2	1000
AsPC-1	2500	SU-DHL-10	5000	MDA-MB-436	2500
PANC-1	5000	Toledo	25000	HCT-116	500
Mia PaCa-2	2000	NK-92	7500	HT29	500
BxPC-3	1500	KHYG-1	2500	SW620	750
SW1990	7500	LN-229	1000	UM-UC-3	1000
AGS	1000	U87 MG	1000	RT-4	2500
NCI-N87	2500	PC-3	1000	RS4;11	100000
NIH: OVCAR-3	2000	SK-HEP-1	2000	NALM-6	100000
SK-OV-3	2000	Hep3B2.1-7	2500	SEM	100000

Tumor cell plating for overnight incubation. The tumor cells were seeded at optimal density (Table 65) in 96-well flat-bottom microtitration plates. Cells were seeded in 90 μ L of drug free minimal essential medium and incubated at 37°C under 5% CO₂. Adherent cells were seeded overnight before treatment whereas cells in suspension were seeded on the day of treatment.

Table 65. Seeding conditions

Cell line	Cells/Well	Cell line	Tissue / Type	Cell line	Tissue / Type
Capan-1	15000	SU-DHL-8	30000	HepG2	3000
AsPC-1	7500	SU-DHL-10	15000	MDA-MB-436	7500

PANC-1	15000	Toledo	75000	HCT-116	1500
Mia PaCa-2	6000	NK-92	22500	HT29	1500
BxPC-3	4500	KHYG-1	7500	SW620	2250
SW1990	22500	LN-229	3000	UM-UC-3	3000
AGS	3000	U87 MG	3000	RT-4	7500
NCI-N87	7500	PC-3	3000	RS4;11	
NIH: OVCAR-3	6000	SK-HEP-1	6000	NALM-6	
SK-OV-3	6000	Hep3B2.1-7	7500	SEM	250000

Compounds were added to the plates containing cells the day following plating. Each compound was tested alone at 6 doses. For Ibrutinib, the doses were 0.0032, 0.016, 0.08, 0.4, 2, and 10 μ M. These doses were prepared from a stock solution of 10 mM in 100% DMSO, with successive 5-fold dilutions in 100% DMSO to provide the stock solutions for the lower concentrations. Ibrutinib was diluted 100-fold from stock solution in culture medium and 10 μ L were added to the cells. The final concentration of DMSO was 0.1%, and 10 μ L of DMSO 0.1% was used as the negative control. For Asparaginase, the doses tested were 0.0032, 0.016, 0.08, 0.4, 2, and 10 U/ml. These were prepared as stock solutions of 10,000 U/ml in DPBS. Successive 5-fold dilutions in DPBS provided the stock solutions for the lower concentrations. Asparaginase was diluted 100-fold from stock solution in culture medium and 10 μ L was added to the cells. The final concentration of DPBS was 0.1%, and 10 μ L of DPBS 0.1% was used as the negative control.

The two active ingredients were also tested in combinations at 6 x 6 doses, with the primary dilutions prepared as above. Ibrutinib was diluted 50-fold from stock solution in culture medium and 5 μ L were added to the cells. The final concentration of DMSO were 0.1%, and 5 μ L of DMSO 0.1% was used as negative control. Asparaginase was diluted 50-fold from stock solution in culture medium and 5 μ L were added to the cells. The final concentration of DPBS was 0.1%, and 5 μ L of DPBS 0.1% were used as negative control. Experiments were performed in duplicate.

Table 66. Plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	CM	CM	CM	CM	CM	CM	CM	CM	CM	CM	CM	CM
B	CM	A0-I0	A1-I0	A2-I0	A3-I0	A4-I0	A5-I0	A6-I0	B-A	B-I	B-1	CM

C	CM	A0-I1	A1-I1	A2-I1	A3-I1	A4-I1	A5-I1	A6-I1	A1	I1	B-A	CM
D	CM	A0-I2	A1-I2	A2-I2	A3-I2	A4-I2	A5-I2	A6-I2	A2	I2	A6I6	CM
E	CM	A0-I3	A1-I3	A2-I3	A3-I3	A4-I3	A5-I3	A6-I3	A3	I3	A6I6	CM
F	CM	A0-I4	A1-I4	A2-I4	A3-I4	A4-I4	A5-I4	A6-I4	A4	I4	B-AI	B-AI
G	CM	A0-I5	A1-I5	A2-I5	A3-I5	A4-I5	A5-I5	A6-I5	A5	I5	B-AI	B-AI
H	CM	A0-I6	A1-I6	A2-I6	A3-I6	A4-I6	A5-I6	A6-I6	A6	I6	w/o cell	w/o cell

CM=culture media

ASNase concentrations (U/ml) (diluted in DPBS)

A0	A1	A2	A3	A4	A5	A6
0	0.0032	0.016	0.08	0.4	2	10

5 Ibrutinib concentrations (μM) (diluted in DMSO)

I0	I1	I2	I3	I4	I5	I6
0	0.0032	0.016	0.08	0.4	2	10

B-A: DPBS only; negative control for ASNase (background)

A1-A6: ASNase only; positive control for ASNase (with DPBS diluent)

10 B-I: DMSO only; negative control for ibrutinib (background)

I1-I6: Ibrutinib only positive control for ibrutinib (with DMSO diluent)

B-AI: PBS + DMSO; negative control for ASNase + ibrutinib (background)

A6-I6 ASNase + Ibrutinib; + control for ASNase + ibrutinib (with DPBS+DMSO diluents)

15 Two protocols of incubation were performed: overnight and over 3 / 4 days as indicated.

Table 67. Seeding conditions

Cell line	Incubation (days)	Cell line	Incubation (days)	Cell line	Incubation (days)
Capan-1	4	SU-DHL-8	4	HepG2	3
AsPC-1	4	SU-DHL-10	4	MDA-MB-436	4
PANC-1	4	Toledo	4	HCT-116	4
Mia PaCa-2	3	NK-92	3	HT29	4
BxPC-3	4	KHYG-1	4	SW620	4
SW1990	4	LN-229	4	UM-UC-3	4
AGS	4	U87 MG	4	RT-4	4
NCI-N87	4	PC-3	4	RS4;11	3
NIH: OVCAR-3	4	SK-HEP-1	3	NALM-6	3
SK-OV-3	4	Hep3B2.1-7	4	SEM	3

Active ingredient efficacy was measured after an overnight incubation time using the Caspase-Glo 3/7™ Assay (Promega). The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The assay was performed as recommended by the manufacturer. Briefly, the plate was read using EnVision

plate reader (PerkinElmer). Compound efficacy after 3-4 days of incubation was monitored using the ApoTox-Glo™ Triplex Assay (Promega). This assay combines three assay chemistries to assess viability, cytotoxicity and apoptosis events in the same cell-based assay well. First, viability and cytotoxicity are determined by measuring two differential protease biomarkers simultaneously with the addition of a single non-lytic reagent containing two peptide substrates. A second reagent containing luminogenic DEVD-peptide substrate for caspase-3/7 and Ultra-Glo™ Recombinant Thermostable Luciferase is added. Caspase-3/7 cleavage of the substrate releases luciferin, which is a substrate for luciferase and generates light. The light output, measured with a luminometer, correlates with caspase-3/7 activation as a key indicator of apoptosis. The assay was performed as recommended by the manufacturer. Briefly, the plate was read using EnVision plate reader (PerkinElmer). Viability was measured in fluorescence units at wavelengths Ex 400 / Em 505. Cytotoxicity was monitored in fluorescence units at wavelengths Ex 485 / Em 520. Apoptosis was measured in luminescence.

RESULTS. The majority of the cell lines demonstrated greater sensitivity to the combination of ASNase and Ibrutinib than they did to either active ingredient alone (i.e. additivity and synergism). In some cases, there is strong evidence of synergistic / supra-additive efficacy. In other cases, the cells were not responsive to Ibrutinib, but were significantly more responsive to the combination than they were to ASNase alone (e.g. Bladder, Glioblastoma and Hepatic cancer cells). In still other cases, the cells were somewhat resistant to ASNase, but that resistance was at least partially overcome by the addition of Ibrutinib.

FIGs. 15 to 23 present % cell viability curves for SU-DHL-10 (DLBCL), NCI-N87 (gastric cancer), AsPC-1/CAPAN-1/BxPC-3 (pancreatic cancer), KHYG-1 (NK Lymphoma), HT-29 (Colorectal cancer), RT4 (bladder cancer) and Hep3B (hepatic cancer) cells. And as indicated in FIGs. 15B-C, the combination of ASNase and Ibrutinib were highly synergistically effective against DLBCL cells. And while NCI-N87 Gastric cancer cells were quite sensitive to each active ingredient alone, they were synergistically sensitive to the combination (FIG. 16).

Taken together, the foregoing results support the broad use of synergistic combinations of amino acid depletion agents (AADA) (e.g. ASNase) and amino acid depletion agent sensitizers

(AADAS) (e.g. BTKi) to kill cancer cells and to treat patients suffering from liquid cancers, solid cancers and lymphomas.

* * *

The invention will now be described in the following non-limiting, numbered
5 embodiments.

1. A pharmaceutical composition, kit or fixed-dose combination comprising:

(a) an effective amount of an amino acid depletion agent (AADA) selected from an asparaginase (ASNase), an arginase (ARGase), an arginine deiminase (ADI), a methioninase / methioninase (METase), an adenosine deaminase (ADA), an IDO, a TDO, fumagillin, a diet low in
10 a selected amino acid, and a glutaminase (GLNase); and

(b) an effective amount of an amino acid depletion agent sensitizer (AADAS), wherein the AADAS is a Bruton's Tyrosine Kinase inhibitor (BTKi);

for use in the treatment of a of disease or condition in a subject or patient in need of treatment thereof, wherein the disease or condition is not effectively treated by either the ADAA or the
15 AADAS alone, or wherein the amounts of the AADA and the AADAS are synergistically effective in treating the disease or condition, or wherein the amount of the AADAS is sufficient to sensitize AADA-resistant cells to AADA, or wherein the amount of the AADAS is sufficient to enable the use of a smaller amount of AADA to treat a disease or condition wherein an effective amount of the AADA would produce unacceptable toxicity in the subject or patient.

20 2. The pharmaceutical combination of embodiment 1, wherein the AADA is ASNase and the AADAS is a small molecule BTKi, and wherein the AADA and AADAS are separate entities, delivered sequentially or simultaneously, and are present in synergistically therapeutically effective amounts.

25 3. The pharmaceutical combination of embodiment 1 or 2, wherein the BTKi is selected from ibrutinib, acalabrutinib, zanabrutinib, tirabrutinib, M7583, vecabrutinib, CT-1530, ARQ 531, DTRMWXHS-12, TG-1701, spebrutinib, CC-292, CG'806, evorbrutinib, RG7845, GDC-0853, poseltinib, LY3337641, HM71224, PRN1008, BMS-986142, PRN2246, TAK-020, AC0058, BIIB-

068, a BTKi having substantially the same *in vivo* PK/PD profile and mechanism of action as any of the foregoing and combinations thereof.

4. The pharmaceutical combination of any one of embodiments 1 to 3, wherein the ASNase is selected from a native *E. coli* asparaginase (e.g. ELSPAR, Lundbeck Inc.), an *E. coli*-
5 derived peg-conjugated ASNase (e.g. ONCASPAR®, Servier), an *E. chrysanthemi* ASNase (e.g. ERWINAZE®, EUSA Pharma), a human-derived ASNase, an ASNase having substantially the same *in vivo* PK/PD profile as any of the foregoing and combinations thereof.

5. A method of treating cancer, comprising administering to a subject in need thereof synergistically effective amounts of an ASNase and a BTKi.

10 6. The method of embodiment 5, wherein the amount of the ASNase would be subtherapeutic for the subject if it were not administered sequentially or simultaneously as a combination therapy with the BTKi.

7. The method of embodiment 5 or 6, wherein the amount of the BTKi would be subtherapeutic for the subject's cancer were it not administered sequentially or simultaneously
15 as a combination therapy with the ASNase.

8. The method of any one of claims 5 to 7, wherein the cancer is acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), DLBCL, Gastric cancer, Pancreatic cancer, NK Lymphoma, Colorectal cancer, Bladder cancer or Hepatic cancer.

9. The method of any one of embodiments 5 to 8, wherein the cancer is resistant to
20 ASNase treatment.

10. The method of any one of embodiments 5 to 9, wherein the ASNase and the BTKi are sequentially administered, preferably wherein the BTKi is administered before the ASNase.

11. The method of any one of embodiments 5 to 10, wherein the cancer comprises a cancer-initiating stem cell.

25 12. The method any one of embodiments 5 to 11, wherein the cancer comprises tumor cells that are resistant to ASNase-induced apoptosis or cell death.

13. The method of any one of embodiments 5 to 7, wherein the cancer comprises a blood-borne, brain, pancreatic, cervical, lung, head and neck, breast or gastro-intestinal cancer.

14. The method of embodiment 13, wherein the cancer comprises a DLBCL or other B cell lymphoma, a pancreatic cancer, a colorectal cancer, a gastric cancer or a triple negative
5 breast cancer.

15. The method of embodiment 14, wherein the cancer comprises a DLBCL or another lymphoma.

16. The method of any one of embodiments 5 to 15, wherein the ASNase and/or the BTKi are administered by injection or wherein the ASNase is administered by injection and the
10 BTKi is administered orally.

17. The method of any one of embodiments 5 to 16, wherein the ASNase is selected from a native *E. coli* asparaginase (e.g. ELSPAR, Lundbeck Inc.), an *E. coli*-derived peg-conjugated ASNase (e.g. ONCASPAR®, Servier), an *E. chrysanthemi* ASNase (e.g. ERWINAZE®, EUSA Pharma), and a human-derived ASNase.

18. The method of any one of embodiments 5 to 17, wherein the ASNase and the BTKi are separate entities.

19. The method of any one of embodiments 5 to 18, wherein the BTKi is a covalent, irreversible BTKi or is a safe and effective agent capable of knocking down or eliminating BTK activity in the cancer cells.

20. The method of any one of embodiments 5 to 19, wherein the ASNase is encapsulated in red blood cells (RBCs) and the BTKi is co-formulated with said encapsulated RBCs.

21. A pharmaceutical composition, kit or fixed dose combination for use in treatment of cancer in subject in need of treatment therefor, comprising a pharmaceutically acceptable
25 carrier and a combination of a BTKi and an ASNase, wherein the combination contains a subtherapeutic dose of the BTKi and a subtherapeutic dose of the ASNase, and neither the dose of the BTKi nor the dose of the ASNase are or would be sufficient alone to treat the cancer.

22. The composition for the use of embodiment 21, comprising at least one dose of the BTKi and at least one dose of the ASNase.

23. The composition for the use of embodiment 21 or 22, comprising from about 0.05 mg to about 1.0 mg of the BTKi and from about 50 to about 500 U of the ASNase.

5 24. The composition for the use of any one of embodiments 21 to 23, wherein the dose of the BTKi is from about 5 to about 50 mg/kg bodyweight of the subject and the dose of the ASNase is about 50 to about 500 IU/kg bodyweight of the subject.

10 25. The composition for the use of any one of embodiments 21 to 24, wherein the dose of the BTKi is from about 10 to about 40 mg/kg and the dose of the ASNase is about 100 to about 400 IU/kg.

26. The composition for the use of any one of embodiments 21 to 25, wherein the dose of the BTKi is from about 15 to about 35 mg/kg and the dose of the ASNase is about 200 to about 400 IU/kg.

15 27. The composition for the use of any one of embodiments 21 to 26, wherein the dose of the BTKi is from about 25 mg/kg and the dose of the ASNase is about 300 IU/kg.

28. The composition for the use of any one of embodiments 21 to 27, wherein the BTKi is ibrutinib and the ASNase is either a PEG-ASNase or an RBC-encapsulated ASNase.

20 29. The composition for the use of any one of embodiment 21 to 28, comprising from about 10 to about 35 mg/kg ibrutinib, preferably dissolved in 5% DMSO + 30% PEG 300 + 5% Tween 80 + ddH₂O; and about 200 to 500 IU/kg PEG-ASNase or RBC-encapsulated ASNase.

30. A pharmaceutical combination comprising (i) a BTKi and (ii) an AADA, or a pharmaceutically acceptable salt thereof, respectively, or a prodrug thereof, respectively, and at least one pharmaceutically acceptable carrier.

25 31. The pharmaceutical combination according to embodiment 30 for simultaneous, separate or sequential use of the components (i) and (ii).

32. The pharmaceutical combination according to embodiment 30 or 31 in the form of a fixed combination.

33. The pharmaceutical combination according to any one of embodiments 30 to 32 in the form or a kit of parts for the combined administration where the BTKi and the AADA may be administered independently at the same time or separately within time intervals, especially where these time intervals allow that the combination partners are jointly active.

34. The pharmaceutical combination according to any one of embodiments 30 to 33, wherein the BTKi is ibrutinib, acalabrutinib, zanabrutinib, tirabrutinib, M7583, vecabrutinib, CT-1530, ARQ 531, DTRMWXHS-12, TG-1701, spebrutinib, CC-292, CG'806, evorbrutinib, RG7845, GDC-0853, poseltinib, LY3337641, HM71224, PRN1008, BMS-986142, PRN2246, TAK-020, AC0058, BIIB-068, a BTKi having substantially the same *in vivo* PK/PD profile and mechanism of action as any of the foregoing, or combinations thereof; and wherein the amino acid depletion agent is ASNase; or a pharmaceutically acceptable salt or prodrug thereof, respectively.

35. The pharmaceutical combination according to any one of embodiments 30 to 34, wherein the ASNase is selected from a native *E. coli* asparaginase (e.g. ELSPAR, Lundbeck Inc.), an *E. coli*-derived peg-conjugated ASNase (e.g. ONCASPAR®, Servier), an *E. chrysanthemi* ASNase (e.g. ERWINAZE®, EUSA Pharma), and a human-derived ASNase.

35. The pharmaceutical combination according to any one of embodiments 30 to 35, further comprising a co-agent, or a pharmaceutically acceptable salt or a prodrug thereof.

36. The pharmaceutical combination according to any one of embodiments 30 to 35 in the form of a co-formulated combination product.

37. Use of the pharmaceutical combination or combination product according to any one of embodiments 30 to 36 for treating cancer that is or has become resistant to treatment with either the BTKi or the ASNase.

38. A combination of (i) a BTKi and (ii) an amino acid depletion agent, for the manufacture of a medicament or a pharmaceutical product, especially a combination or combination product according to embodiment 30, for treating cancer.

39. A pharmaceutical product or a commercial package comprising a combination or combination product according to embodiment 30, in particular together with instructions for

simultaneous, separate or sequential use thereof in the treatment of a BTKi and an AADA for the treatment of cancer.

40. A pharmaceutical combination according to embodiment 30, for use in the treatment of cancer or as a medicine.

5 41. A method of inducing apoptosis in a tumor cell *in vivo* in a mammalian subject, wherein the tumor cell is resistant to treatment with an AADA, or the tumor cell that has only been rendered quiescent by said AADA, comprising administering an effective amount of an AADAS, administering said AADA, and allowing sufficient time for the tumor cells to undergo apoptosis, thereby inducing the apoptosis in the tumor cell.

10 42. The method of embodiment 41, wherein the AADAS is administered before the AADA.

43. The method of embodiment 41 or 42, wherein the AADAS is administered 1, 2, 3, 4, 5 or more days prior to the administration of the AADA.

15 44. The method of any one of embodiments 41 to 43, wherein the AADAS is administered in an amount from about 5 to about 50 mg/kg bodyweight of the subject.

45. The method of any one of embodiments 41 to 44, wherein the AADAS is administered in an amount from about 10 to about 40 mg/kg.

46. The method of any one of embodiments 41 to 45, wherein the AADAS is administered in an amount from about 20 to about 30 mg/kg .

20 47. The method of any one of embodiments 41 to 46, wherein the AADAS is a BTKi and the AADA is an ASNase.

48. The method of any one of embodiments 40 to 47, wherein the BTKi is administered in an amount from about 15 to about 35 mg/kg and the ASNase is administered in an amount from about 200 to about 400 IU/kg.

25 49. The method of any one of embodiments 40 to 48, wherein the BTKi is administered in an amount from about 20 to about 30 mg/kg and the ASNase is administered in an amount from about 200 to about 400 IU/kg.

50. The method of any one of embodiments 40 to 49, wherein the BTKi is ibrutinib and the ASNase is encapsulated in enucleated RBCs.

51. A method of treating a subject or patient suffering from cancer and previously unsuccessfully treated with ASNase, wherein the subject or patient exhibited hypersensitivity to and/or silent inactivation of said ASNase, comprising administering a sensitizing-effective amount of an AADAS and an apoptosis-inducing effective amount of an AADA.

52. The method of embodiment 51, comprising the step of administering an effective amount of the composition of any one of embodiments 1-4 or any one of embodiments 21-40.

53. The method of embodiment 51 or 52, wherein the AADAS is administered in an amount from about 5 to about 50 mg/kg bodyweight of the subject.

