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(54) Title: RESISTANT CELL LINES AND METHODS OF TREATING CANCER USING COMBINATION OF HDAC6 INHIBITOR AND BTK PATHWAY INHIBITOR

(57) Abstract: Provided herein is a new therapeutic regimen comprising an HDAC6 inhibitor and a BKT inhibitor for the treatment of lymphoma. More specifically, the regimen comprises treatment with ACY-1215 and ibrutinib. Furthermore, the invention provides a lymphoma cell line resistant to the HDAC6 selective inhibition.



RESISTANT CELL LINES AND METHODS OF TREATING CANCER USING COMBINATION OF HDAC6 INHIBITOR AND BTK PATHWAY INHIBITOR

CROSS-REFERENCE TO RELATED APPLICATIONS

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The present application claims priority to U.S. Provisional Patent Application Ser. No. 62/415,733 filed November 1, 2016, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

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The instant application contains a Sequence Listing which has been filed electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 30, 2017, is named 01001_005317-WO0_ST25.txt and is 2,895 bytes in size.

FIELD OF THE INVENTION

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The present invention relates to a combination therapy for the treatment of lymphoma, particularly to combinations of an HDAC6 inhibitor and a BTK inhibitor, wherein the combination therapy shows enhanced anti-tumor effect. The invention also provides a lymphoma cell line that has been developed to be resistant to the HDAC6 inhibition.

BACKGROUND

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Pan-class histone deacetylase (HDAC) inhibitors have proven successful in the treatment of lymphomas but their clinical application has been restricted predominantly to hematologic malignancies of T-cell lineage. Since 2009, there have been FDA approvals of a number of HDAC inhibitors including: vorinostat, romidepsin, belinostat, and chidamide (China) in T-cell lymphomas and panobinostat for the treatment of multiple myeloma [1-5].

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Additionally, there have been discrete scenarios where HDAC inhibitors have demonstrated utility in B-cell lymphomas. One example is in B-cell lymphoma of germinal center origin which takes advantage of an accumulation of epigenetic derangements to drive lymphomagenesis. Targeting epigenetic programs with HDAC inhibitors in germinal center derived B-cell lymphoma has been shown to abrogate BCL6, the master regulator of the

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germinal center, and activate p53 leading to apoptosis [6].

Despite these and other findings, defining the precise role of individual HDACs in lymphoma has been elusive. Recently, isoform selective HDAC inhibitors have been developed with the initial goal of eliminating unwanted off-target effects aiming to decrease side effects known to accompany pan-class HDAC inhibitors. ACY-1215 (ricolinostat) is a first in class selective HDAC6 inhibitor. HDAC6 belongs to the class 2b family of HDACs and differs from other HDACs in that it resides predominantly in the cytoplasm. It is known to play a role in protein homeostasis and the unfolded protein response (UPR) [7, 8]. HDAC6 inhibition has demonstrated activity in preclinical models of lymphoma and multiple myeloma and is currently being studied in the clinical setting both as a single agent and in combination. Although very well tolerated clinically, activity as a single agent has been limited due to the development of resistance. Thus, combination strategies have had a greater therapeutic impact on treating disease. Combinations of ACY-1215 are presently in clinical study for patients with multiple myeloma. Examples of drug partners include revlimid, pomalidomide and bortezomib [9-12].

The development of chemotherapy resistant models of disease has been employed for over half a century. In 1950, Burchenal and colleagues developed a sub-strain of a mouse implanted with the leukemia cell line Ak4[13]. This mouse strain was engineered to be resistant to treatment with 4-amino-N¹⁰-methyl-pteroylglutamic acid. The purpose of this experiment was to evaluate the mechanism of eventual lack of response and the investigators hypothesized that this resistant leukemia was either able to synthesize its own PGA, deaminate the anti-metabolite, or “increase the ability of the host organism to detoxify the compound.” Again in the 1970s, a group of investigators studied the effects of high exposure of methotrexate in sarcoma cells which were sensitive and those that were resistant[14]. They identified that the resistant cell lines increased their rate of dihydrofolate reductase levels by 200-fold compared to sensitive cell lines. This increase was correlated with increased mRNA which was associated with increased gene copy number. Efforts from these studies have paved the way for improving the effectiveness of drugs and led to the development of combination chemotherapies for the majority of malignancies.

There is an ongoing need for new therapeutics for lymphoma and multiple myeloma, as well as for improved models for predicting and avoiding the development of drug resistance by the cancer cells.

SUMMARY OF INVENTION

In certain embodiments, the present invention relates to an isolated diffuse large B-cell lymphoma cell line resistant to an HDAC6 inhibitor. In some embodiments, the isolated diffuse large B-cell lymphoma cell line is R10-OCI-LY10 (R10). In certain embodiments, the HDAC6 inhibitor is ACY-1215. In other embodiments, the HDAC6 inhibitor is vorinostat.