54. The method of embodiment 53, wherein the AADAS is administered in an amount from about 10 to about 40 mg/kg.

55. The method of embodiment 54, wherein the AADAS is administered in an amount from about 20 to about 30 mg/kg .

56. The method of embodiment 55, wherein the AADAS is a BTKi and the AADA is an ASNase.

57. The method of embodiment 56, wherein the BTKi is administered in an amount from about 15 to about 35 mg/kg and the ASNase is administered in an amount from about 200 to about 400 IU/kg.

58. The method of embodiment 57, wherein the BTKi is administered in an amount from about 20 to about 30 mg/kg and the ASNase is administered in an amount from about 200 to about 400 IU/kg.

59. The method of any one of embodiments 51 to 58, wherein the BTKi is ibrutinib and the ASNase is encapsulated in enucleated RBCs.

60. The method of any one of embodiments 56 to 59, wherein the BTKi and the ASNase are administered to the subject or patient in amounts that, if give separately, would not induce apoptosis in a majority of the cancer cells.

61. The method of any one of the preceding embodiments, wherein the AADA is a diet low in methionine or asparagine.

62. The method of embodiment 61, wherein the AADA is a diet low in methionine and asparagine.

5 63. The method of embodiment 61, wherein the low methionine diet is begun about 7 days after the administration of the AADAS.

64. The method of embodiment 61, wherein the low asparagine diet is begun about 7 days after the administration of the AADAS.

10 65. The method of embodiment 62, wherein the low methionine and low asparagine diet is begun about 7 days after the administration of the AADAS.

66. The composition or method of any one of the preceding embodiments comprising or making use of no traditional chemotherapeutic drug.

67. A method for preventing ASNase resistance (silent inactivation) comprising the following steps:

15 1) determining the emergence of a predetermined level of ASNase blocking and/or inhibitory anti-ASNase antibodies in a patient who is receiving ASNase therapy, said level being indicative of an early phase in the development of resistance against ASNase;

2) administering to the patient an effective amount of a BTKi to reduce and/or prevent the levels of blocking and/or inhibitory anti-ASNase antibodies past a predetermined level; and

20 3) continuing to administer effective amounts of ASNase in combination with a resistance-blocking effective amount of BTKi.

68. The method of embodiment 67, comprising the steps of discontinuing the administration of the BTKi when the anti-ASNase blocking and/or inhibitory antibody levels have reduced to below the initial predetermined level of step 1; periodically monitoring the patient for the re-emergence of anti-ASNase antibody levels in excess of the predetermined level; and, in the event of re-emergence of anti-ASNase antibodies at or above the predetermined level, repeating steps 2 to 5 as needed, until the ALL is in complete remission.

69. A method of reversing ASNase resistance in a patient suffering from a cancer that has ceased to be sensitive to ASNase therapy as a result of antibody induced inactivation, wherein the cancer is optionally a leukemia, a lymphoma or a solid cancer, comprising administering to the patient an effective amount of a BTKi to re-sensitize the patient's cancer to ASNase therapy, thereby reversing the resistance.

70. The method of any one of embodiments 67 to 69, wherein the BTKi is administered in an amount from about 5 to about 50 mg/kg, about 10 to about 40 mg/kg, or about 20 to about 30 mg/kg bodyweight of the patient.

71. The method of any one of embodiments 67 to 70, wherein the amount of BTKi is sufficient to attenuate or completely inhibit ASNase-induced increases in ASNS expression.

72. The method of embodiment 71, wherein BTKi inhibits ASNase-induced ASNS expression by attenuating or inhibiting ASNase-induced increases in ATF4 expression.

73. A method of blocking an in culture or *in vivo* cell from mounting an effective amino acid deprivation stress response (AADSR) comprising treating the cell, or administering to a patient or subject comprising the cell, an effective amount of an AADA and an effective amount of an AADAS, thereby blocking the cell from mounting the effective AADSR; optionally wherein the AADAS is a BTKi and the AADA is an ASNase.

74. The method of embodiment 73, wherein the amount of ASNase would be sufficient to induce GCN2-mediated, ATF4-mediated increases in ASNS expression, in absence of the BTKi.

75. The method of any of the foregoing embodiments, wherein the amount of BTKi administered to the patient or subject is sufficient to reverse ASNase hypersensitivity, wherein the hypersensitivity is optionally selected from acute allergic reactions to ASNase ranging from mild symptoms to systemic anaphylaxis.

* * *

The invention will now be described in the following non-limited claims.

WHAT IS CLAIMED:

1. A pharmaceutical composition, kit or fixed-dose combination comprising:

(a) an effective amount of an amino acid depletion agent (AADA) selected from an asparaginase (ASNase), an arginase (ARGase), an arginine deiminase (ADI), a methionase (METase), an adenosine deaminase (ADA), an IDO, a TDO, fumagillin, a diet low in a selected amino acid, and a glutaminase (GLNase); and

(b) an effective amount of an amino acid depletion agent sensitizer (AADAS), wherein the AADAS is a Bruton's Tyrosine Kinase inhibitor (BTKi);

for use in the treatment of a disease or condition in a subject or patient in need of treatment thereof, wherein the disease or condition is not effectively treated by either the AADA or the AADAS alone, or wherein the amounts of the AADA and the AADAS are synergistically effective in treating the disease or condition, or wherein the amount of the AADAS is sufficient to sensitize AADA-resistant cells to AADA, or wherein the amount of the AADAS is sufficient to enable the use of a smaller amount of AADA to treat a disease or condition wherein an effective amount of the AADA would produce unacceptable toxicity in the subject or patient.

2. The pharmaceutical combination of claim 1, wherein the AADA is ASNase and the AADAS is a small molecule BTKi, and wherein the AADA and AADAS are separate entities, delivered sequentially or simultaneously, and are present in synergistically therapeutically effective amounts.

3. The pharmaceutical combination of claim 1 or 2, wherein the BTKi is selected from ibrutinib, acalabrutinib, zanabrutinib, tirabrutinib, M7583, vecabrutinib, CT-1530, ARQ 531, DTRMWXHS-12, TG-1701, spebrutinib, CC-292, CG'806, evorbrutinib, RG7845, GDC-0853, poseltinib, LY3337641, HM71224, PRN1008, BMS-986142, PRN2246, TAK-020, AC0058, BIIB-068, a BTKi having substantially the same *in vivo* PK/PD profile and mechanism of action as any of the foregoing and combinations thereof.

4. The pharmaceutical combination of any one of claims 1 to 3, wherein the ASNase is selected from a native *E. coli* asparaginase (e.g. ELSPAR, Lundbeck Inc.), an *E. coli*-derived peg-

conjugated ASNase (e.g. ONCASPAR®, Servier), an *E. chrysanthemi* ASNase (e.g. ERWINAZE®, EUSA Pharma), a human-derived ASNase, an ASNase having substantially the same in vivo PK/PD profile as any of the foregoing and combinations thereof.

5. A method of treating cancer, comprising administering to a subject in need thereof synergistically effective amounts of an ASNase and a BTKi.

6. The method of claim 5, wherein the amount of the ASNase would be subtherapeutic for the subject if it were not administered sequentially or simultaneously as a combination therapy with the BTKi.

7. The method of claim 5 or 6, wherein the amount of the BTKi would be subtherapeutic for the subject's cancer were it not administered sequentially or simultaneously as a combination therapy with the ASNase.

8. The method of any one of claims 5 to 7, wherein the cancer is acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), DLBCL, Gastric cancer, Pancreatic cancer, NK Lymphoma, Colorectal cancer, Bladder cancer, Hepatic cancer or a Glioma or Glioblastoma.

9. The method of any one of claims 5 to 8, wherein the cancer is resistant to ASNase treatment.

10. The method of any one of claims 5 to 9, wherein the ASNase and the BTKi are sequentially administered, preferably wherein the BTKi is administered before the ASNase.

11. The method of any one of claims 5 to 10, wherein the cancer comprises a cancer-initiating stem cell.

12. The method any one of claims 5 to 11, wherein the cancer comprises tumor cells that are resistant to ASNase-induced cell death optionally selected from apoptosis.

13. The method of any one of claims 5 to 7, wherein the cancer comprises a blood-borne, brain, pancreatic, cervical, lung, head and neck, breast or gastro-intestinal cancer.

14. The method of claim 13, wherein the cancer comprises a DLBCL or other B cell lymphoma, a pancreatic cancer, a colorectal cancer, a gastric cancer or a triple negative breast cancer.

15. The method of claim 14, wherein the cancer comprises a DLBCL or another lymphoma.

16. The method of any one of claims 5 to 15, wherein the ASNase and/or the BTKi are administered by injection or wherein the ASNase is administered by injection and the BTKi is administered orally.

17. The method of any one of claims 5 to 16, wherein the ASNase is selected from a native *E. coli* asparaginase (e.g. ELSPAR, Lundbeck Inc.), an *E. coli*-derived peg-conjugated ASNase (e.g. ONCASPAR®, Servier), an *E. chrysanthemi* ASNase (e.g. ERWINAZE®, EUSA Pharma), and a human-derived ASNase.

18. The method of any one of claims 5 to 17, wherein the ASNase and the BTKi are separate entities.

19. The method of any one of claims 5 to 18, wherein the BTKi is a covalent, irreversible BTKi or is a safe and effective agent capable of knocking down or eliminating BTK activity in the cancer cells.

20. The method of any one of claims 5 to 19, wherein the ASNase is encapsulated in red blood cells (RBCs) and the BTKi is co-formulated with said encapsulated RBCs.

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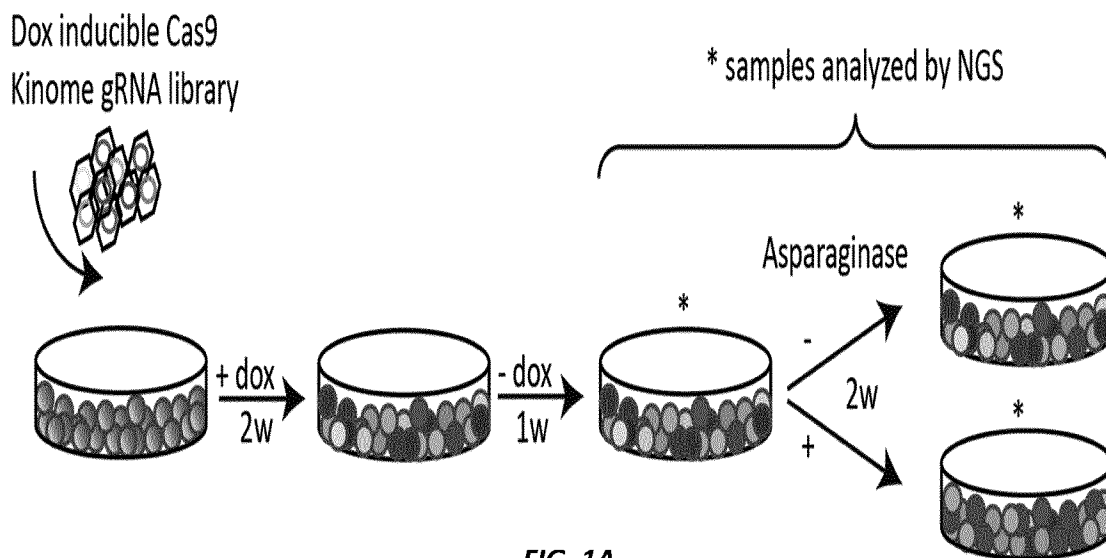