In further embodiments, the isolated diffuse large B-cell lymphoma cell line of the present invention exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN. In yet further embodiments, the isolated diffuse large B-cell lymphoma cell line of the present invention exhibits down-regulation of SH3BP5 and LCK. In more specific embodiments, the isolated diffuse large B-cell lymphoma cell line of the present invention exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.

In some aspects, the present invention relates to a kit comprising diffuse large B-cell lymphoma cell line (R10-OCI-LY10 (R10)) and a culture medium. In certain aspects, the kit further comprises the parental OCI-Ly10 cell line.

In certain embodiments, the present invention relates to an isolated diffuse large B-cell lymphoma cell line (R10-OCI-LY10 (R10)) resistant to a proteasome inhibitor. In some embodiments, the proteasome inhibitor is bortezomib.

In some embodiments, the present invention relates to a method for treating lymphoma in a patient comprising administering to a patient a therapeutically effective amount of (i) an HDAC6 inhibitor and (ii) a BTK inhibitor. In certain embodiments, the HDAC6 inhibitor is selected from the group consisting of ACY-1215, ACY-241, Tubacin, Tubastatin A, ST-3-06, ST-2-92, Nexturastat A, and Nexturastat B, and optionally combinations thereof. In further embodiments, the HDAC6 inhibitor is ACY-1215. In some embodiments, the BTK inhibitor is selected from the group consisting of ibrutinib, HM-71224, BGB-3111, CG-036806, CC-292, ACP-196, GDC-0834, ONO-4049, RN-486, SNS-062, TAS-5567, AVL-101, AVL-291, PCI-45261, HCI-1684, and PLS-123, and optionally combinations thereof. In yet further embodiments, the BTK inhibitor is ibrutinib. In particular embodiments, the HDAC6 inhibitor is ACY-1215 and the BTK inhibitor is ibrutinib.

In some embodiments, the lymphoma is selected from the group comprising of small lymphocytic lymphoma, lymphoplasmacytic B cell lymphoma, Waldenström macroglobulinemia, splenic marginal zone lymphoma, plasmacytoma, extranodal marginal

zone B cell lymphoma, MALT lymphoma, nodal marginal zone B cell lymphoma (NMZL), follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma (DLBCL), mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt lymphoma, chronic lymphocytic lymphoma, classical Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, adult T cell lymphoma, nasal type extranodal NK/T cell lymphoma, enteropathy-type T cell lymphoma, hepatosplenic T cell lymphoma, blastic NK cell lymphoma, mycosis fungoide, Sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, angioimmunoblastic T cell lymphoma, unspecified peripheral T cell lymphoma, and anaplastic large cell lymphoma.

In certain embodiments, the lymphoma is selected from the group consisting of DLBCL, mantle cell lymphoma, chronic lymphocytic lymphoma, lymphoplasmacytic B-cell lymphoma, and nodal marginal zone lymphoma. In yet further embodiments, the lymphoma is diffuse large B-cell lymphoma (DLBCL).

In some embodiments, the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN. In certain embodiments, the lymphoma exhibits down-regulation of SH3BP5 and LCK. In further embodiments, the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.

In some aspects, the present invention relates to a method of selecting a patient diagnosed with lymphoma for a method of treatment comprising administering to a patient a therapeutically effective amount of (i) an HDAC6 inhibitor and (ii) a BTK inhibitor, wherein the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN. In certain aspects, the lymphoma exhibits down-regulation of SH3BP5 and LCK. In further aspects, the patient's lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-D are graphs showing the development and characterization of selective HDAC6 inhibitor resistant cell line. The DLBCL cell line, OCI-LY10 was exposed to increasing concentrations of ACY-1215 over time. Fig. 1A are graphs showing the concentration effect relationships established for resistant and parental Ly10 at 48 and 72 hours following exposure to ACY-1215. The resistant line has a 10-fold higher IC50. The 48 hour concentration effect relationship of increasing concentrations of ACY-1215 in the