FIG. 1A

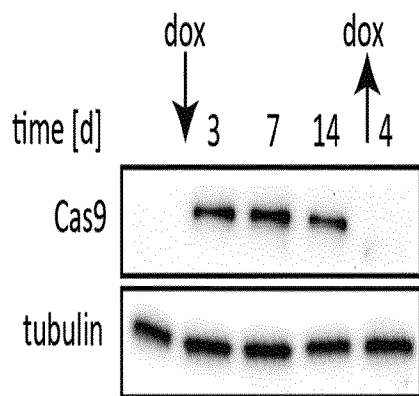


FIG. 1B

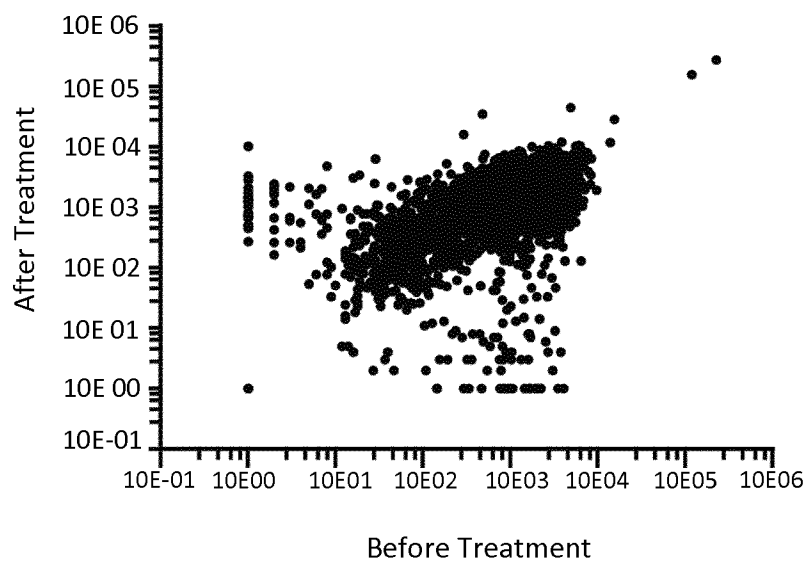


FIG. 1C

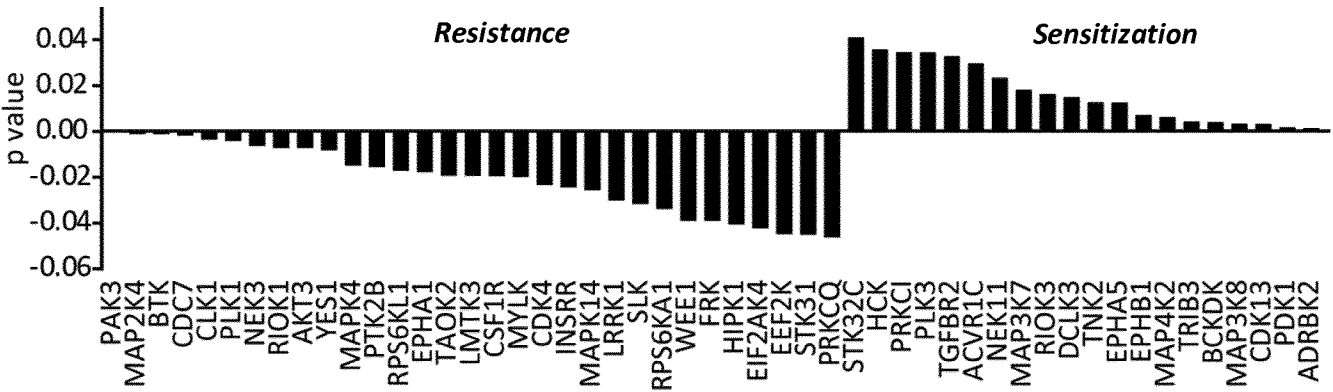


FIG. 1D

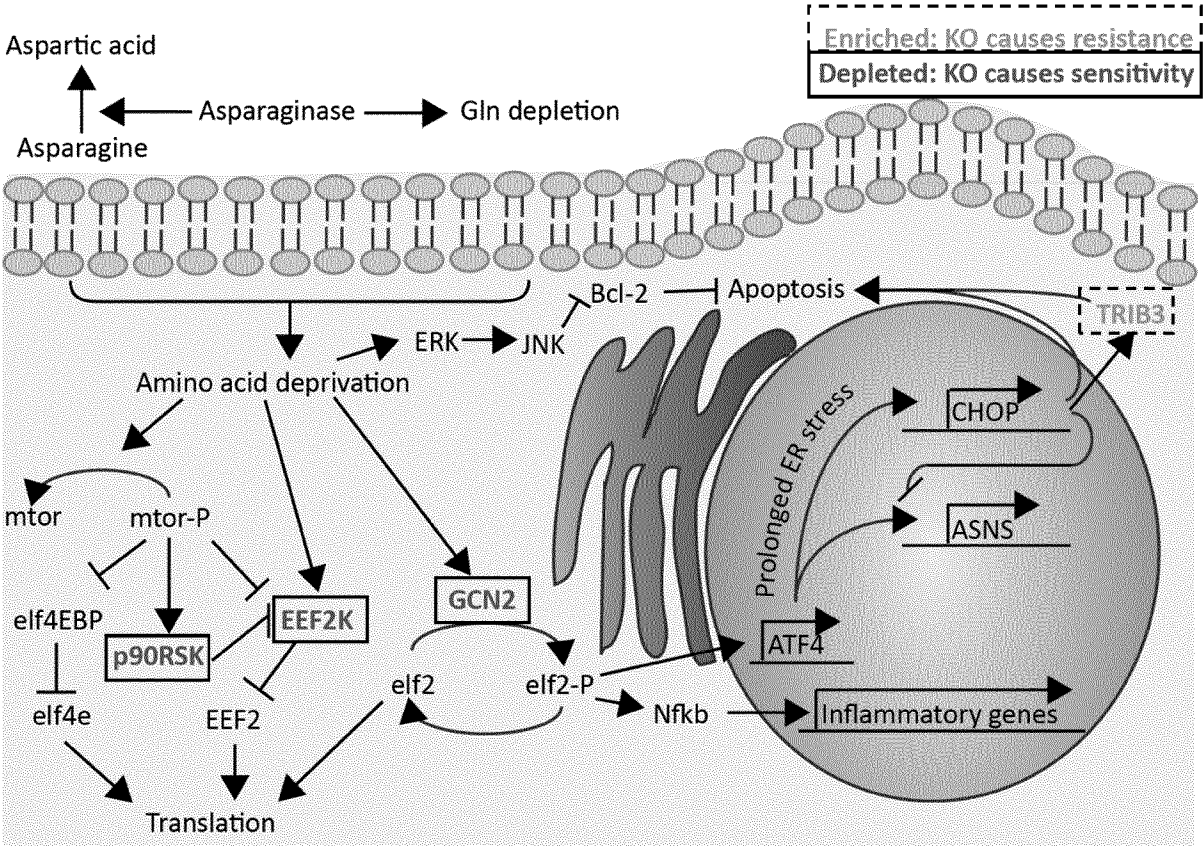


FIG. 2A

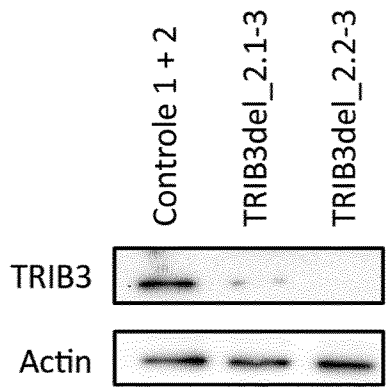


FIG. 2B

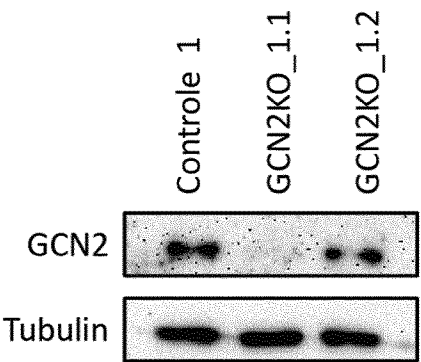


FIG. 2C

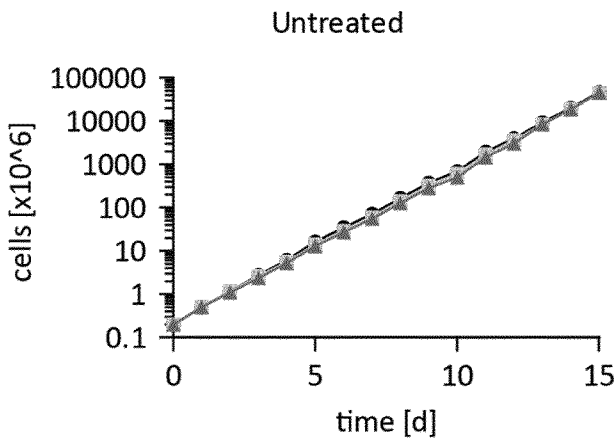


FIG. 2D

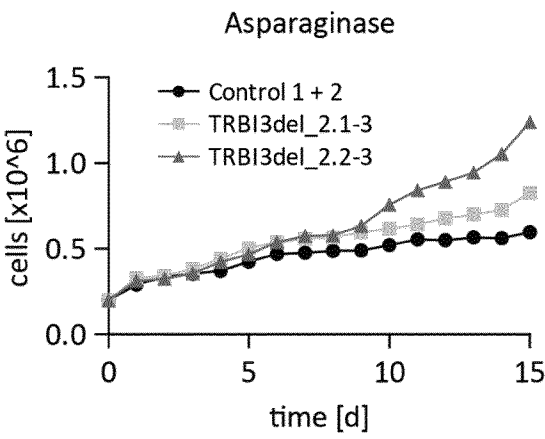


FIG. 2E

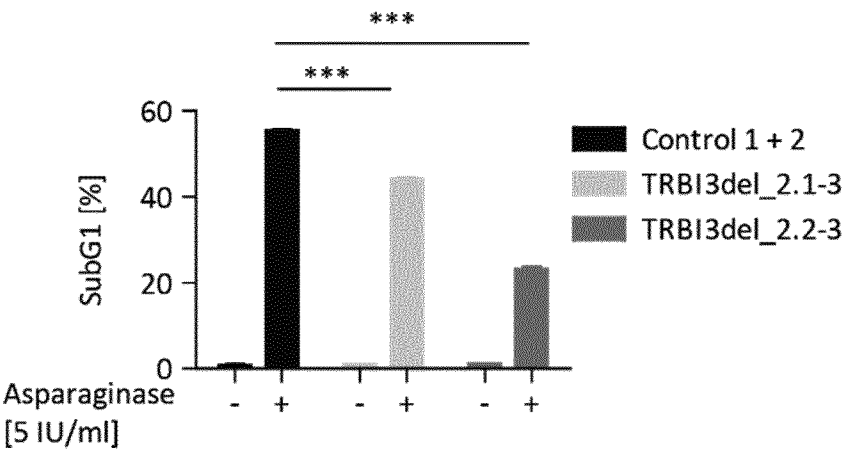


FIG. 2F

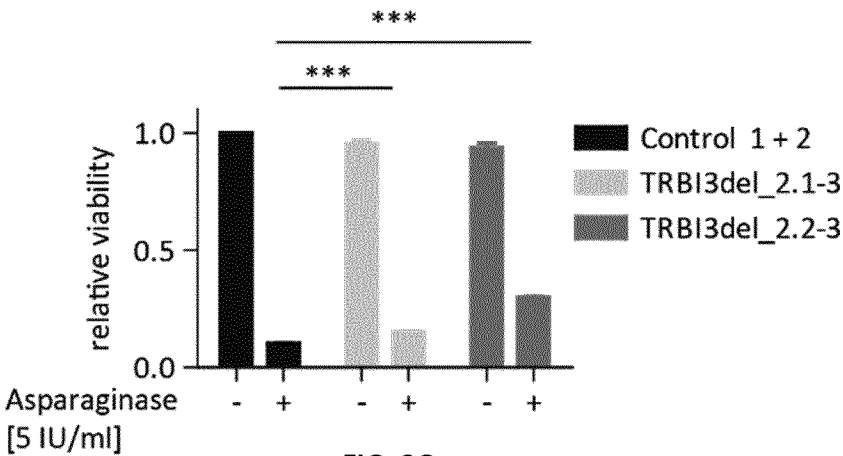
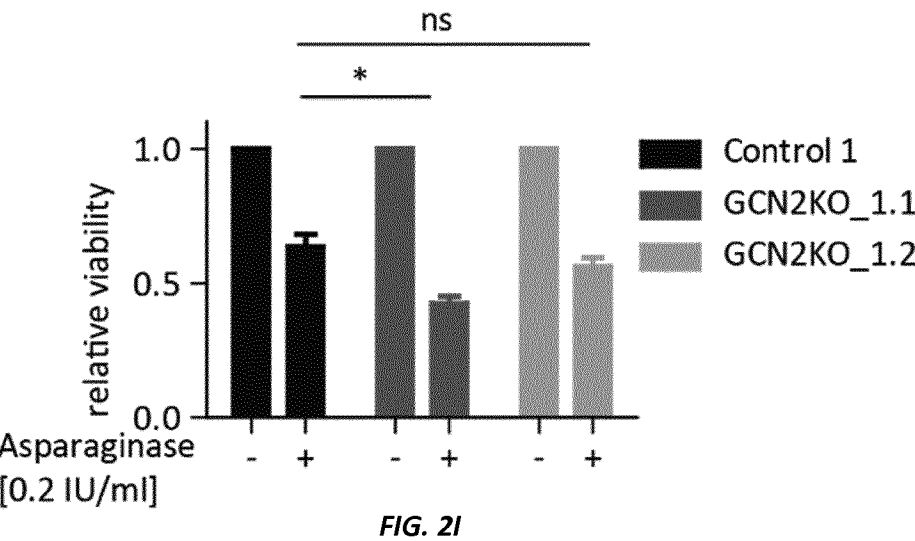
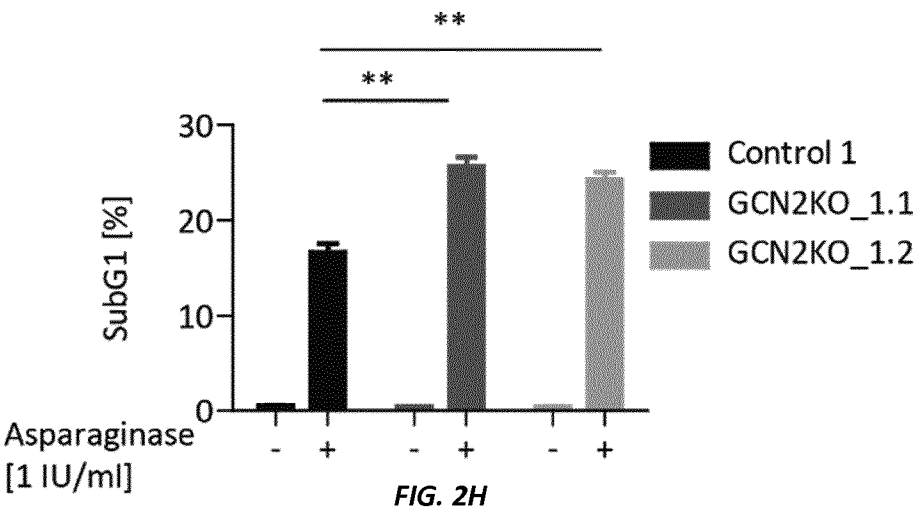


FIG. 2G

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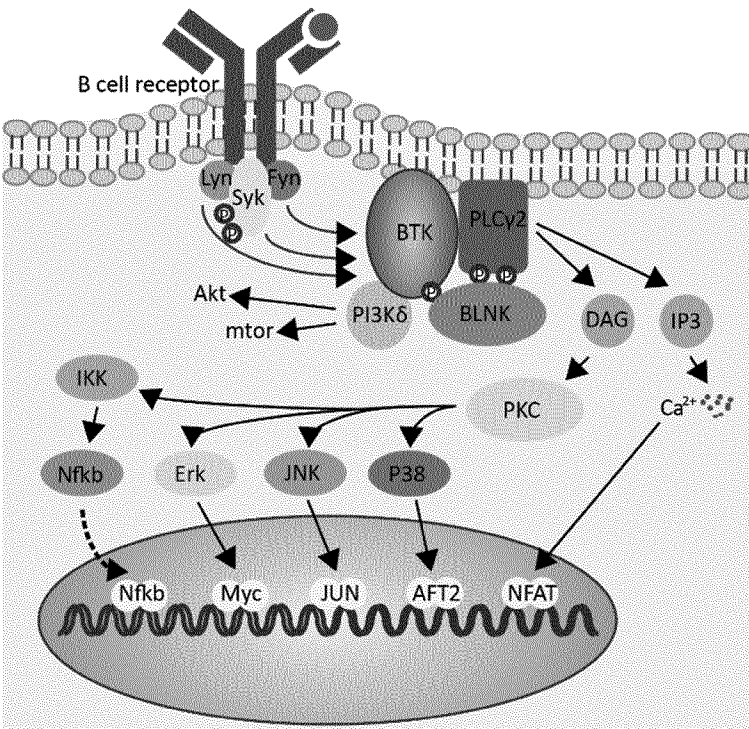


FIG. 3A

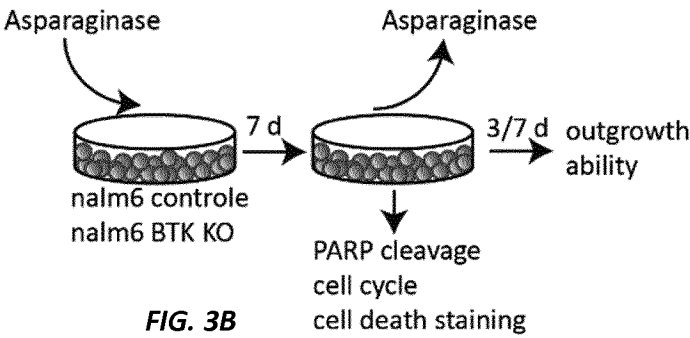


FIG. 3B

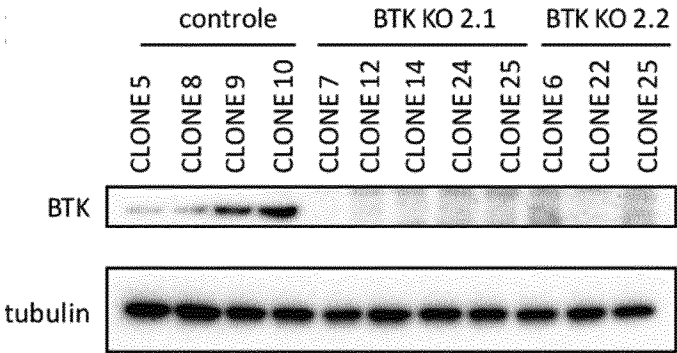


FIG. 3C

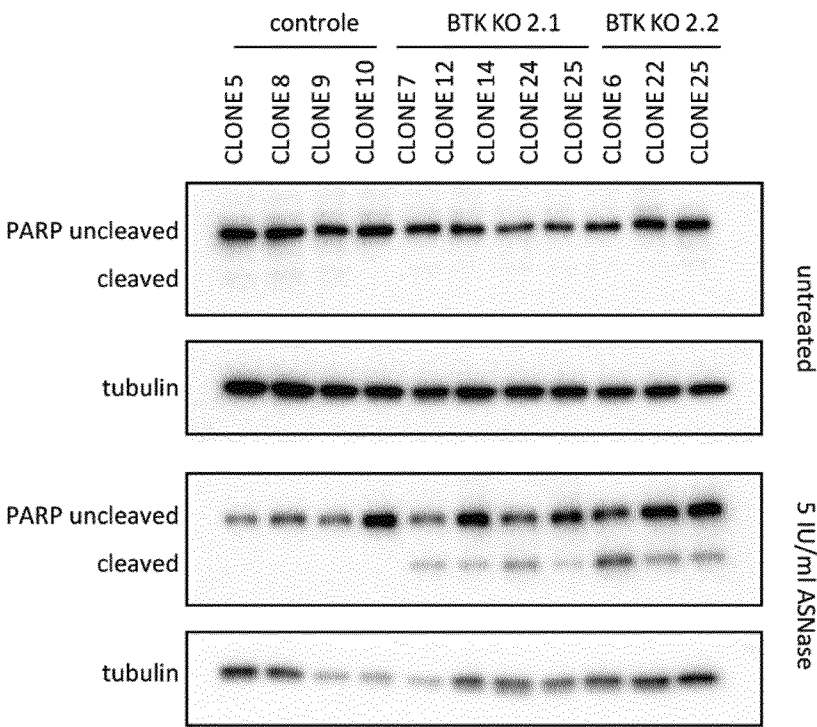


FIG. 3D

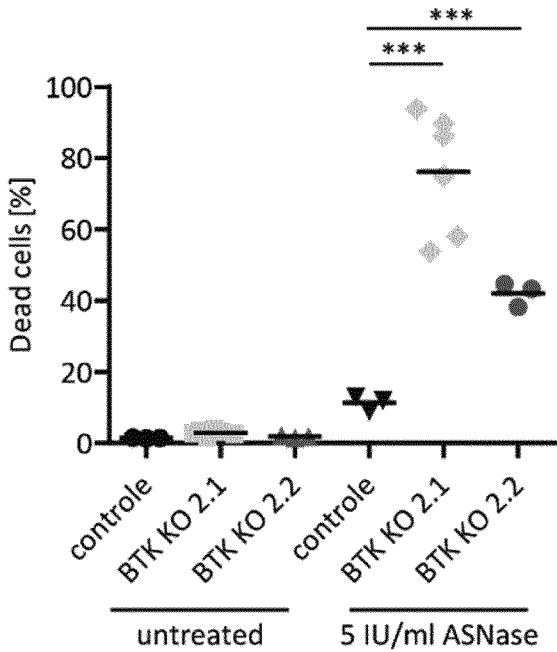


FIG. 3E

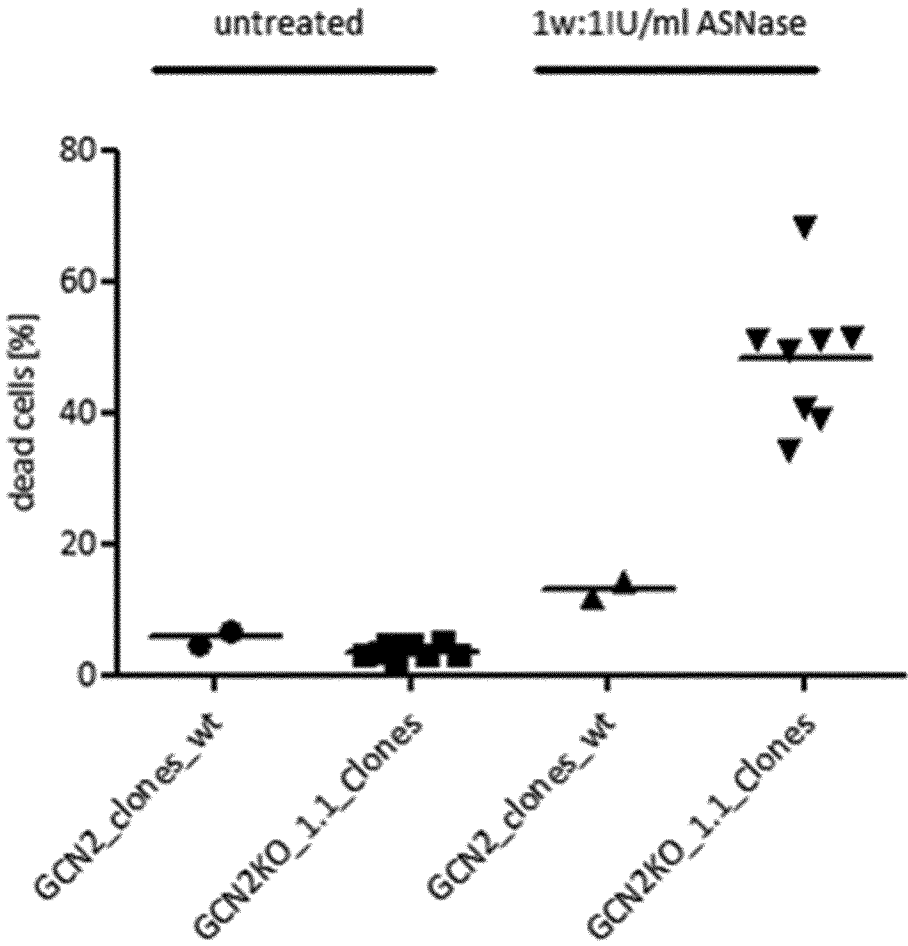


FIG. 3F

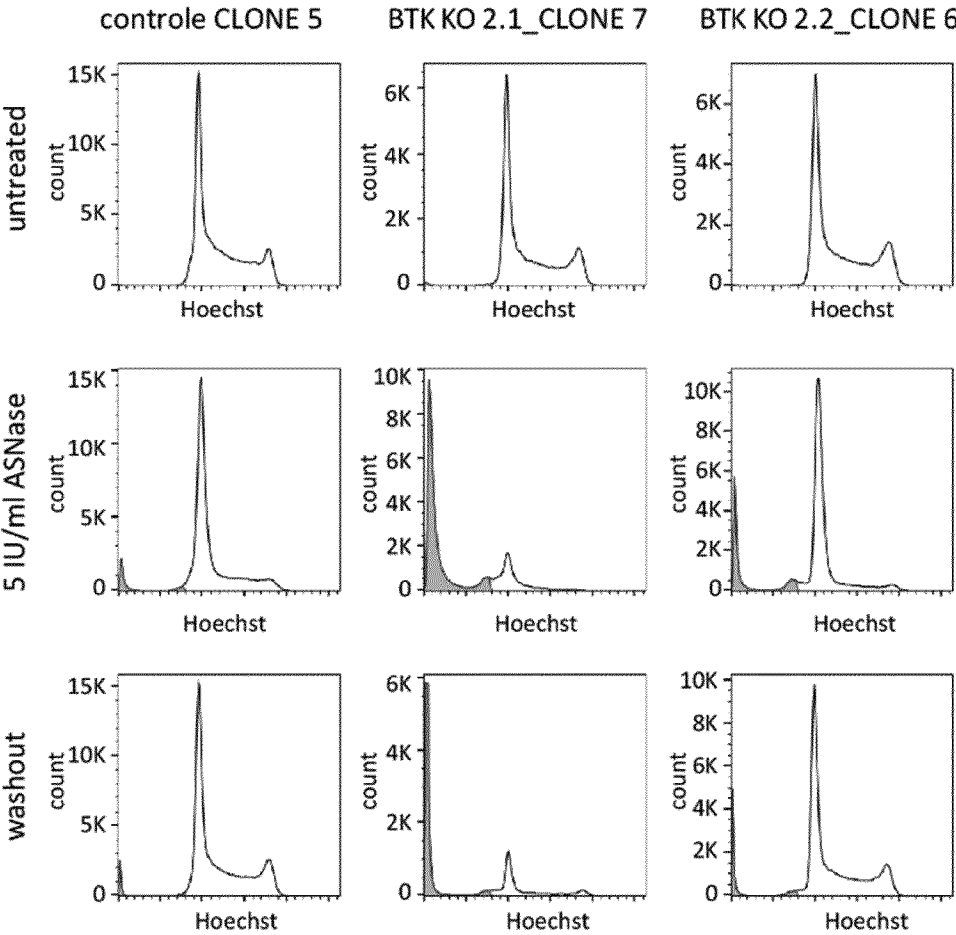


FIG. 3G

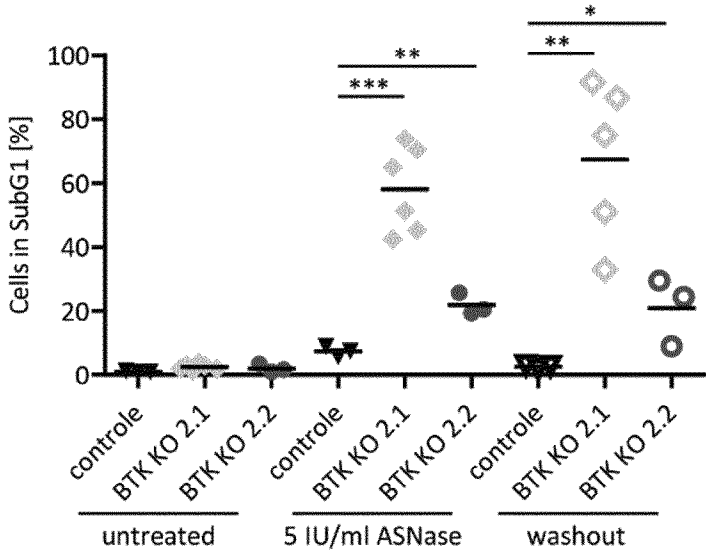


FIG. 3H

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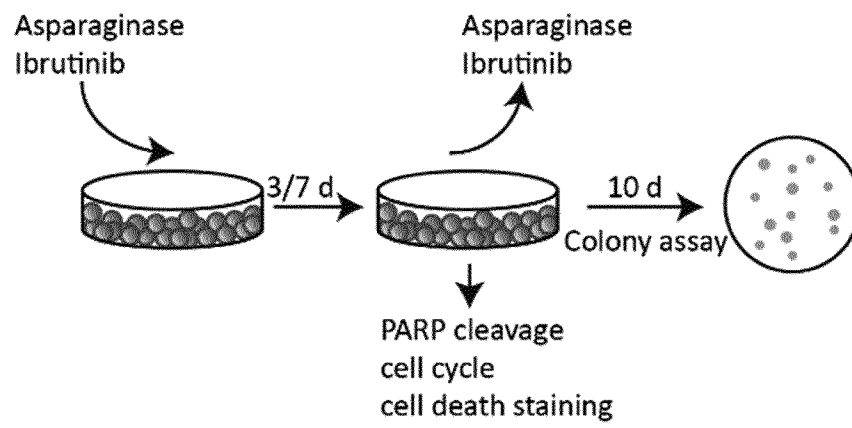


FIG. 4A

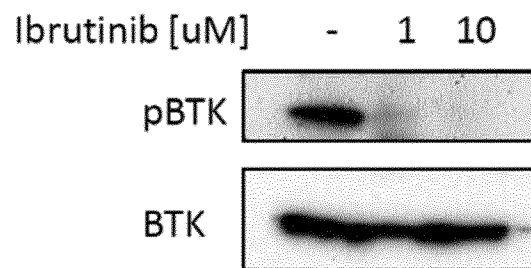


FIG. 4B

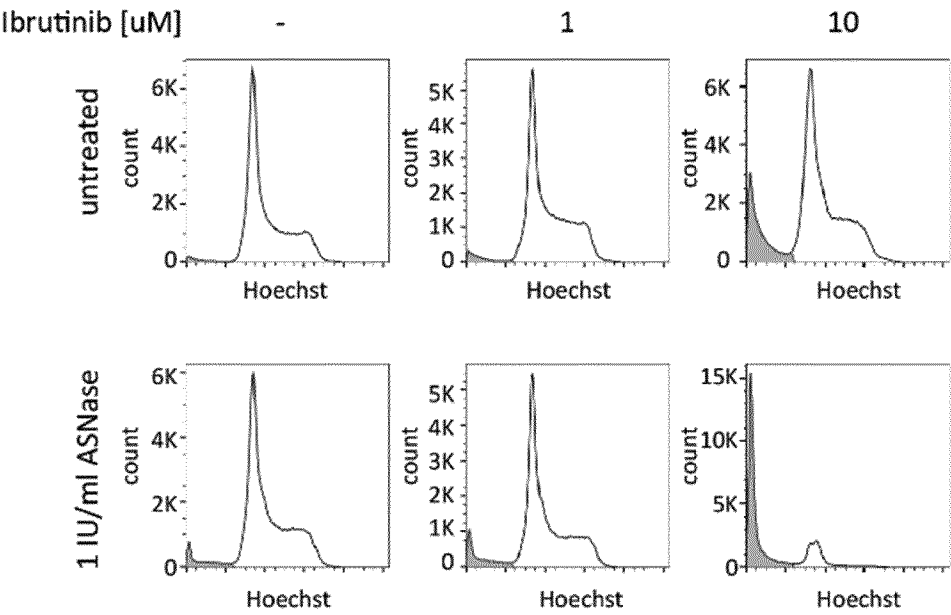


FIG. 4C

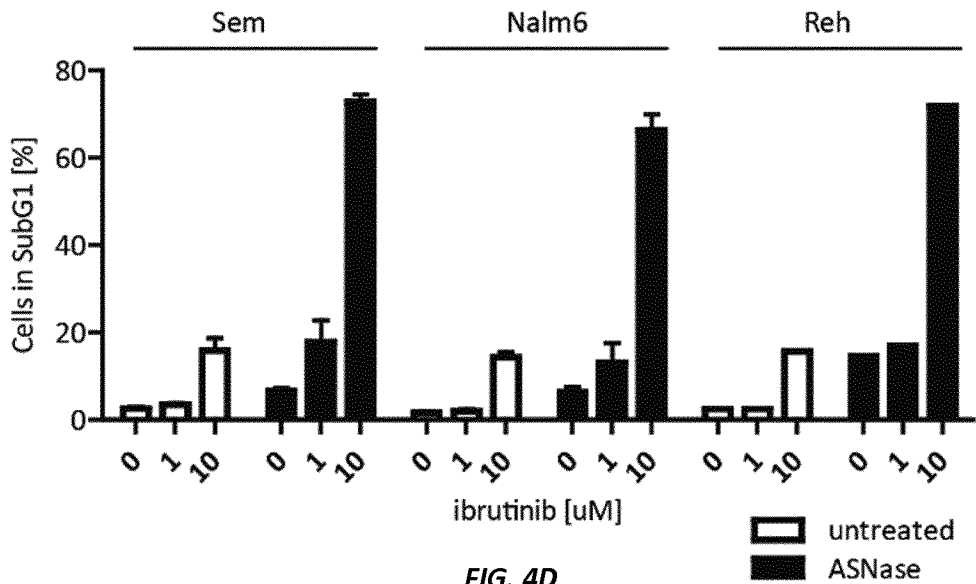


FIG. 4D

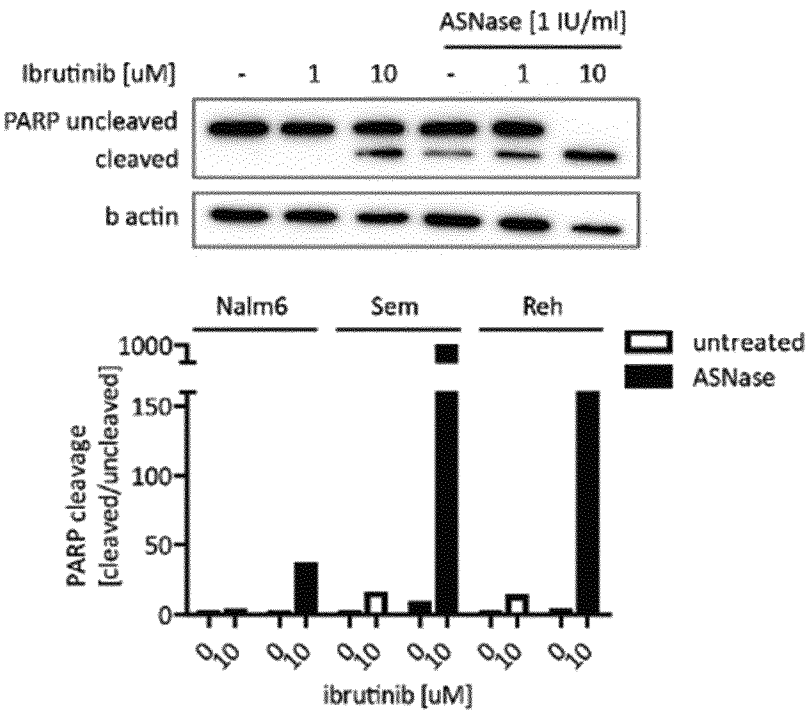


FIG. 4E

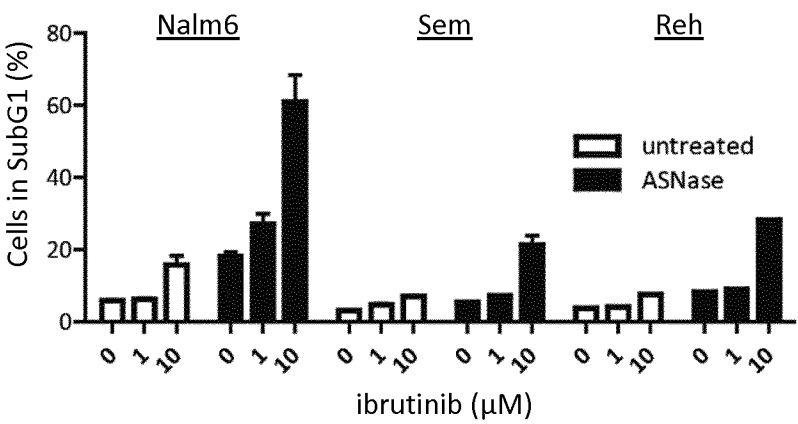


FIG. 4F

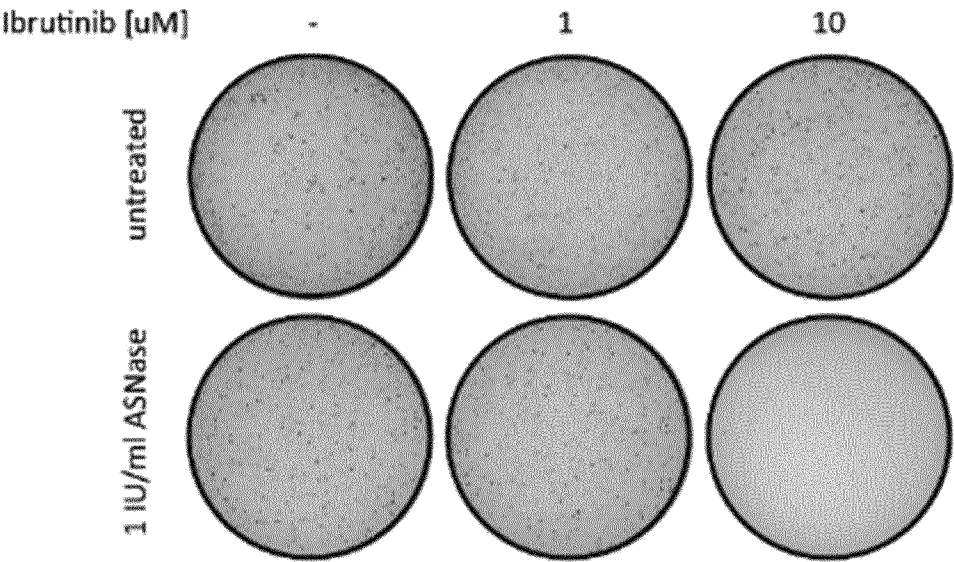


FIG. 4G

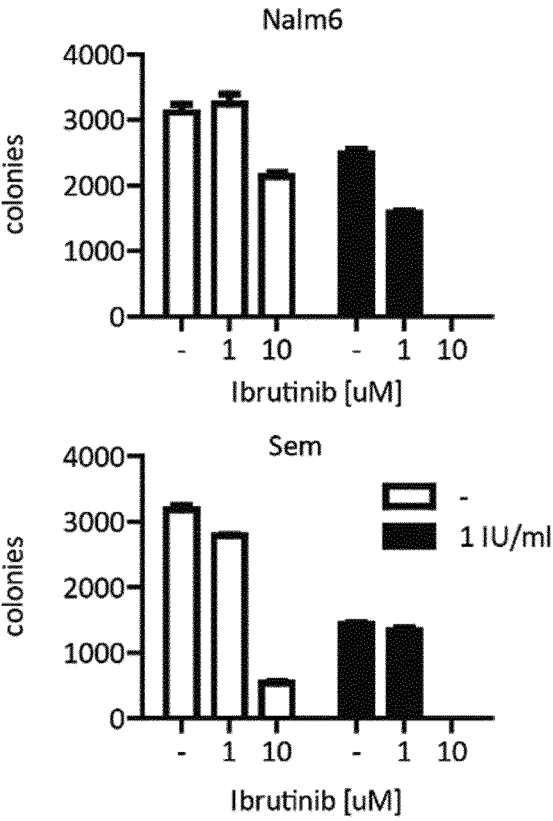


FIG. 4H

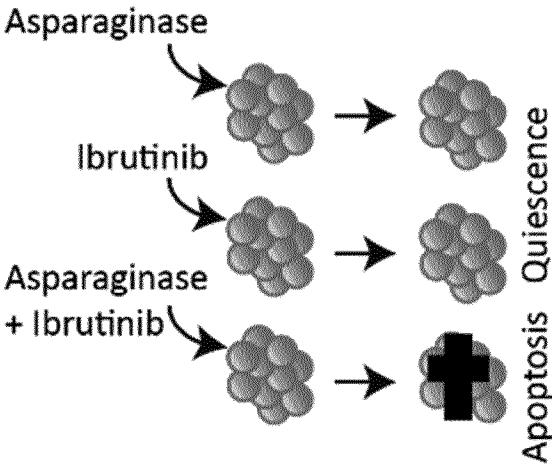


FIG. 4I

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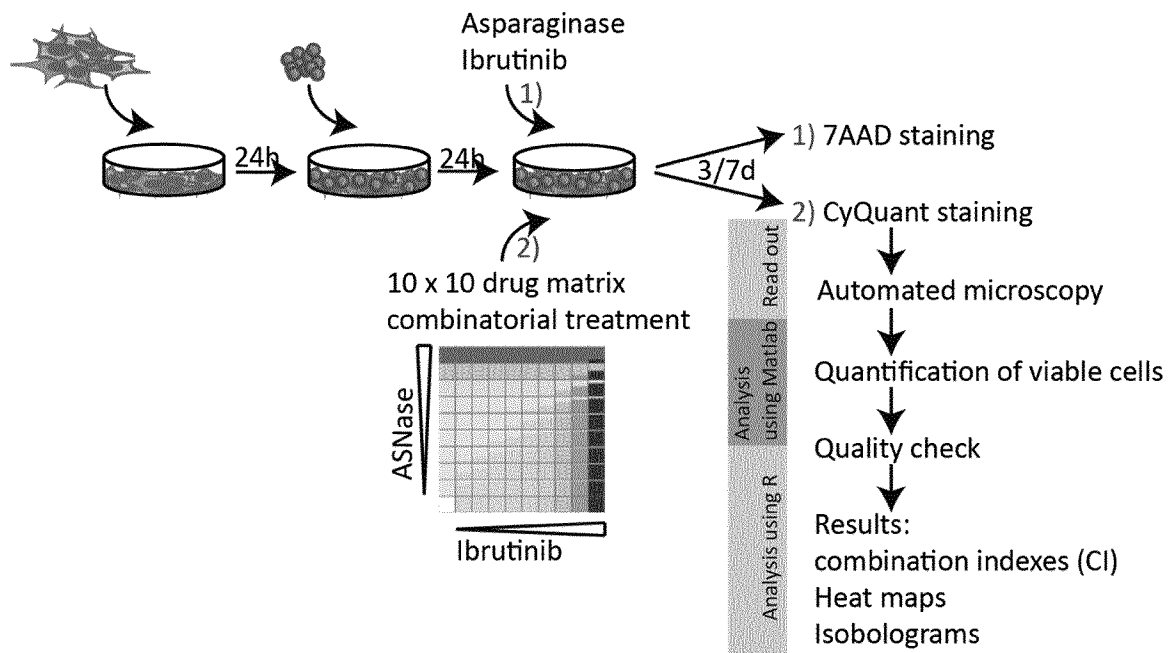