resistant line are shown after immediate exposure and after a wash-out period of 1 month. Resistance is maintained over time. The concentration effect relationship was determined with ACY-1215 alone or in combination with verapamil 20 μ M to inhibit efflux pumps. Resistance was maintained after 48 hour exposure in the presence of verapamil and is not
5 influenced by drug efflux pumps. Fig. 1B are graphs showing concentration effect relationships for resistant and parental Ly10 at 48 and 72 hours following exposure to: vorinostat, bortezomib, and romidepsin. The resistant line maintains resistance to vorinostat (potent HDAC6 inhibitor), bortezomib (inhibitor of protein degradation) but not romidepsin (potent HDAC 1, 2, 3 inhibitor). In Fig. 1C SCID-beige mice were injected with LY10 10^7 in
10 their flanks and treated with ACY-1215 at 50 mg/kg on days 1-5, 8-12, 15-19 via the intraperitoneal route. The resistant cell line maintained resistance following xenograft. Treatment with ACY-1215 had little effect on tumor growth. The tumor doubling time was calculated with the resistant cohort tumors growing the fastest. Treatment with ACY-1215 led to a significant tumor growth delay in the parental line only. Fig. 1D is the Kaplan Meier
15 Curve calculated for the resistant control mice as compared to the resistant ACY-1215 mice, parental control and treatment cohorts. Survival was shortest for the resistant cohorts and longest in the ACY-1215 treated parental cohort.

Figures 2A-D are graphs and immunoblots showing that the IRE-1/XBP-1 pathway is upregulated in cells resistant to ACY-1215. Fig. 2A is a Western blot analysis of a panel of
20 lymphoma cell lines for Bcl2 and Bim, which was compared to the IC50 in these cell lines. The Bcl2:Bim ratio correlated with IC50 with cell lines relative low Bcl2:Bim ratios demonstrating higher sensitivity to ACY-1215. Fig. 2B is a scatter plot showing mitochondrial membrane potential measured following 48 hour exposure of cells to ACY-1215 2.5 μ M via flow cytometry. Apoptosis was not induced in the resistant line following
25 exposure as compared to parental cells. Fig. 2C are bar graphs illustrating BH3 profiling performed on the parental and resistant cell lines. There was no difference in the cellular dependence on anti-apoptotic proteins for evasion of cell death. Fig. 2D are Western blots illustrating the baseline characteristics of the resistant line compared to the parental line with respect to the unfolded protein response (UPR). The IRE-1/XBP-1 pathway but not the
30 PERK pathway was upregulated.

Figures 3A-E are graphical representations and blots showing that the resistant cell line has a differentially expressed gene profile as compared to parental line and demonstrates that the BCR pathway is upregulated in resistant cells. Fig. 3A is a heat map showing the

evaluations of the resistant (R) cells and parental cells (P) by RNA Seq for gene expression. The two cells lines were distinct as demonstrated by principle component analysis. The heat map represents the top 100 genes with significant overall 2-log fold change between the resistant and parental lines. Fig. 3B shows the gene set enrichment analysis (GSEA) for comparing enrichment of pathways in resistant verses parental lines. Pathways of the B-cell receptor pathway were upregulated in the resistant line. Fig. 3C are immunoblot images of differentially expressed genes of interest, which were confirmed via PCR for the resistant line as compared to the parental line. Fig. 3D are immunoblots illustrating protein expression of genes of interest. Fig. 3E is a graph showing the concentration effect relationships of ibrutinib in resistant and parental lines overlap with similar IC50 values at 48 and 72 hours.

Figures 4A-C are heat maps and expression analyses illustrating that ibrutinib plus ACY-1215 is synergistic in cell lines and primary human lymphoma samples. Fig. 4A is a heat map representing the viability of a panel of cell lines following treatment with ACY-1215, ibrutinib or the combination at 24, 48, and 72 hours. Red boxes indicate lower viability. The combination is synergistic in ABC-DLBCL and MCL but not in T-cell lymphoma as represented by a heat map representing synergy co-efficients. Synergy was calculated by the relative risk ratio (RRR). $RRR < 1$ connotes synergy and is represented by red boxes. Fig. 4B are viability and synergy heat maps of primary human lymphoma samples, chronic lymphocytic leukemia (CLL), lymphoplasmacytic lymphoma (LPL), and 17p deleted nodal marginal zone lymphoma (MZL), treated with ACY-1215, ibrutinib or the combination over 24 to 96 hours. Viability was measured and synergy was calculated by RRR and represented in the heat maps. Synergy was observed across all subtypes of lymphoma. Fig. 4C are immunoblots showing that treatment with the combination of ibrutinib and ACY-1215 led to modulation of the IRE-1 and BTK pathways.

Figures 5A-D are graphs and blots showing that the combination of ACY-1215 and ibrutinib leads to statistically significant tumor growth delay compared to single agent treatment in a xenograft mouse model of lymphoma. Fig. 5A is a schematic representation showing a treatment regimen of SCID-beige mice injected with LY10 10⁷ in their flanks and treated with ACY-1215 50 mg/kg days 1-5, 8-12, 15-19, ibrutinib 3 mg/kg days 1-20, or the combination via the intraperitoneal route. Fig. 5B is a graph of weight (g) over time (days) of animals, where mice were weighed every 3-4 days as a measurement for toxicity. The combination was well tolerated with mice treated with the combination having similar weight loss to those treated with ACY-1215 alone. Weights returned back to baseline by day 20.