FIG. 5A

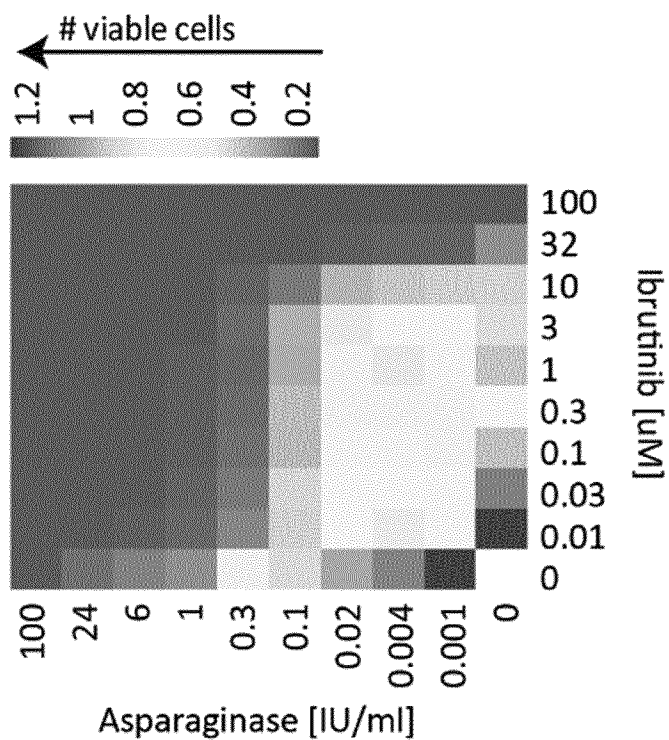


FIG. 5B

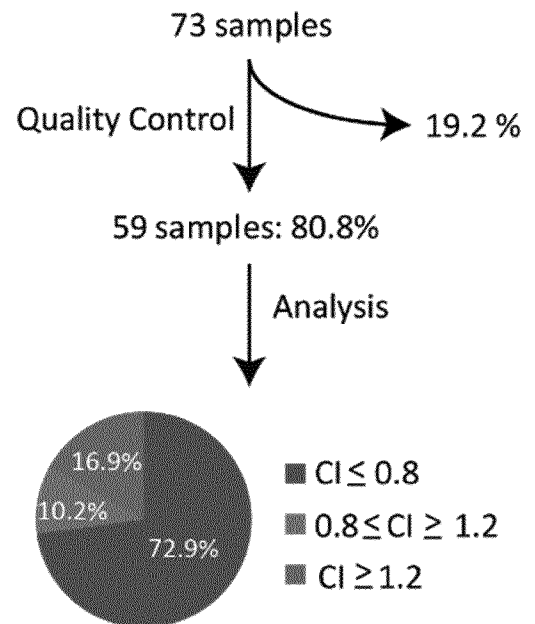


FIG. 5C

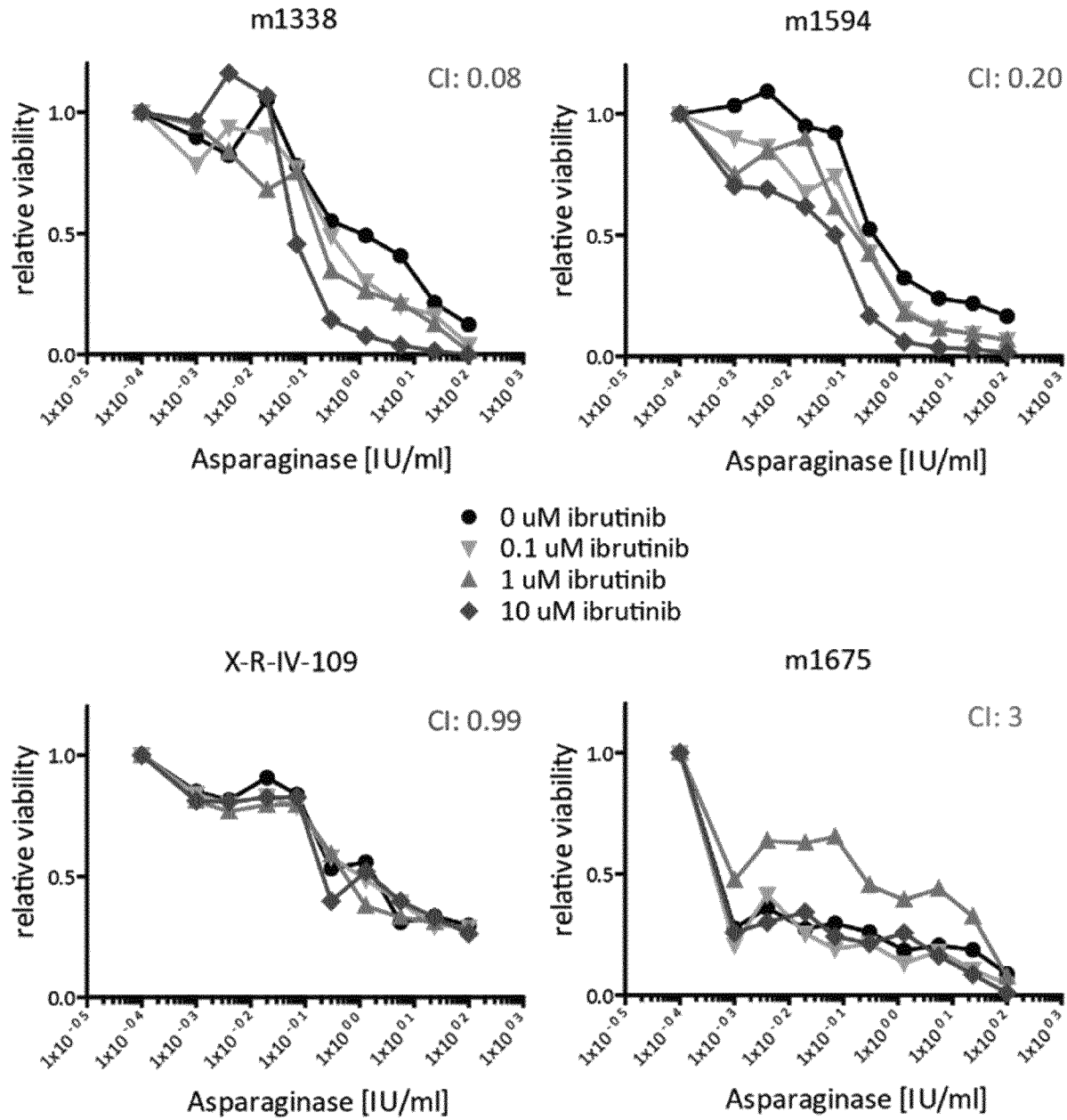


FIG. 5D

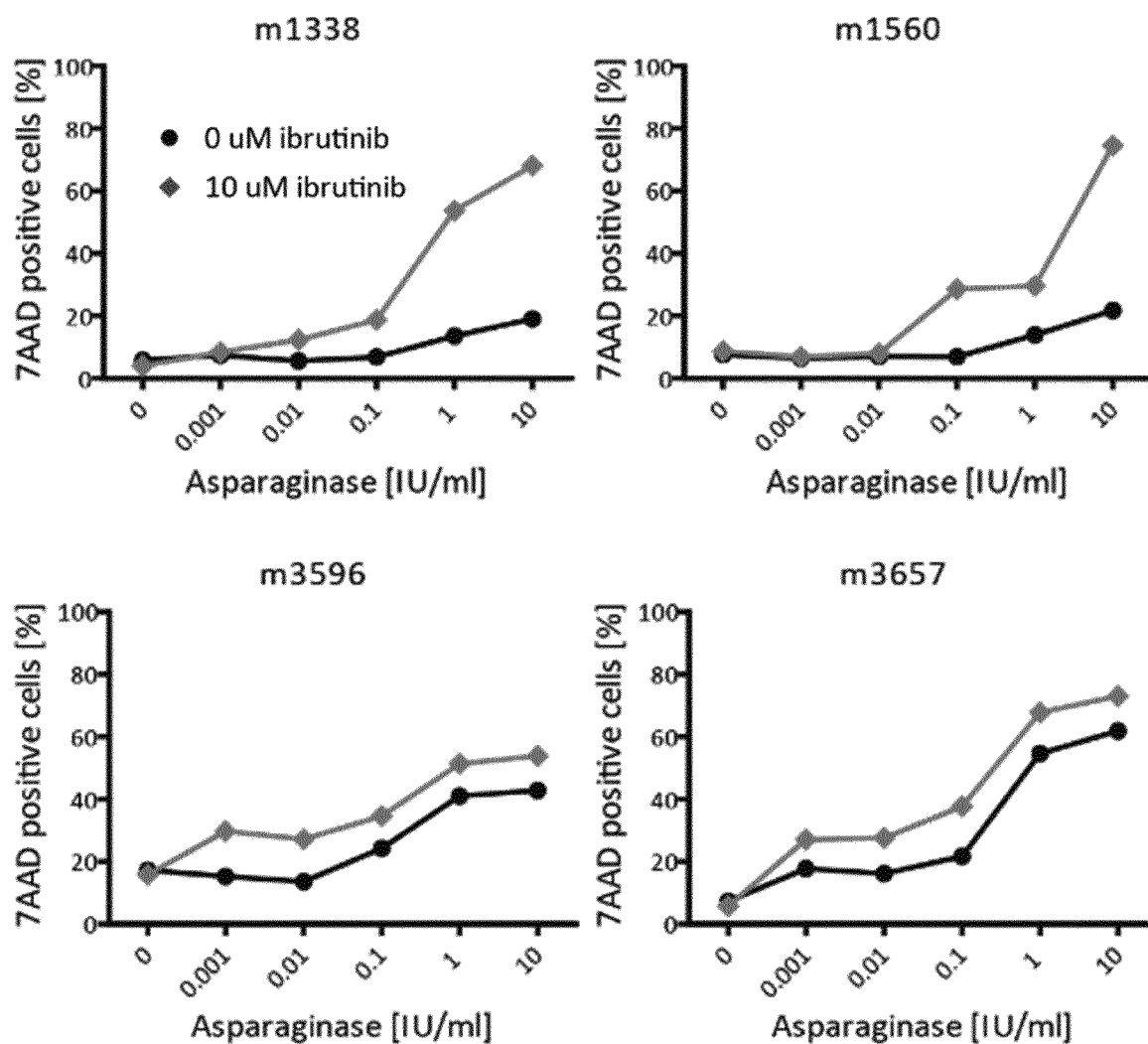


FIG. 5E

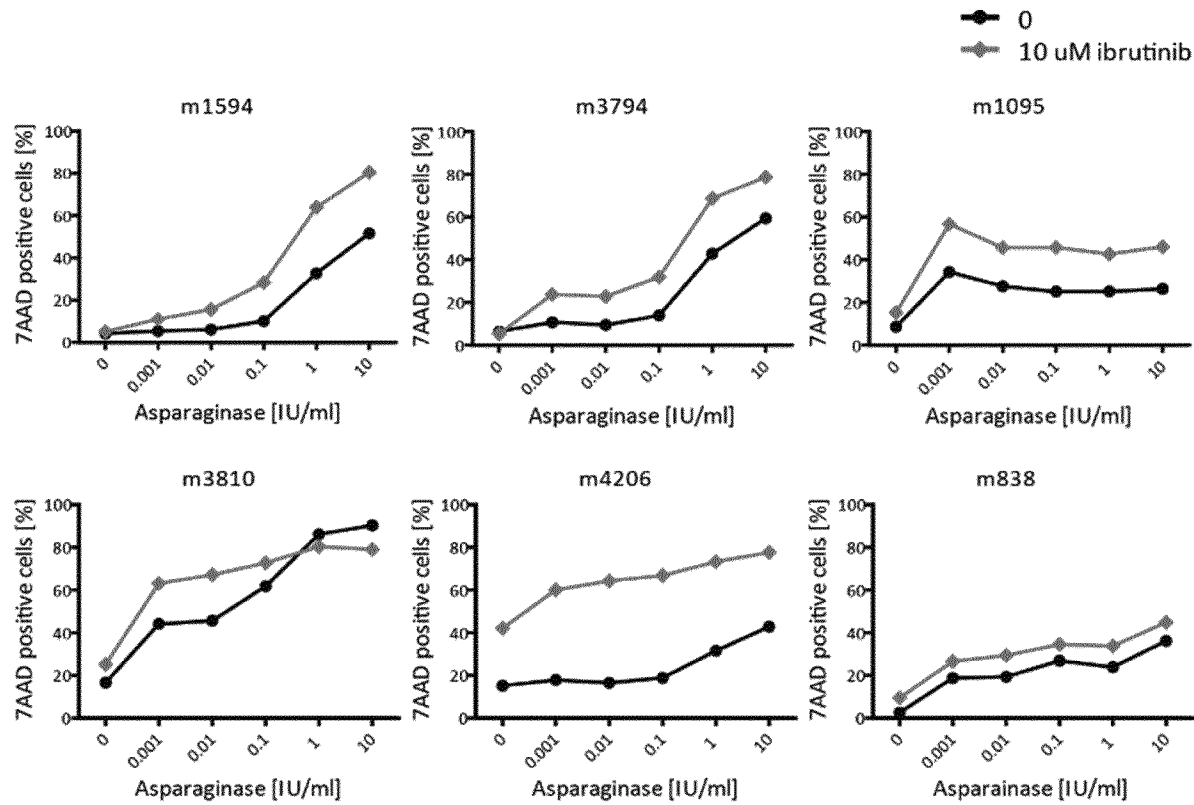


FIG. 5E (Continued)

Cytogenetics	ALL Xenograft	Index #	Treatment time (d)	Combination Index (CI)
	4206	1	3	0.01
t(4;11) MLL-AF4	838	2	3	0.01
ETV6-RUNX1 (t(12;21);	1095	3	3	0.01
EBF1del1-16,PAX5del2-8,ETV6del1-2,IL3del,CSF2RA,Ikzf1del4-7	XX-IV-113	4	3	0.02
E2A-HLF_t(17;19)	3657	5	7	0.03
E2a-PBX1; T(1;19)	3810	6	3	0.05
Ikzf1del4-7,CDKN2A del	XX-IV-182	7	3	0.10
	4138	8	7	0.12
E2A-HLF_t(17;19)	1307	9	7	0.16
ABL+,CDKN2A/BDel,PAX5Del,Ikzf1 del1-8, P53Del	XX-IV-153	10	3	0.25
MLL	3596	11	3	0.27
t (1;19)	1717	12	3	0.27
	4138	8	3	0.32
t (1;19)	X-SK-6231	13	3	0.40
TP53 unknown (del+R248Q in relapse)	X-SK-14316	14	3	0.43
t1;19	1594	15	7	0.44
	3153	16	7	0.52
TP53 del+R280T	X-SK-5486-R	17	3	0.52
E2A-HLF_t(17;19)	3657	5	3	0.53
t1;19	1594	15	3	0.57
	1371	18	7	0.57
E2a-PBX1; T(1;19)	1600	19	3	0.58
t(1;19) E2A-PBX1, t(2;12), complex	877	20	3	0.59
E2A-HLF_t(17;19)	3794	21	7	0.63
EBF1del1-16,PAX5del2-8,ETV6del1-2,IL3del,CSF2RA,Ikzf1del4-7	XX-IV-113	4	7	0.65
MLL	3596	11	7	0.66
	1560	22	3	0.71
E2A-HLF_t(17;19)	1307	9	3	0.8
	3147	23	7	0.81
t(1;19) E2A-PBX1	1730	24	3	1.06
E2A-HLF_t(17;19)	3794	21	3	1.09
	1560	22	7	1.16
Ikzf1del4-7,CDKN2A del	XX-IV-182	7	7	1.27
ABL+,CDKN2A/BDel,PAX5Del,Ikzf1 del1-8, P53Del	XX-IV-153	10	7	1.42
	1371	18	3	2.04

FIG. 5F

Xenograft	Index #	Days treatment		CI calculated without 100µM Ibrutinib data
		3d	7d	
m3810	6		x	0.01
m764	25	X		0.01
m838	2	X		0.01
m1095	3	x		0.01
m4136	26	x		0.010569414
m1600	19	x		0.017229945
m877	20	x		0.034712177
m3657	5		x	0.036302521
m1338	27		x	0.079824089
m4206	1	x		0.0864618
m1717	12	x		0.095580455
m3810	6	x		0.104794365
m4206	1		x	0.11288078
X-SK-5864D	28		x	0.113530923
m1560	22	x		0.135229298
XX-SK-15723	29		x	0.157892193
X-SK-14316	14	x		0.183278526
m3596	11	x		0.190249768
XX-IV-182	7	x		0.190287405
m1594	15	x		0.198412666
XX-SK-15723	29	x		0.213526713
X-SK-6731	30	x		0.214204639
m3596	11		x	0.233152667
m3657	5	x		0.245183105
m1307	9	x		0.273101293
m1307	9		x	0.30144226
m4138	8		x	0.311627821
m1106	31	x		0.313055545
m1371	18		x	0.408872082
m4138	8	x		0.427192959
m3320	32	x		0.471233083
XX-IV-113	4		x	0.476491859
m3794	21	x		0.493958606
m3147	23		x	0.495611173
m1594	15		x	0.507673327
m3794	21		x	0.592319951
m3153	16		x	0.616423624
m1560	22		x	0.633028962

FIG. 5G

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Xenograft	Index	Days treatment		Xenograft
		3d	7d	
XX-IV-182	7		x	0.669460172
m1600	19		x	0.697427491
XX-IV-153	10	x		0.719234708
X-SK-8266	33	x		0.730022236
X-SK-8266	33		x	0.78131993
m1730	24		x	0.979596467
X-R-IV-109	34		x	0.993400191
XX-IV-153	10		x	1.008430703
X-SK-5486-R	17		x	1.040659463
X-SK-6731	30		x	1.116768453
m1371	18	x		1.153994341
m1730	24	x		1.206177013
m877	20		x	1.318501801
m3320	32		x	1.638005798
X-R-IV-109	34	x		1.951201839
m1339	35	x		3
m3285	36		x	3
m1675	37	x		3
XX-IV-113	4	x		3
m1717	12		x	3
X-SK-5486-R	17	x		3

FIG. 5G (Continued)

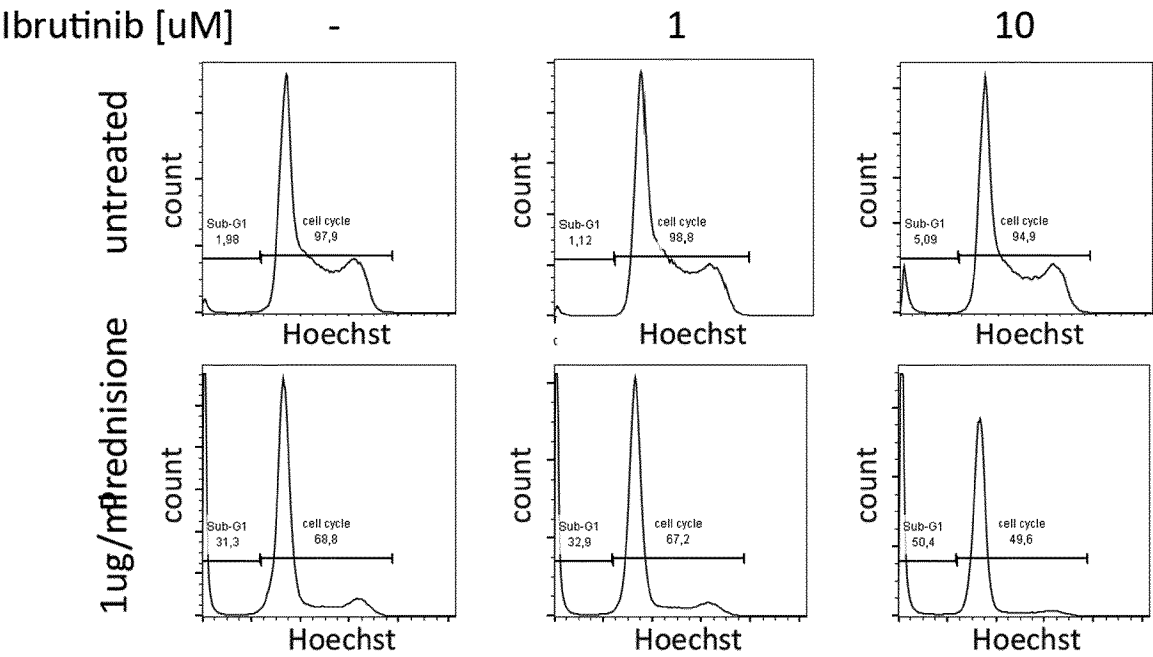


FIG. 6A

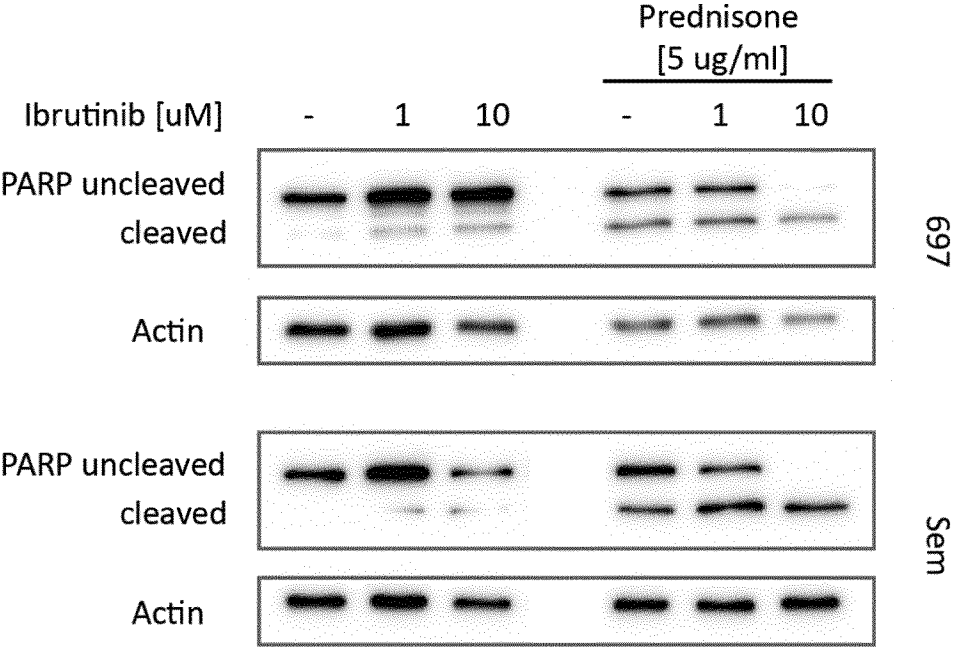


FIG. 6B

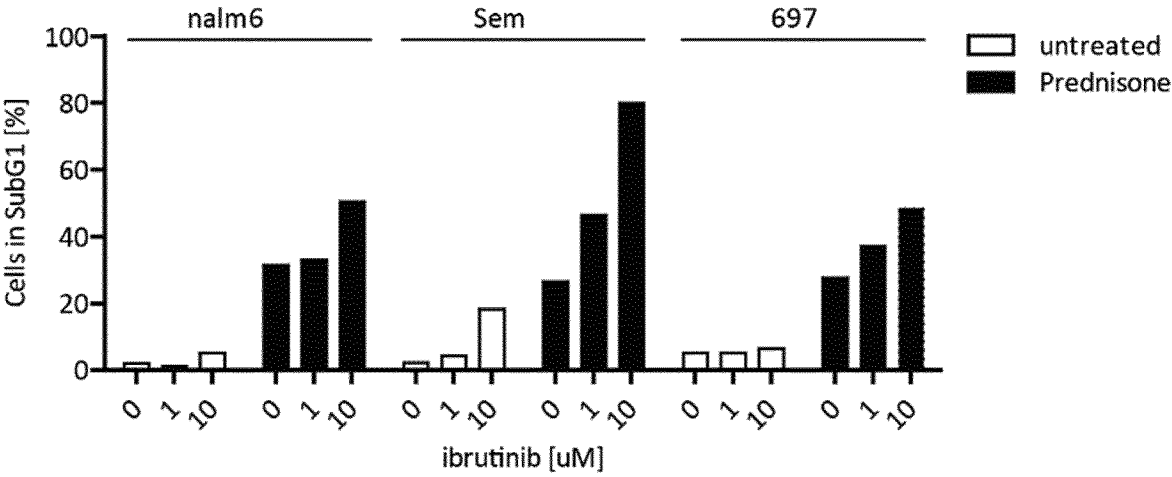


FIG. 6C

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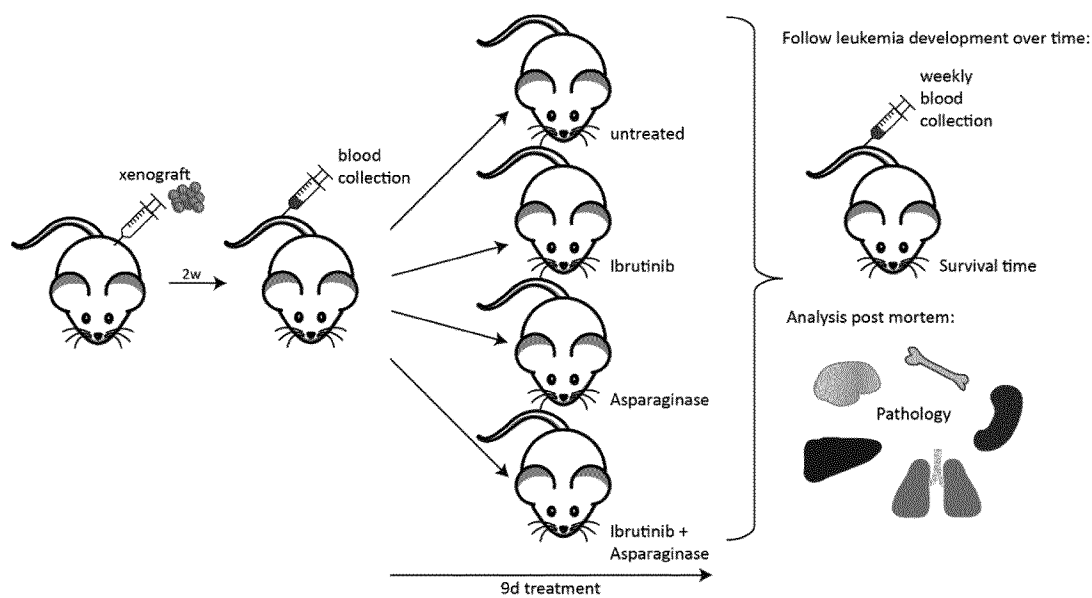