Fig. 5C is a graph of tumor volume over time showing that treatment with the combination of ACY-1215 and ibrutinib led to a significant tumor growth delay as compared to either agent alone or control ($p < 0.0006$). Fig. 5D is a graph showing the Kaplan Meier Curve calculated for the resistant control mice as compared to the parental control and treatment cohorts. Survival was shortest for the resistant cohort and longest in the combination cohort.

Figures 6A-D are graphical illustrations and immunoblots showing pharmacokinetic and pharmacodynamics effects of ACY-1215 in combination with ibrutinib in mice. Serum and tumor tissue was collected from mice at sequential time points and analyzed for concentration of ACY-1215 and ibrutinib by LC-MS/MS. Mice were treated with ACY-1215 at 50 mg/kg alone and ACY-1215 50 mg/kg with ibrutinib 3 mg/kg. Drug concentrations are represented as mean values. Fig. 6A is a graph of the ACY-1215 concentration over time analyzed in serum and tumor tissue. Mice received one dose of ACY-1215 50 mg/kg or ACY-1215 50 mg/kg plus ibrutinib 3 mg/kg for analysis of serum concentration of ACY-1215. For analysis of drug concentration in tumor tissue, ACY-1215 was administered at 50 mg/kg with or without ibrutinib. Fig. 6B are graphs of ibrutinib concentration over time analyzed in serum and tumor tissue. Mice received one dose of ibrutinib 3 mg/kg via i.p. route. Fig. 6C is a chart summarizing the pharmacokinetic data for ACY-1215 and ibrutinib. Fig. 6D is immunoblot analysis of the IRE1 pathway of the UPR and the BTK pathway from whole cell lysates of mouse tumor tissue treated with ACY-1215, ibrutinib or the combination. Mice were treated with a single i.p. injection and analyzed at 6 hours.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a mammalian cancer cell line developed to be resistant to HDAC6 inhibition. In a particular embodiment, the present invention concerns the diffuse large B-cell lymphoma cell line resistant to HDAC6 inhibitor. In a further embodiment, the present invention concerns the diffuse large B-cell lymphoma (DLBCL) cell line resistant to HDAC6 inhibitor ACY-1215.

The present invention also provides methods of treatment of lymphoma comprising administering to a patient, a therapeutically effective amount of HDAC6 inhibitor and a BTK inhibitor. In one aspect, the present invention provides methods of treatment of lymphoma comprising administering to a patient, a therapeutically effective amount of the HDAC6 inhibitor ACY-1215 and a BTK inhibitor. The BTK inhibitor may be any one of the following: ibrutinib, HM-71224, BGB-3111, CG-036806, CC-292, ACP-196, GDC-0834,

ONO-4049, RN-486, SNS-062, TAS-5567, AVL-101, AVL-291, PCI- 45261, HCI-1684, or PLS-123. More specifically, the BTK inhibitor is ibrutinib. Thus, in a more specific aspect, the present invention provides methods of treatment of lymphoma comprising administering to a patient, a therapeutically effective amount of an HDAC6 inhibitor and ibrutinib.

5 In particular embodiments, the present invention provides methods of treatment of lymphoma comprising administering to a patient, a therapeutically effective amount of the HDAC6 inhibitor ACY-1215 and ibrutinib.

 In further aspects, the present invention provides methods of treatment of lymphoma, wherein lymphoma is diffuse large B-cell lymphoma. Thus, in particular aspects, the present invention provides methods of treatment of diffuse large B-cell lymphoma comprising
10 administering to a patient, a therapeutically effective amount of HDAC6 inhibitor and a BTK inhibitor. An example of an HDAC6 inhibitor is ACY-1215. An example of BTK inhibitor is ibrutinib. Thus, in particular embodiments, the present invention provides methods of treatment of diffuse large B-cell lymphoma comprising administering to a patient, a
15 therapeutically effective amount of the HDAC6 inhibitor ACY-1215 and ibrutinib.

 The present invention provides a combination therapy for treating various types of lymphoma. Particularly, the present invention provides compositions and methods combining an effective amount of HDAC6 inhibitor and a BTK inhibitor. In some embodiments, the combination therapy demonstrates significant enhancement of the anti-tumor effect compared
20 to either agent alone. In particular embodiments, the enhancement of the anti-tumor effect is synergistic. An example of HDAC6 inhibitor is ACY-1215. An example of BTK inhibitor is ibrutinib. Thus, in particular embodiments, the present invention provides compositions and methods combining an effective amount of the HDAC6 inhibitor ACY-1215 and ibrutinib.