FIG. 7A

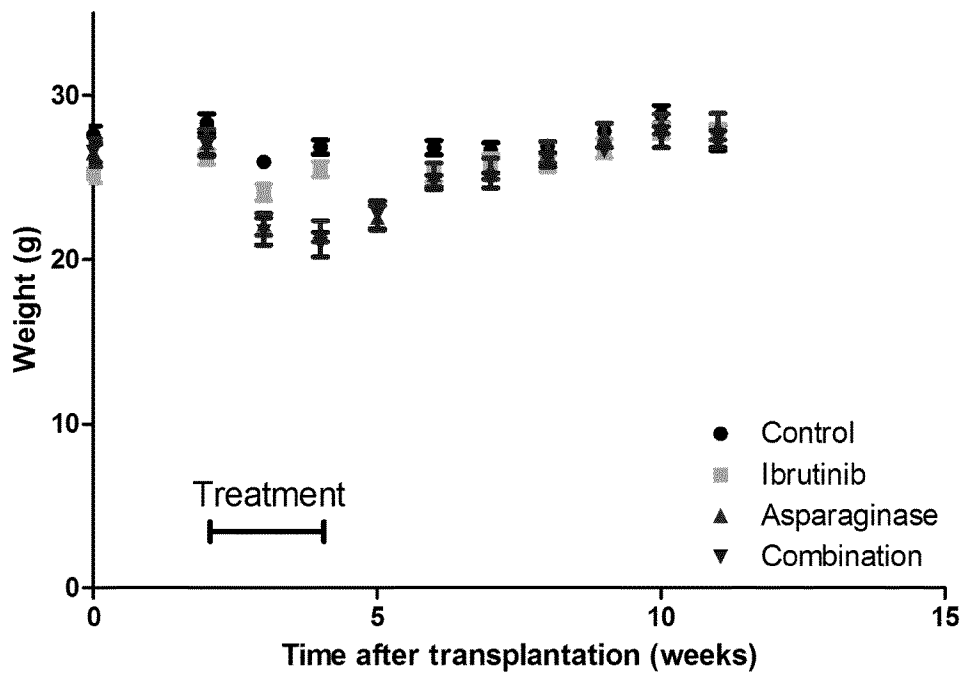


FIG. 7B

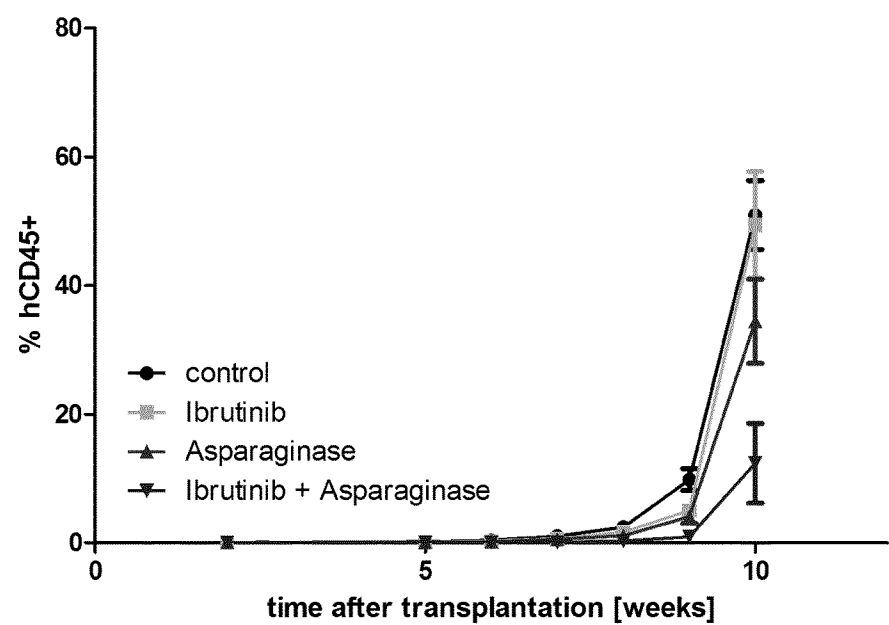


FIG. 7C

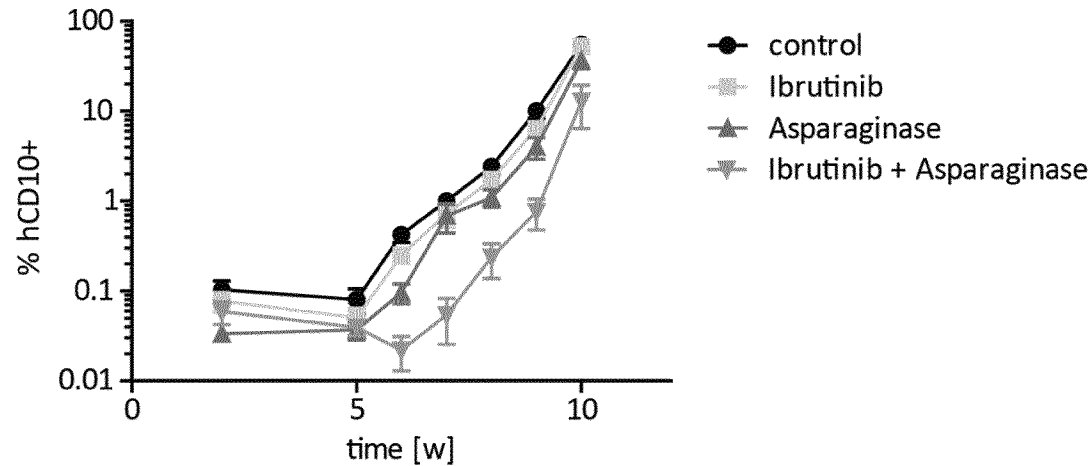


FIG. 7D

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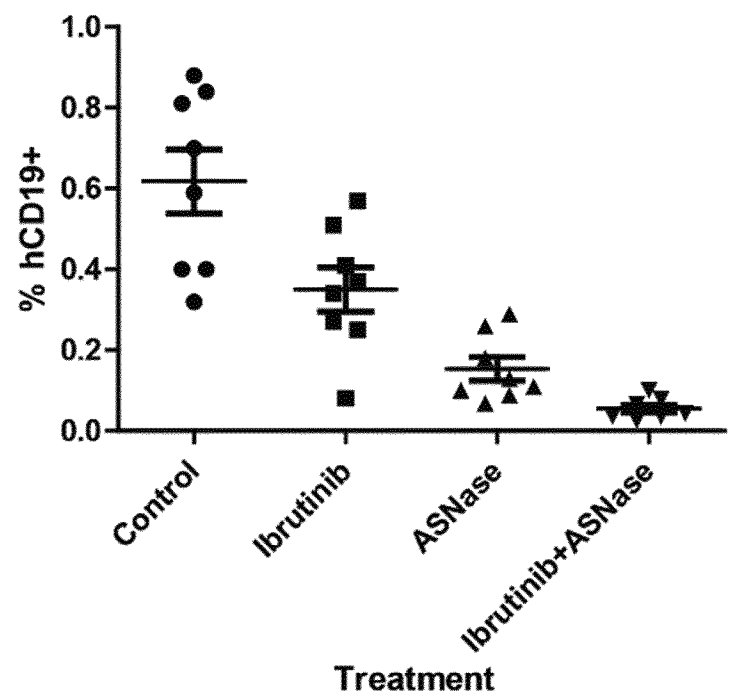


FIG. 7E

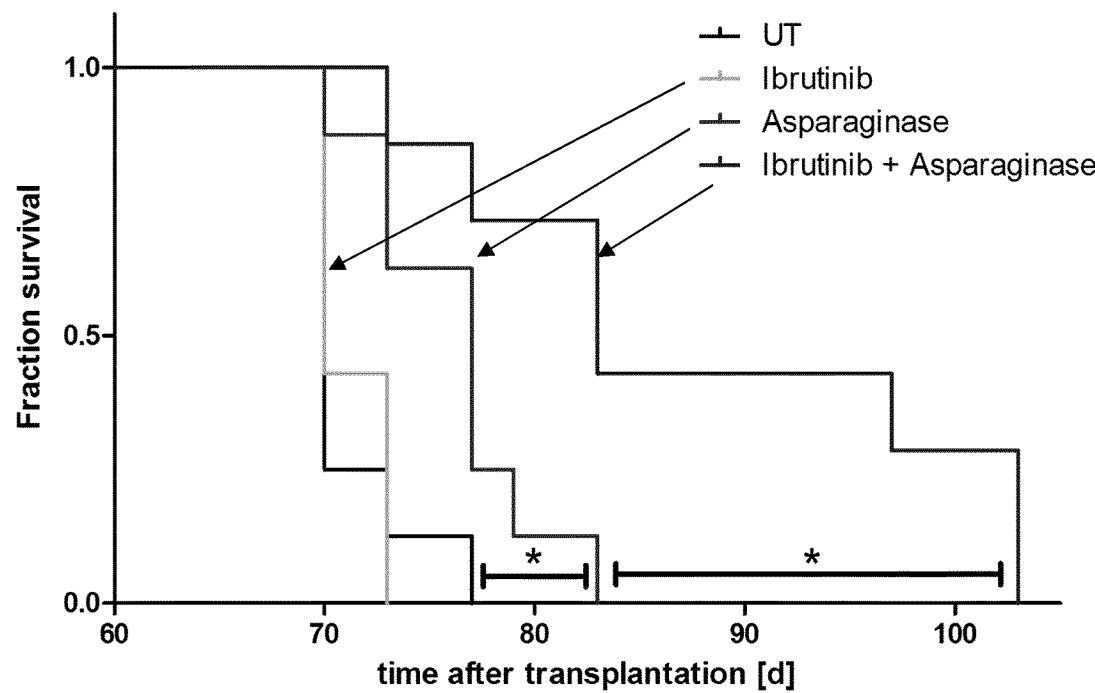


FIG. 7F

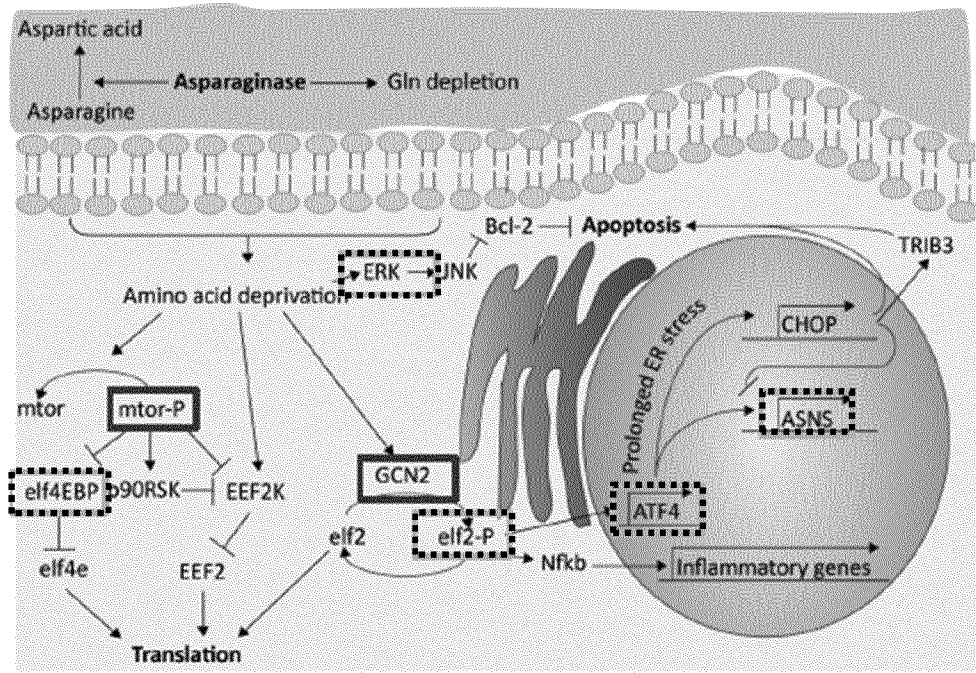


FIG. 8A

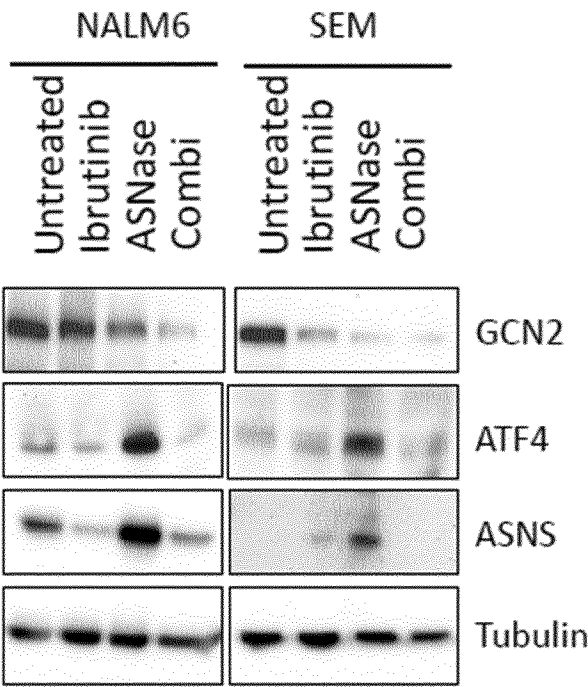


FIG. 8B

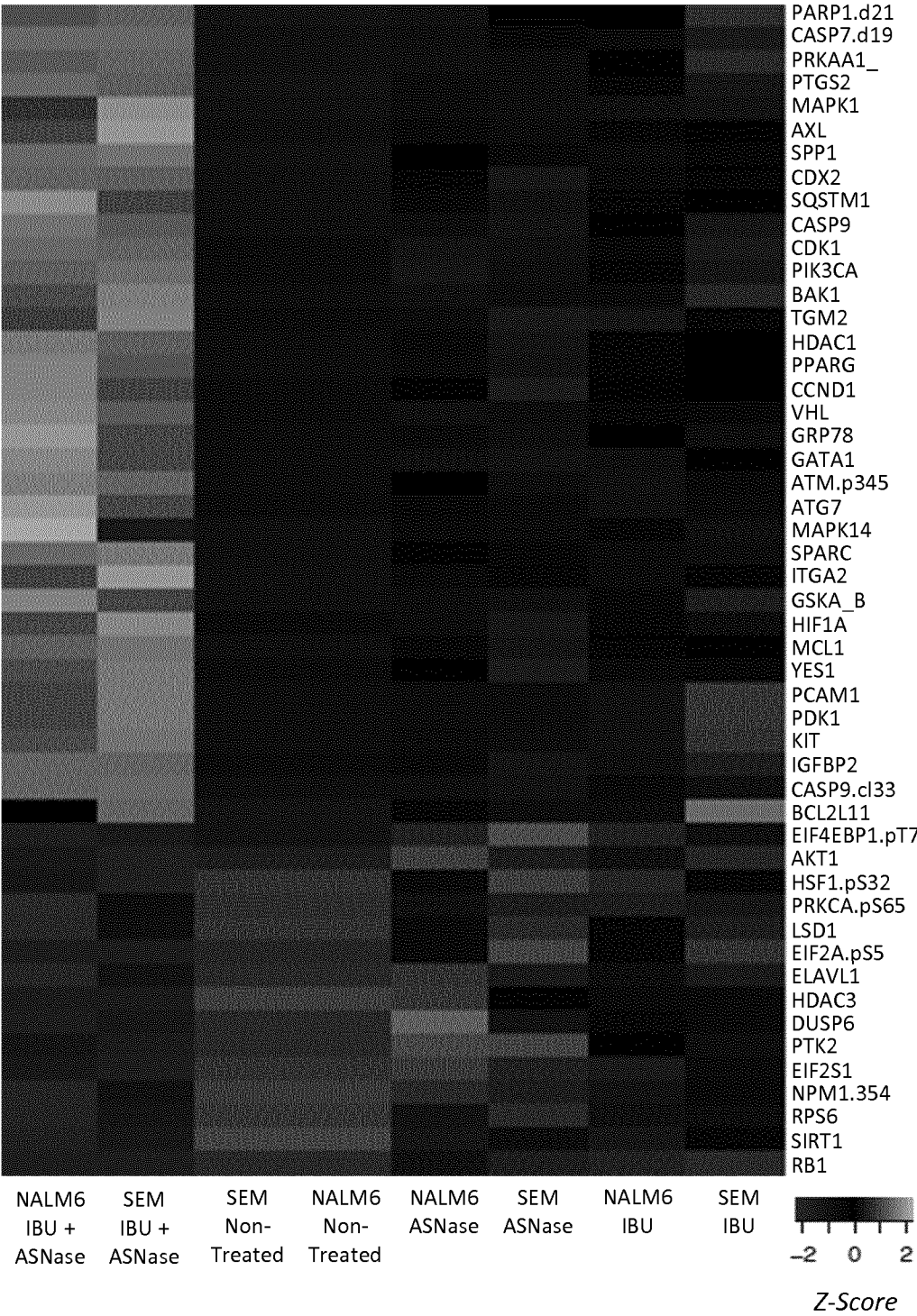


FIG. 9

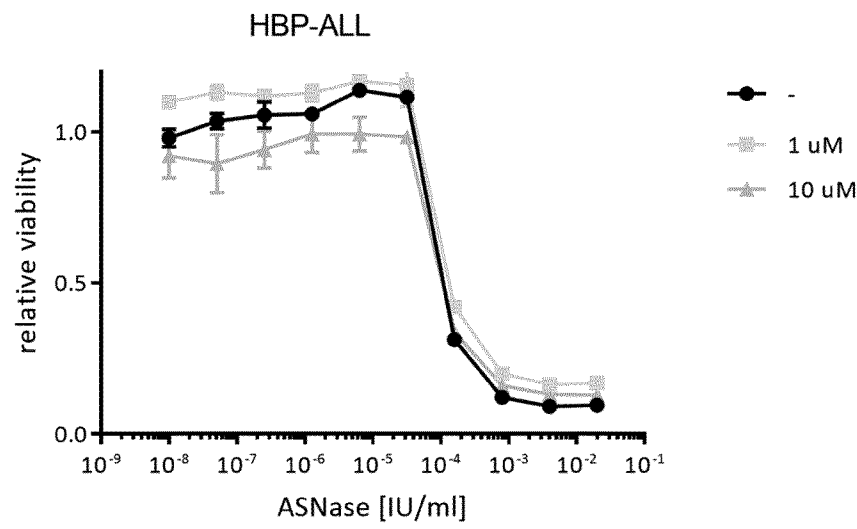


FIG. 10A

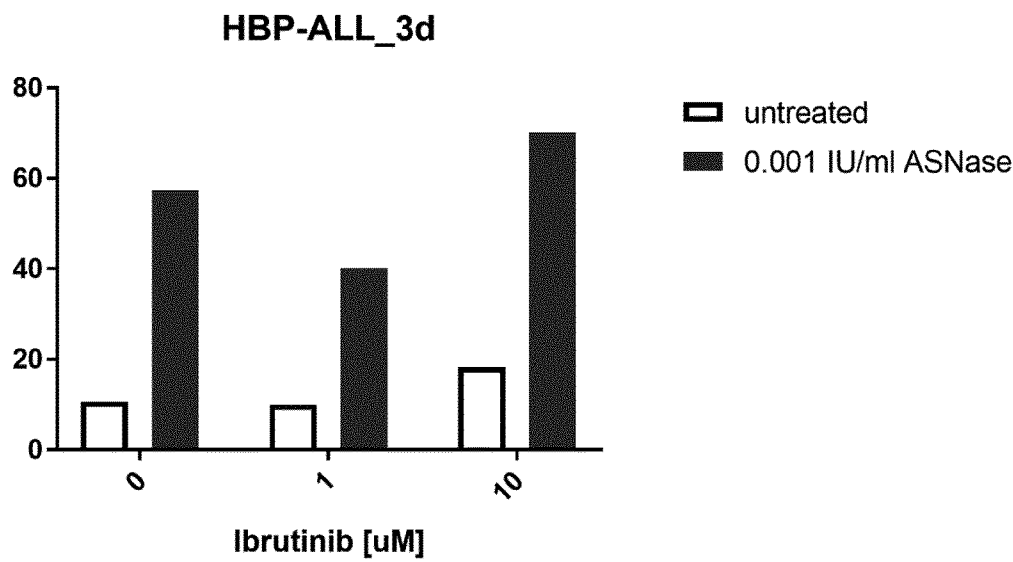


FIG. 10B

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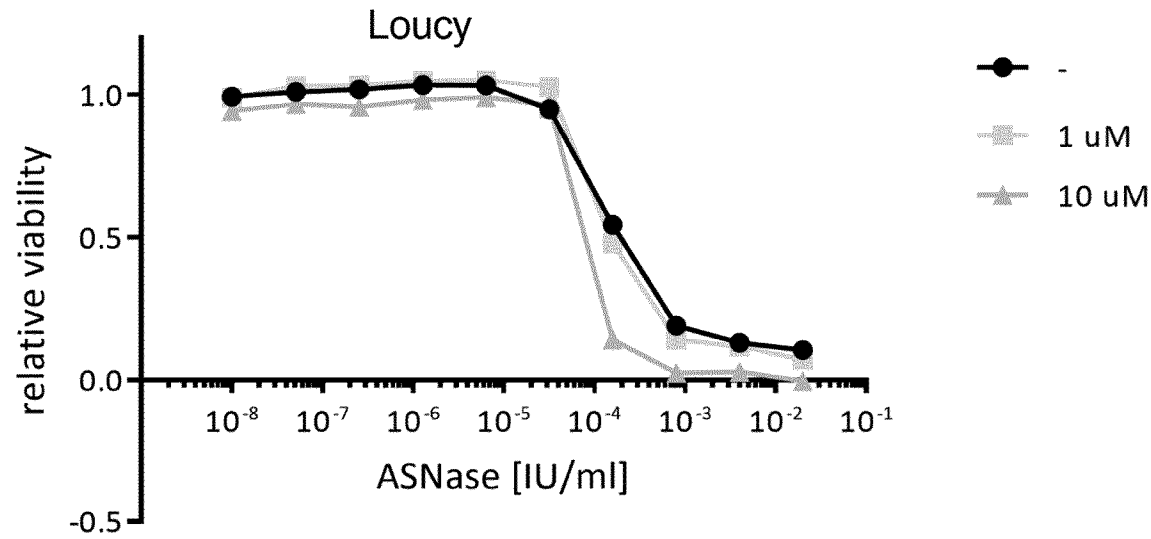


FIG. 11A

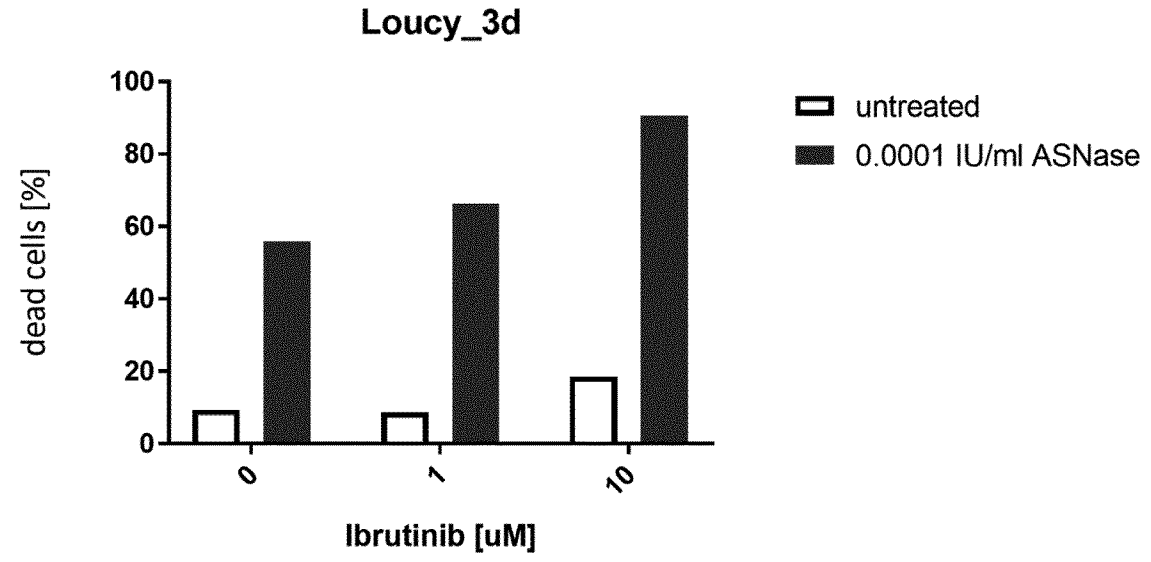


FIG. 11B

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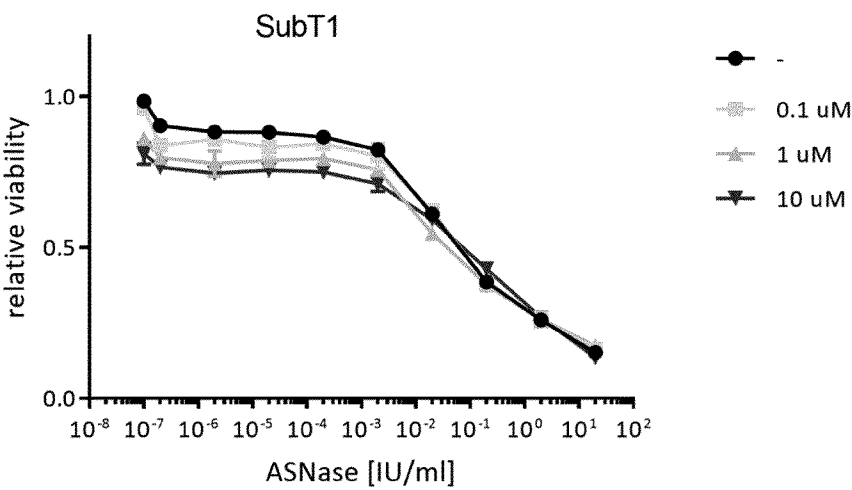


FIG. 12A

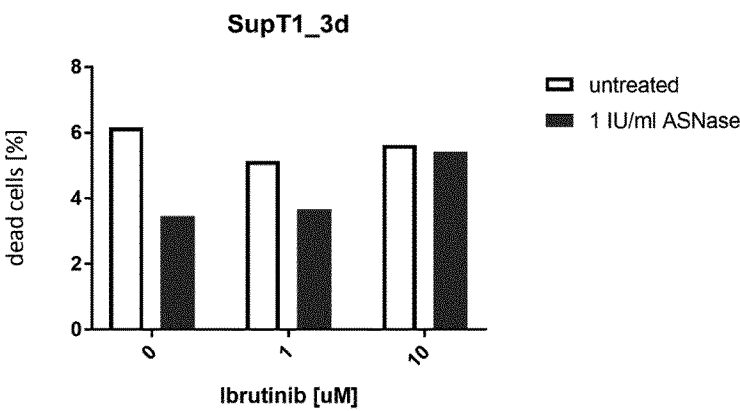


FIG. 12B

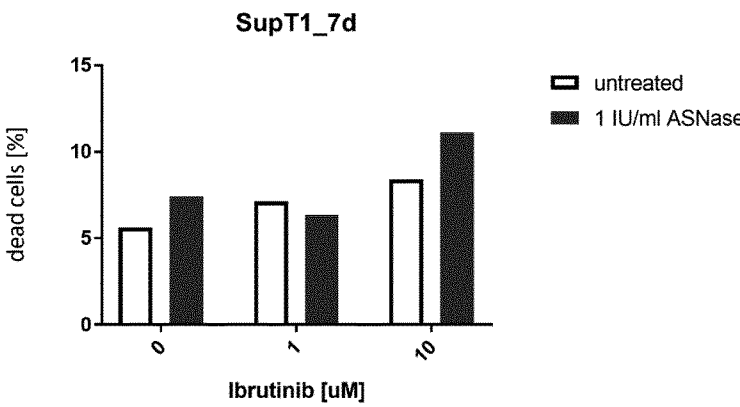


FIG. 12C

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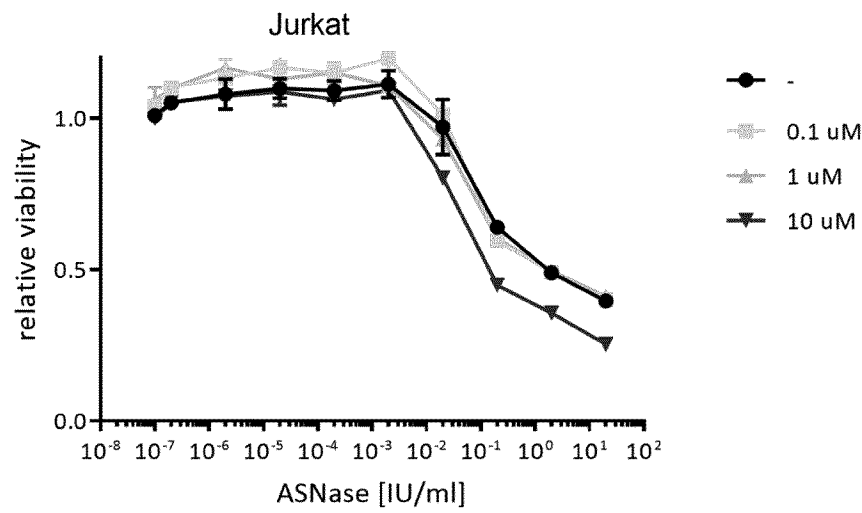


FIG. 13A

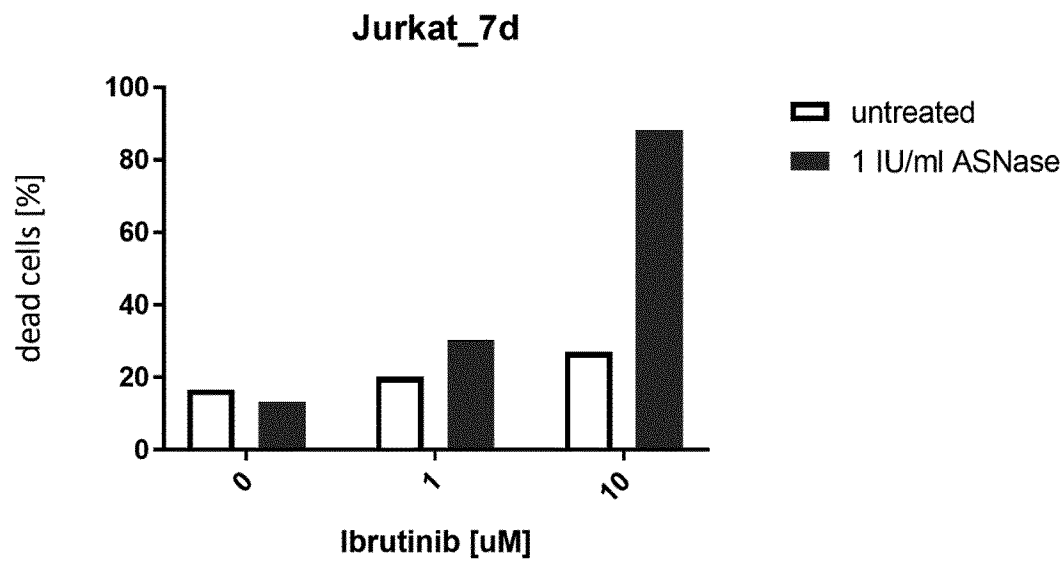


FIG. 13B

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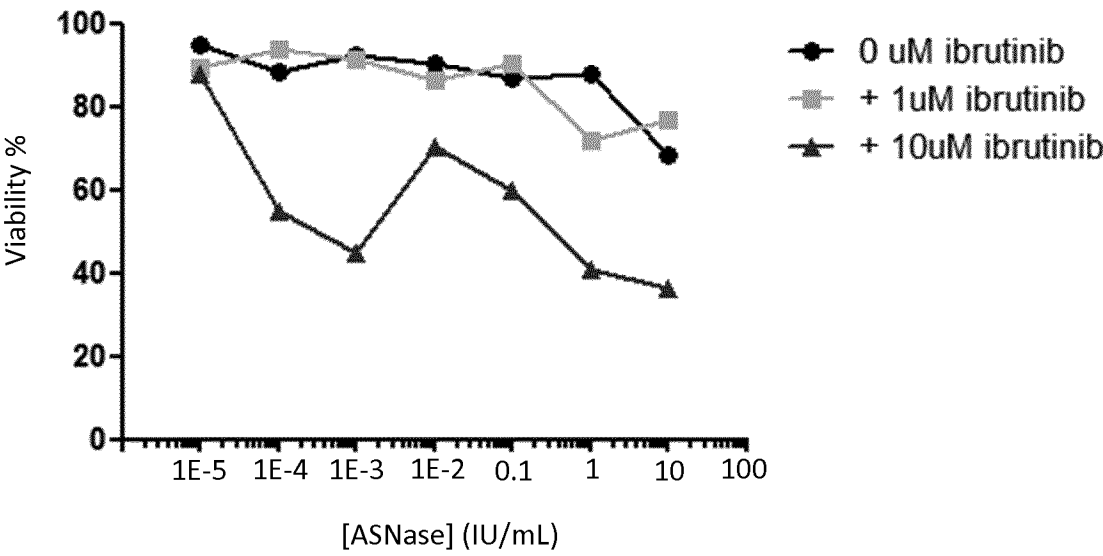


FIG. 14A

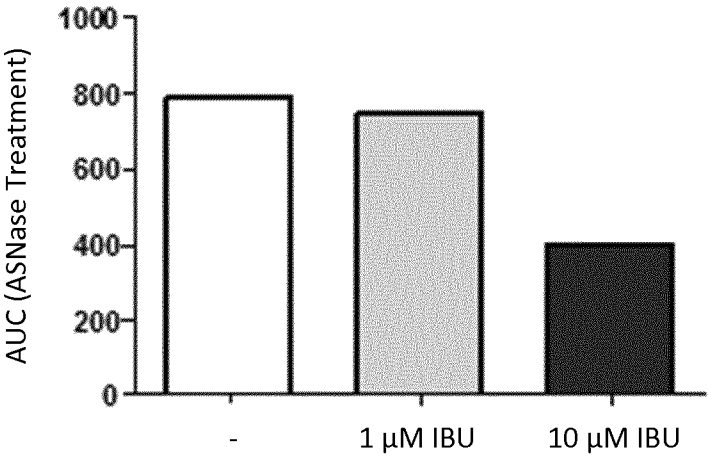


FIG. 14B

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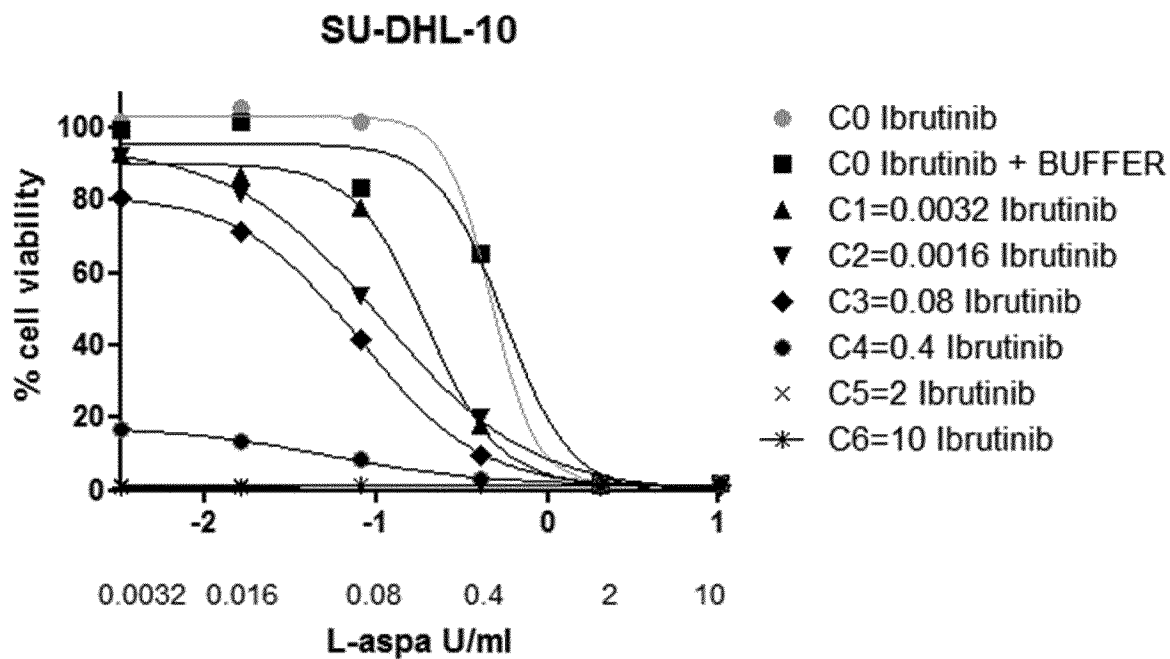