25 **Definitions**

 As used herein, the terms “patient” or “subject” are used interchangeably and mean a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline. Preferably, the patient is a human.

 The term “lymphoma” as used herein is a cancer of lymphatic cells of the immune
30 system. Lymphomas typically present as a solid tumor. Exemplary lymphomas include: small lymphocytic lymphoma, lymphoplasmacytic B-cell lymphoma, Waldenström macroglobulinemia, splenic marginal zone lymphoma, plasmacytoma, extranodal marginal zone B cell lymphoma, MALT lymphoma, nodal marginal zone B cell lymphoma (NMZL),

follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma (DLBCL), mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt lymphoma, chronic lymphocytic lymphoma, classical Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, adult T cell lymphoma, nasal type extranodal NK/T cell lymphoma, enteropathy-type T cell lymphoma, hepatosplenic T cell lymphoma, blastic NK cell lymphoma, mycosis fungoide, Sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, angioimmunoblastic T cell lymphoma, unspecified peripheral T cell lymphoma, and anaplastic large cell lymphoma.

As used herein, the terms “reduce or inhibit” refer to the ability to cause an overall decrease of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or greater. Reduce or inhibit can refer to the symptoms of the disorder being treated, the presence or size of metastases, or the size of the primary tumor.

As used herein, the phrases “treating cancer” and “treatment of cancer” and “treatment of tumors” mean to decrease, reduce, or inhibit the replication of cancer cells; decrease, reduce or inhibit the spread (formation of metastases) of cancer; decrease tumor size; decrease the number of tumors (i.e. reduce tumor burden); lessen or reduce the number of cancerous cells in the body; prevent recurrence of cancer after surgical removal or other anti-cancer therapies; or ameliorate or alleviate the symptoms of the disease caused by the cancer.

As used herein, the term “synergistic” refers to an interaction of an HDAC6 inhibitor and a BTK inhibitor, wherein the observed effect (e.g., reduction of tumor volume) in the presence of the combination of compounds together is higher than the sum of the individual effects of each compound administered separately. In one embodiment, the observed combined effect of the compounds is significantly higher than the sum of the individual effects.

As used herein, the term “therapeutically effective” means that the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

As used herein, the term “a Bruton's Tyrosine Kinase (BTK) inhibitor” refers to a compound which targets, decreases or inhibits human and murine B cell development. Examples of a BTK inhibitor include, but are not limited to, ibrutinib, HM-71224, BGB-

3111, CG-036806, CC-292, ACP- 196, GDC-0834, ONO-4049, RN-486, SNS-062, TAS-5567, AVL-101, AVL-291, PCI- 45261, HCI-1684 and PLS-123.

As used herein, the term “HDAC6 specific” means that the compound binds to HDAC6 to a substantially greater extent, such as 5X, 10X, 15X, 20X greater or more, than to any other type of HDAC enzyme, such as HDAC1 or HDAC2. That is, the compound is selective for HDAC6 over any other type of HDAC enzyme.

HDAC6 inhibitor ACY-1215 (See U.S. Patent Nos. 8,148,526 and 8,609,678) is a specific inhibitor of the Class IIB histone deacetylase enzyme, HDAC6. Inhibition of HDAC6 versus other isoforms uniquely preserves normal gene expression in cells, thereby minimizing patient toxicity. At the same time, HDAC6 inhibition severely disrupts diseased cells’ ability to produce normal proteins through disruption of the HSP-90 protein chaperone system, and to dispose of damaged misfolded proteins through modification of microtubules and disruption of the aggresome protein disposal pathway. Metabolically active cancer and autoimmune cells produce large amounts of misfolded proteins and inhibition of HDAC6 further increases the generation and accumulation of protein “trash”, triggering self-destruction of diseased cells via programmed cell death and leading to regression of disease.

Vorinostat, also known as suberanilohydroxamic acid (suberoyl+anilide+hydroxamic acid abbreviated as SAHA) is a member of a larger class of compounds that inhibit histone deacetylases (HDAC). Vorinostat is marketed under the name Zolinza by Merck for the treatment of cutaneous manifestations in patients with cutaneous T cell lymphoma (CTCL) when the disease persists, gets worse, or comes back during or after two systemic therapies (See: U.S. Patent Nos: 7,399,787; 7,456,218; and 8,067,472).

As used herein, the expressions “cell,” “cell line,” and “cell culture” are used interchangeably and all such designations include progeny. In particular, by progeny is also intended cell clones obtained by limit dilution of the cell lines of the invention. As certain modifications may occur in succeeding generations due to mutation or environmental influences, or clonal selection, such progeny may not be identical to the parent cell, but is still included within the scope of the term as used herein.