FIG. 15A

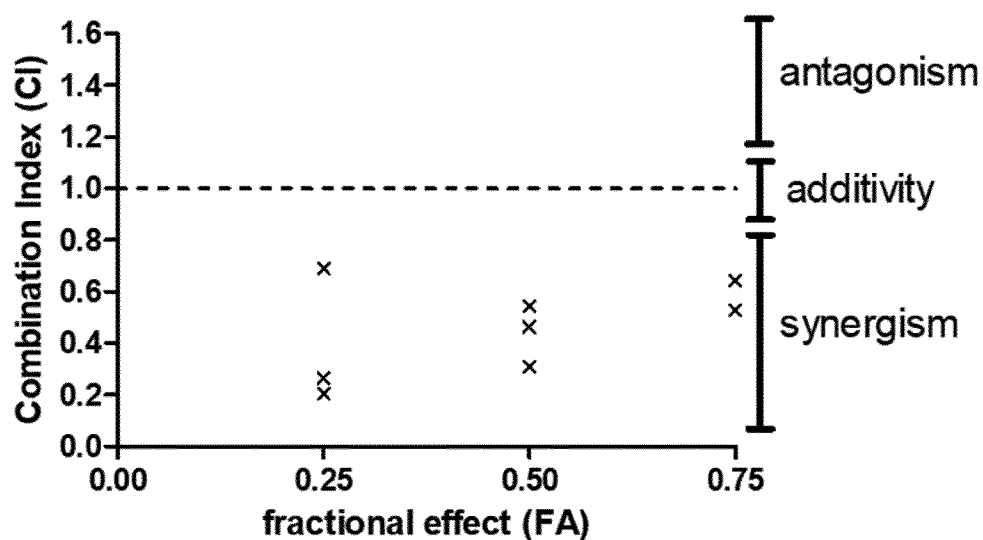


FIG. 15B

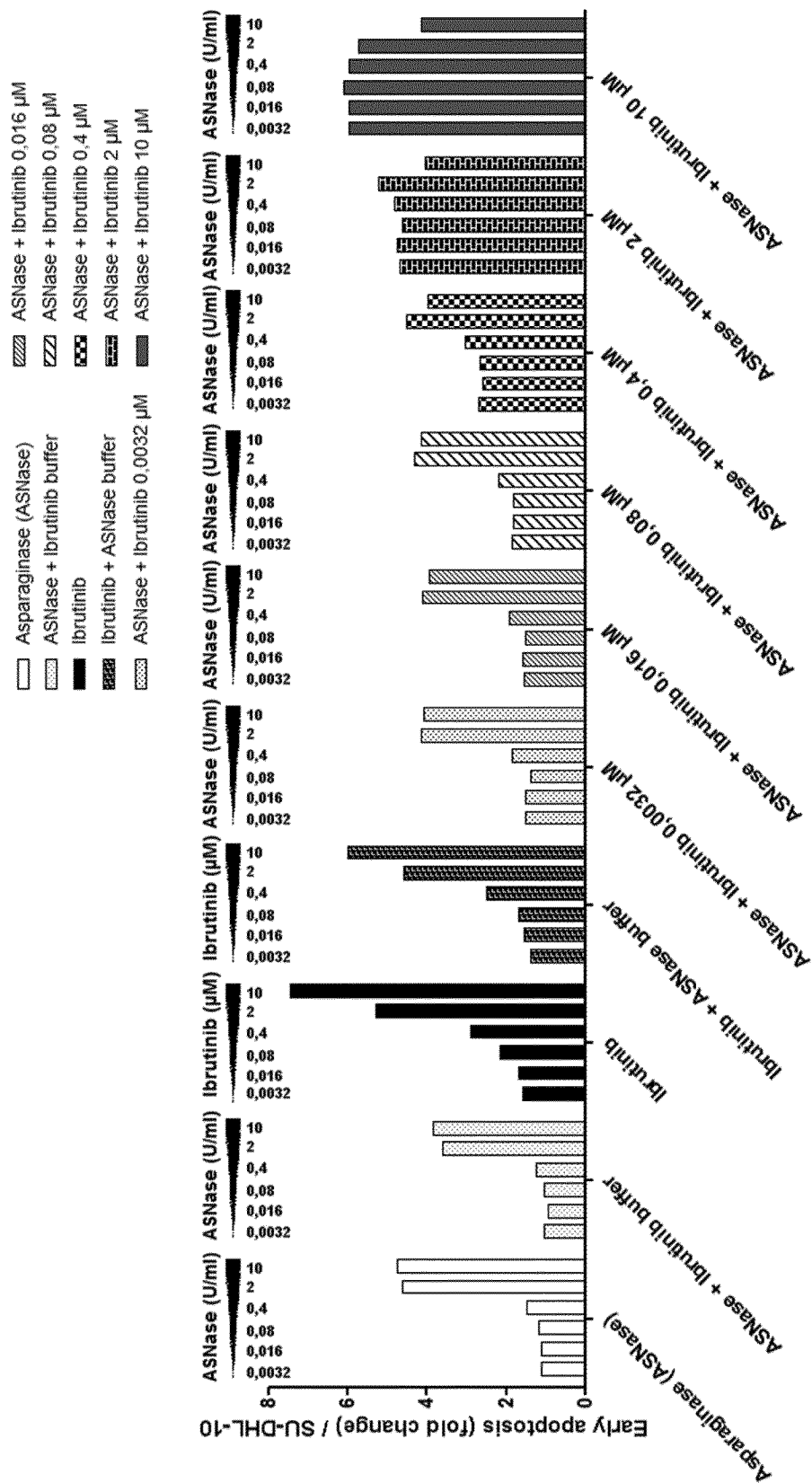


FIG. 15C

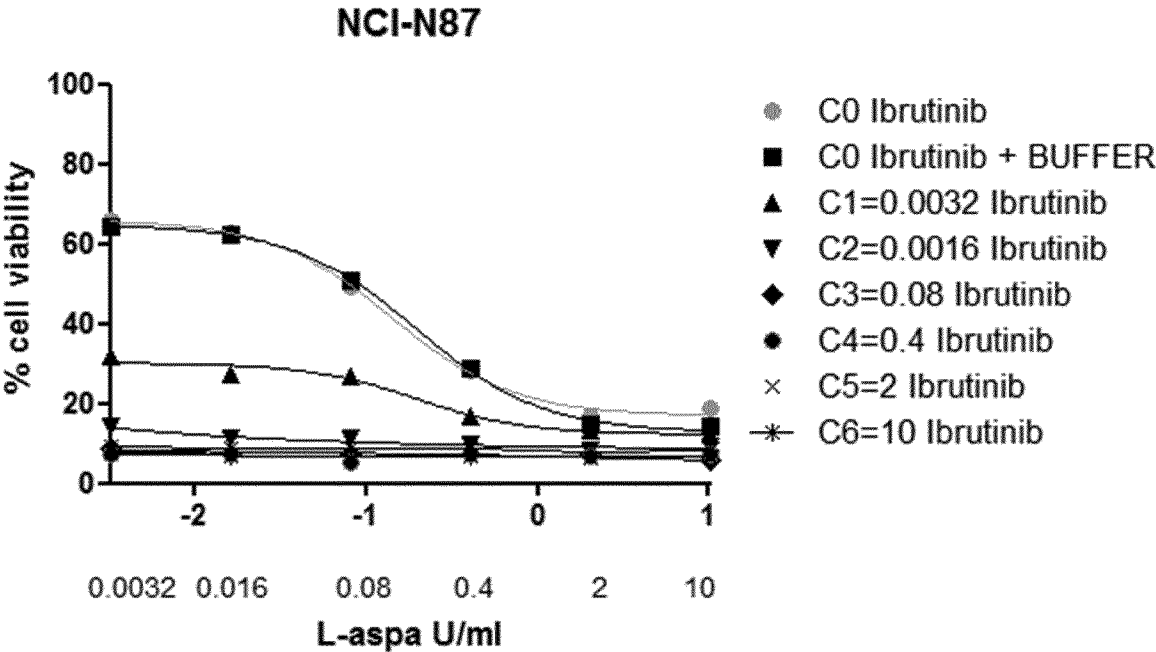


FIG. 16

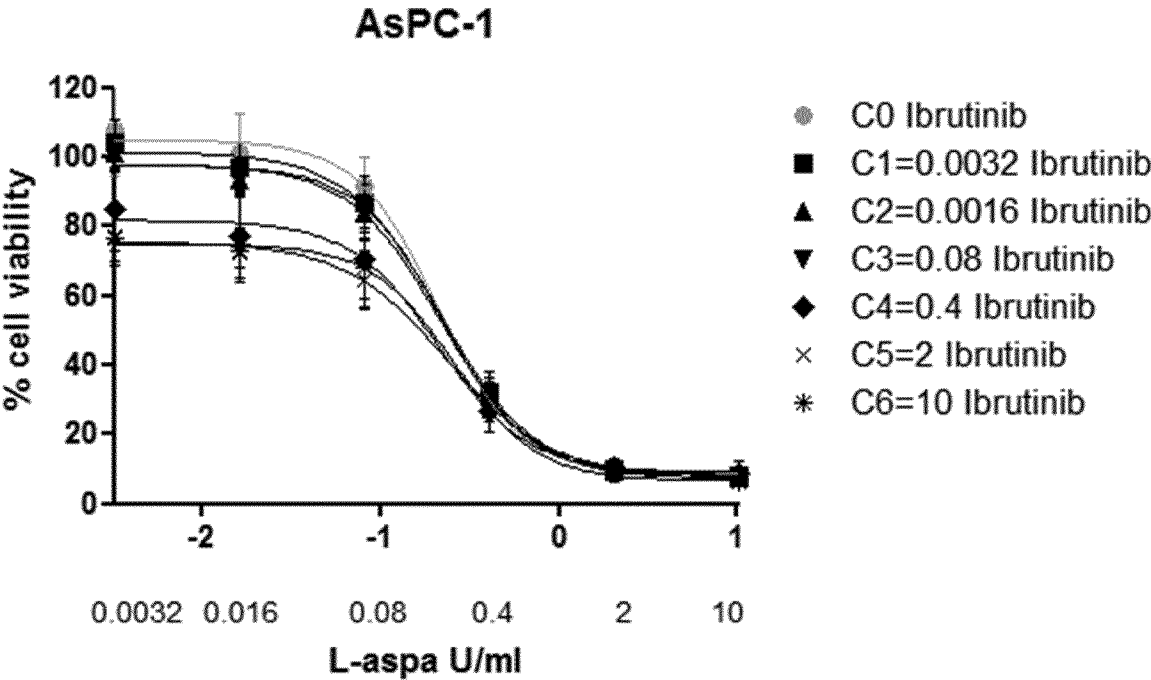


FIG. 17

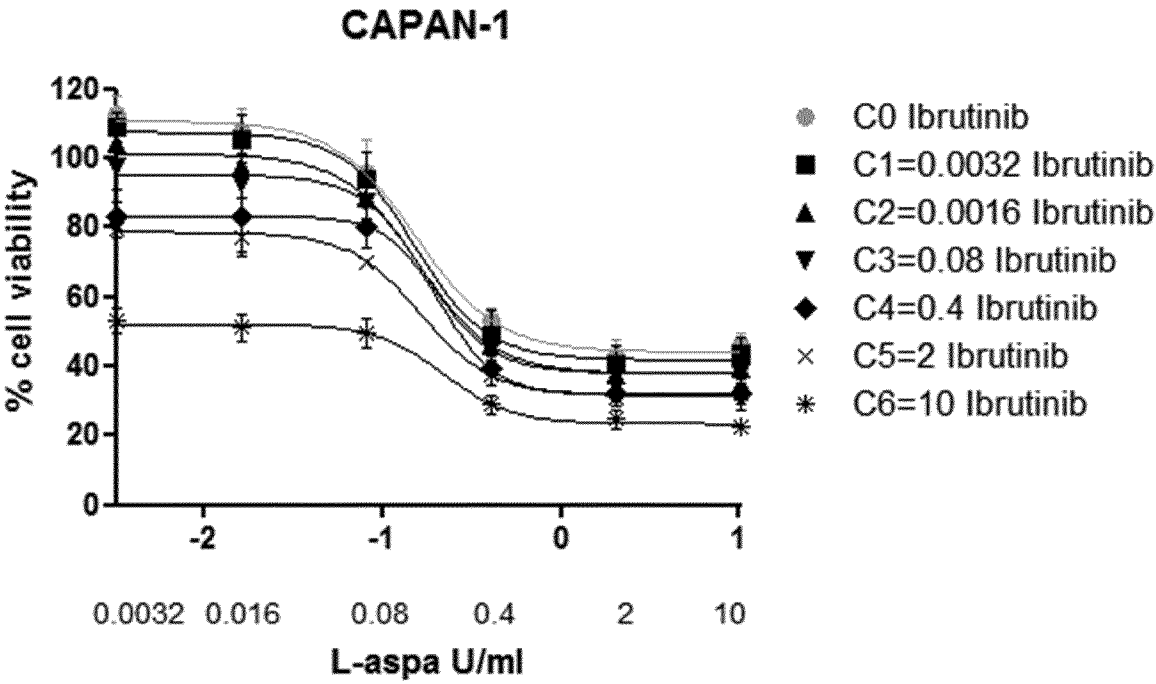


FIG. 18

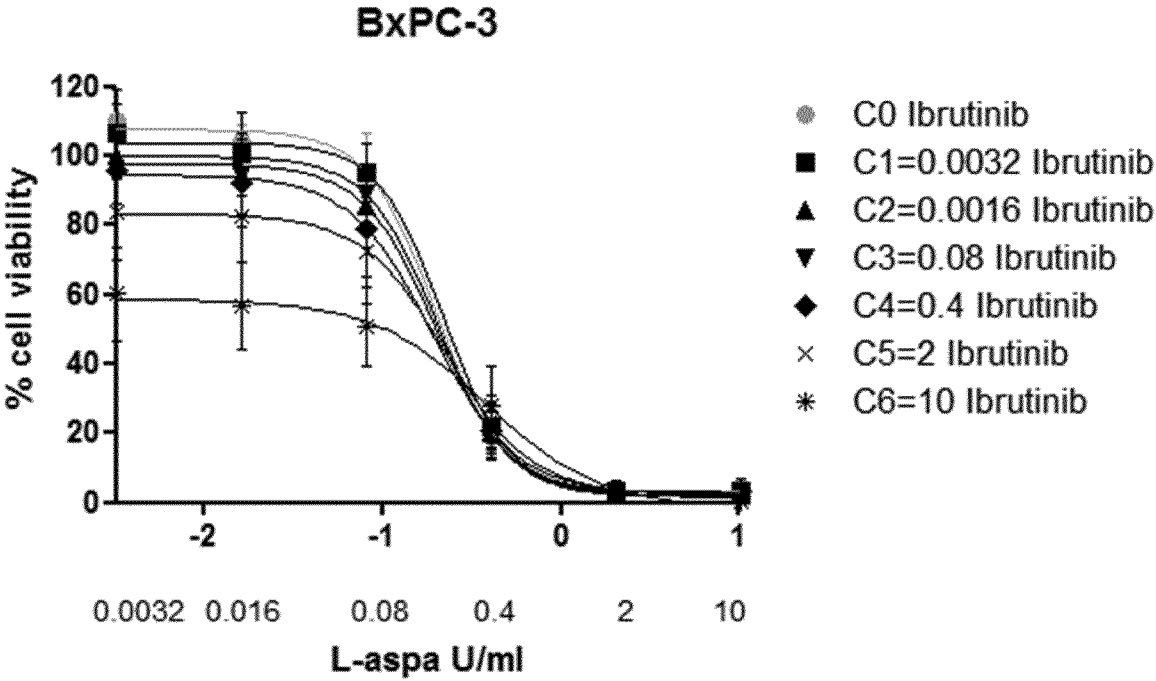


FIG. 19

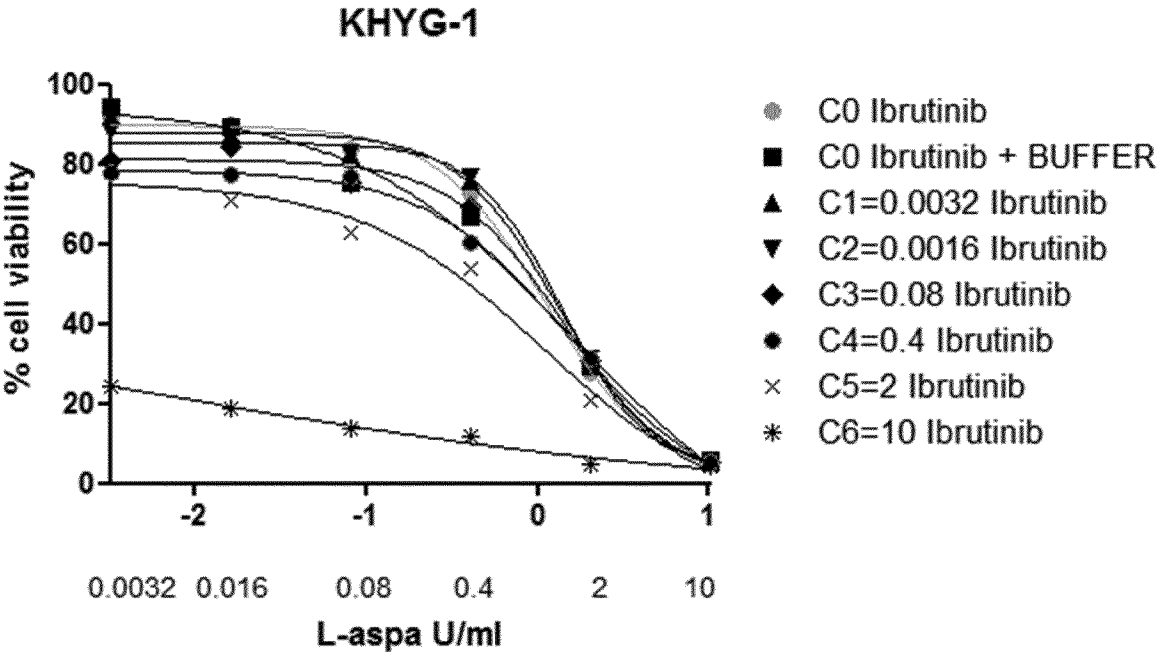


FIG. 20

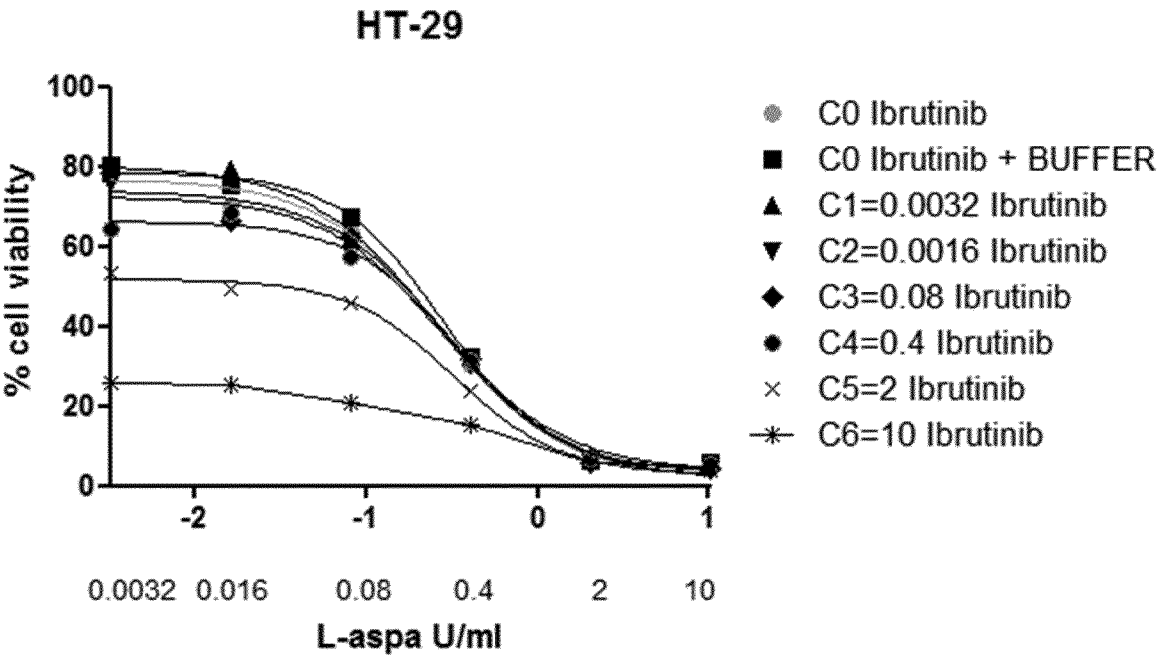


FIG. 21

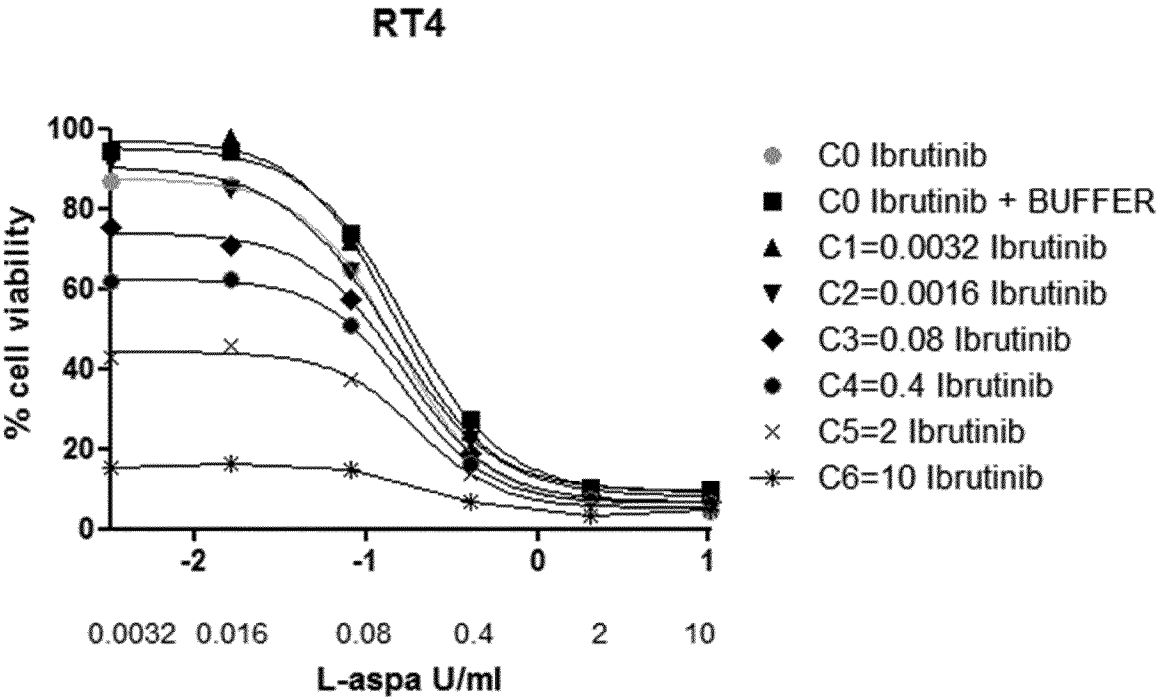


FIG. 22

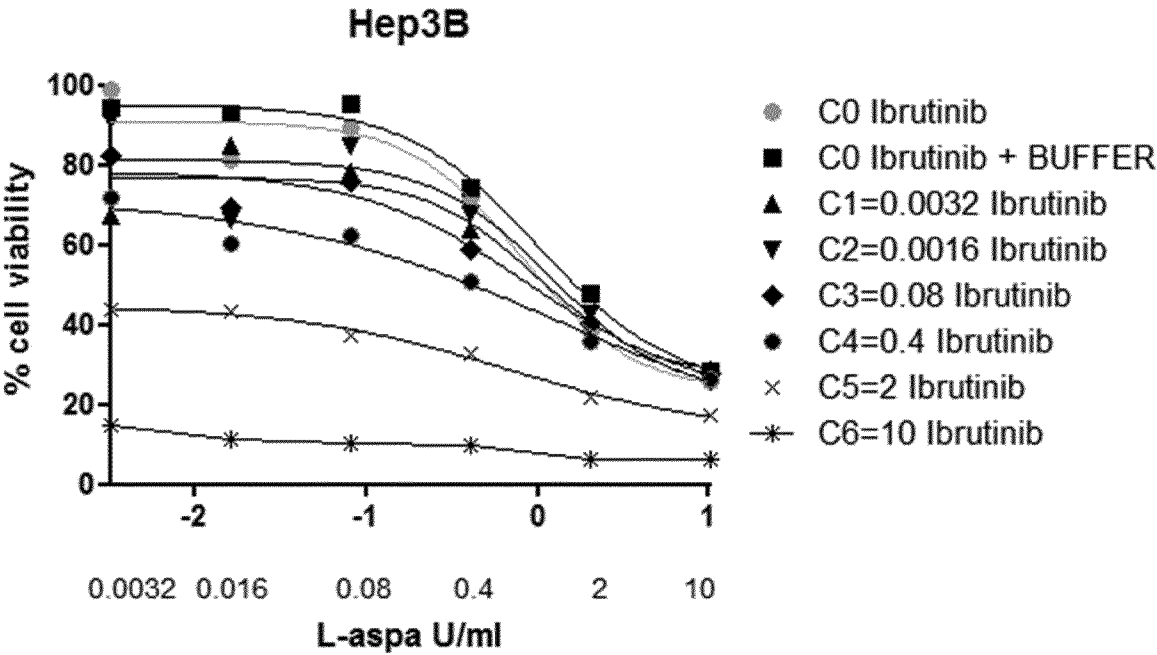


FIG. 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/083893

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/50 A61K45/06 A61K31/519 A61P35/00
ADD.

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)

A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Butler ET AL: "A CRISPR/Cas9 Based Kinome Screen Identifies Bruton Tyrosine Kinase (BTK) As an Important Determinant of Asparaginase Treatment Response in Acute Lymphoblastic Leukemia Blood Journal", Blood, 1 December 2017 (2017-12-01), XP055564057, Retrieved from the Internet: URL:http://www.bloodjournal.org/content/130/Suppl_1/34 [retrieved on 2019-03-04] figure 1 ----- -/-	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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"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

4 March 2019

Date of mailing of the international search report

15/03/2019

Name and mailing address of the ISA/

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Authorized officer

Dolce, Luca

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/083893

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Van Der Meer ET AL: "Crispr/Cas9 Based Kinome Screen Identifies Novel Targets That Determine Sensitivity for Asparaginase Therapy in Acute Lymphoblastic Leukemia", Blood, 3 December 2015 (2015-12-03), XP055564067, Retrieved from the Internet: URL: http://www.bloodjournal.org/content/126/23/2629?sso-checked=true [retrieved on 2019-03-04] figure 2	1-20
A	----- WO 2008/039218 A2 (PHARMACYCLICS INC [US]; HONIGBERG LEE [US]; VERNER ERIK [US]; PAN ZHEN) 3 April 2008 (2008-04-03) paragraph [0373] - paragraph [0375] -----	1-20
A	WO 2014/168975 A1 (PHARMACYCLICS INC [US]; JANSSEN PHARMACEUTICA NV [BE]; CHANG BETTY [US]) 16 October 2014 (2014-10-16) paragraph [0005] -----	1-20
A	WO 2017/151707 A1 (UNIV ILLINOIS [US]) 8 September 2017 (2017-09-08) paragraph [0003] - paragraph [0004] -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/083893

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