HDAC6 Resistant Lymphoma Cell Line

A lymphoma cell line was developed to be resistant to the HDAC6 inhibition. More specifically, the diffuse large B-cell lymphoma cell line OCI-Ly10 was developed to be resistant to the HDAC6 selective inhibitor ACY-1215 (Example 1). This resistant cell line

served as a tool to gain insights into the role of HDAC6 in lymphoma and uncover novel pathways that may be synergistic with ACY-1215.

Thus, the present invention provides a diffuse large B-cell lymphoma (DLBCL) cell line resistant to the HDAC6 specific inhibition. More specifically, diffuse large B-cell lymphoma cell line described herein is resistant to HDAC6 specific inhibitor ACY-1215, wherein the cell line is referred to as R10-OCI-LY10. Additionally, R10-OCI-LY10 was shown to be cross resistant to other HDAC6 inhibitors. For example, as shown in Example 1 (Figure 1B), R10-OCI-LY10 is resistant to vorinostat, which strongly inhibits HDAC6. Thus, the cell line described herein can be used to study the resistance to HDAC6 inhibition broadly, and is not limited to ACY-1215 resistance. Given the findings described herein, in certain embodiments, the present invention relates to a cell line (R10-OCI-LY10) resistant to HDAC6 inhibitors including ACY-1215 and vorinostat.

In addition to being resistant to HDAC6 inhibition, the cell line of the present invention showed partial resistance to bortezomib (also known as Velcade, MG-341, and PS-341), a proteasome inhibitor which effectively inhibits proteasome activity (Example 1). This is likely due to the fact that both drug mechanisms (HDAC6 inhibition and proteasome inhibition) converge on the processing of misfolded proteins. Thus, the cell line described herein (R10-OCI-LY10) is also at least partially resistant to proteasome inhibitors.

The HDAC6 inhibition resistant cell line described herein exhibits an IC₅₀ of 10 μ M (for ACY-1215) as compared to parental IC₅₀ of 0.9 μ M. Importantly, the resistance is maintained even in the absence of drug (ACY-1215).

The resistant cell line described herein can be used as a drug discovery tool to identify novel compounds that can be used to treat lymphomas as well as to identify rational and synergistic drug:drug combinations. Furthermore, comparing the parental and resistant cell lines as described herein can be useful for determining or identifying biomarkers for resistance to the cytotoxic drug, such as an HDAC6 inhibitor.

The ACY-1215 resistant lymphoma cell line of the present invention is also useful for screening potential therapeutic agents. Accordingly, the present invention concerns a kit for screening potential therapeutic agents comprising a lymphoma cell line of the invention. The kit can further comprise a culture medium suitable for the diffuse large B-cell lymphoma cell line. In a particular embodiment, the kit can comprise both the parental OCI-Ly10 and ACY-1215 resistant R10-OCI-LY10 line. The present invention also relates to the use of a

DLBCL line of the invention or a combination thereof for screening potential therapeutic agents.

The potential therapeutic agents can be a molecule having a cytotoxic or cytostatic effect of the cells. Alternatively, the potential therapeutic agents can be a molecule increasing the efficacy of a drug, increasing or restoring the sensitivity of cells to a drug to which they are resistant, a molecule preventing, reducing, or delaying the appearance in cells of a resistance to a drug.

In one embodiment, the present invention relates to a method for determining whether a candidate agent inhibits proliferation of the resistant DLBCL line described herein. In another embodiment, the present invention relates to a method for determining whether a candidate agent increases the sensitivity to a cytotoxic drug of the resistant DLBCL line described herein. In yet another embodiment, the present invention relates to a method for determining whether a candidate agent is able to prevent, reduce, or delay the appearance of resistance to a cytotoxic drug in the resistant DLBCL line of the present invention.

The development and characterization of ACY-1215-resistant cell line of the present invention can be used as a tool to understand the functional role of HDAC6 in lymphoma.

Baseline characteristics of the resistant line were compared to the parental line with respect to the unfolded protein response (UPR), which showed upregulation of the IRE-1/XBP-1 pathway (Example 2). Furthermore, gene expression profiling of the resistant line was compared to the parental line and revealed increased expression of pathways known to drive lymphomagenesis of activated B-cell (ABC) lymphoma such as the BTK pathway (Example 3). In addition to changes in the UPR, differentially expressed genes in the resistant line included up-regulation of MAPK10, HELIOS, HDAC9 and FYN, as well as down-regulation of SH3BP5 (a negative regulator of BTK) and LCK.

HDAC6 Inhibitors

Histone deacetylases (HDACs) are a family of 18 enzymes that play diverse roles in mammalian cell homeostasis and in tumor growth. For some time, 'broad-spectrum' HDAC inhibitors have been available clinically for the treatment of certain uncommon malignancies. More recently, drug discovery efforts have focused on the development of isoform-specific inhibitors, more specifically on the development of HDAC6 inhibitors due to its unique functions within the cell. HDAC6 has been implicated in the modulation of immune responses (Serrador et al, Immunity 20:417-428 (2004); Kalin et al, J Med Chem. (2012)).

The combinations and methods of the present invention comprise a histone deacetylase (HDAC) 6 inhibitor. A variety of HDAC6 inhibitors have been investigated (Butler et al, "Rational Design and Simple Chemistry Yield a Superior, Neuroprotective HDAC6 Inhibitor, Tubastatin A," J Am Chem Soc 2010, 132(31): 10842-10846; Kalin et al, "Second-Generation Histone Deacetylase 6 Inhibitors Enhance the Immunosuppressive Effects of Foxp3+ T-Regulatory Cells," J Med Chem 2012, 55(2):639-651). Non-limiting examples include rocilinostat (ACY- 1215), ACY-241, Tubacin, Tubastatin A, ST-3- 06, ST-2-92, Nexturastat A, and Nexturastat B. Batchu et al. provide a detailed discussion regarding HDAC6 inhibitors (Clinical Science (2016) 130, 987–1003).

Thus, in one embodiment, the HDAC6 inhibitor of the present invention is selected from the group comprising ACY-1215, ACY-241, Tubacin, Tubastatin A, ST-3-06, ST-2-92, Nexturastat A, and Nexturastat B.

BTK inhibitors

Bruton's tyrosine kinase (BTK), a member of the Tec family of cytoplasmic tyrosine kinases, is involved in multiple signal-transduction pathways regulating survival, activation, proliferation, and differentiation of B-lineage lymphoid cells.

There are a number of BTK inhibitors in the clinic. One molecule currently approved is ibrutinib (1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one disclosed in WO2008/039218).

In some embodiments, the BTK inhibitor is selected from ibrutinib, PCI-45292, PCI-45466, AVL-101/CC-101 (Avila Therapeutics/Celgene Corporation), AVL-263/CC-263 (Avila Therapeutics/Celgene Corporation), AVL-292/CC-292 (Avila Therapeutics/Celgene Corporation), AVL-291/CC-291 (Avila Therapeutics/Celgene Corporation), CNX 774 (Avila Therapeutics), BMS-488516 (Bristol-Myers Squibb), BMS-509744 (Bristol-Myers Squibb), CGI-1746 (CGI Pharma/Gilead Sciences), CGI-560 (CGI Pharma/Gilead Sciences), CTA-056, GDC-0834 (Genentech), HY-11066 (also, CTK4I7891, HMS3265G21, HMS3265G22, HMS3265H21, HMS3265H22, 439574-61-5, AG-F-54930), ONO-4059 (Ono Pharmaceutical Co., Ltd.), ONO-WG37 (Ono Pharmaceutical Co., Ltd.), PLS-123 (Peking University), RN486 (Hoffmann-La Roche), HM71224 (Hanmi Pharmaceutical Company Limited) or LFM-A13. In some embodiments, the BTK inhibitor is ibrutinib.

HDAC6 and BTK Inhibition Therapy

Gene expression profiling of the resistant line was compared to the parental line and revealed modulation of the B-cell receptor (BCR) pathway such as down-regulation of the

negative regulator of BTK (SH3BP5). This discovery led to the evaluation of the combination of ACY-1215 with ibrutinib, a first in class BTK inhibitor, which demonstrated strong synergy. This synergy was demonstrated in the parental DLBCL line, mantle cell lymphoma cell lines, primary human lymphoma samples, and an *in vivo* murine xenograft model of lymphoma (Examples 4 and 5).

The combination therapy described herein can be used for the treatment of lymphoma. Non-limiting examples of preferred types of cancers/tumors for treatment include small lymphocytic lymphoma, lymphoplasmacytic B-cell lymphoma, Waldenström macroglobulinemia, splenic marginal zone lymphoma, plasmacytoma, extranodal marginal zone B cell lymphoma, MALT lymphoma, nodal marginal zone B cell lymphoma (NMZL), follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma (DLBCL), mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt lymphoma, chronic lymphocytic lymphoma (CLL), classical Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, adult T cell lymphoma, nasal type extranodal NK/T cell lymphoma, enteropathy-type T cell lymphoma, hepatosplenic T cell lymphoma, blastic NK cell lymphoma, mycosis fungoide, Sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, angioimmunoblastic T cell lymphoma, unspecified peripheral T cell lymphoma, and anaplastic large cell lymphoma. Additionally, the present disclosure includes refractory or recurrent malignancies whose growth may be inhibited using the combinations described herein.

As shown in Example 4, synergy between ACY-1215 and ibrutinib was shown in DLBCL line, mantle cell lymphoma cell lines, as well as primary patient samples. In patient samples, the inventors observed synergy in various types of lymphoma, including chronic lymphocytic lymphoma (CLL), lymphoplasmacytic B-cell lymphoma (LPL), and nodal marginal zone lymphoma (MZL). Thus, in one embodiment, the combination therapy described in the present disclosure can be used for the treatment of DLBCL, mantle cell lymphoma, chronic lymphocytic lymphoma, lymphoplasmacytic B-cell lymphoma, and nodal marginal zone lymphoma.

The combination therapy described in the present invention is particularly advantageous, since not only the anti-tumor effect is enhanced compared to the effect of each compound alone, but it is anticipated that the dosage of each agent in a combination therapy can be reduced as compared to monotherapy with each agent, while still achieving an overall

anti-tumor effect. In addition, due to the synergistic effect, the total amount of compounds administered to a patient can advantageously be reduced, which may result in decreased side effects.

It has been observed that patients relapsing off of ibrutinib have highly aggressive and often explosive disease. The methods of the present invention provide that combining ibrutinib with ACY-1215 is a potent synergy and that this combination is effective at inhibiting the most aggressive subtypes of lymphoma.

Furthermore, the present invention relates to a pharmaceutical composition comprising a therapeutically effective amounts of (i) an HDAC6 inhibitor, or a pharmaceutically acceptable salt thereof, and (ii) a BTK inhibitor or a pharmaceutically acceptable salt thereof. In certain embodiments, the HDAC6 inhibitor is ACY-1215. In some embodiments, the BTK inhibitor is ibrutinib.

As discussed above, it was shown that the resistant line exhibits up-regulation of MAPK10, HELIOS, HDAC9 and FYN, as well as down-regulation of SH3BP5 and LCK. These molecular characteristics can be used for the selection of patients suffering from lymphoma that are likely to respond to combination therapy described herein.

Pharmaceutical Compositions and Administration

The compounds disclosed herein can be formulated according to known methods for preparing pharmaceutically acceptable compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, Remington 's Pharmaceutical Science by E.W. Martin (1995) describes formulations that can be used in connection with the disclosed methods. In general, the compounds disclosed herein can be formulated such that an effective amount of the compound is combined with a suitable carrier in order to facilitate effective administration of the compound. The compositions used can also be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspension, suppositories, injectable and infusible solutions, and sprays. The preferred form depends on the intended mode of administration and therapeutic application. The compositions also preferably include conventional pharmaceutically-acceptable carriers and diluents which are known to those skilled in the art. Examples of carriers or diluents for use with the compounds include ethanol, dimethyl sulfoxide, glycerol, alumina, starch, saline, and equivalent carriers and diluents. To provide for the administration of such dosages for the desired therapeutic treatment, compositions disclosed herein can advantageously comprise

between about 0.1% and 99%, and especially, 1 and 15% by weight of the total of one or more of the subject compounds based on the weight of the total composition including carrier or diluent.

Formulations suitable for administration include, for example, aqueous sterile
5 injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze dried (lyophilized) condition requiring only
10 the condition of the sterile liquid carrier, for example, water for injections, prior to use.

The various components of the combination therapy described in the present disclosure may be administered concurrently with, or separately from, other components in a treatment regimen. For the treatment of oncological disorders, the compounds disclosed herein can be administered to a patient in need of treatment in combination with other anti-
15 tumor or antic-ancer substances and/or with radiation and/or photodynamic therapy and/or with surgical treatment to remove a tumor. These other substances or treatments can be given at the same as or at different times from the compounds disclosed herein.

The components of the combination therapy described herein can be administered simultaneously, separately or sequentially. In some embodiments, the HDAC6 inhibitor
20 (ACY-1215) is administered simultaneously with BTK inhibitor (ibrutinib). Simultaneous administration typically means that both compounds enter the patient at precisely the same time. However, simultaneous administration also includes the possibility that the HDAC6 inhibitor and BTK inhibitor enter the patient at different times, but the difference in time is sufficiently short that the first administered compound is not provided the time to take effect
25 on the patient before entry of the second administered compound. Such delayed times typically correspond to less than 1 minute, and more typically, less than 30 seconds. In one example, wherein the compounds are in solution, simultaneous administration can be achieved by administering a solution containing the combination of compounds. In another example, simultaneous administration of separate solutions, one of which contains the
30 HDAC6 inhibitor (ACY-1215) and the other of which contains BTK inhibitor (ibrutinib) can be employed. In one example wherein the compounds are in solid form, simultaneous administration can be achieved by administering a composition containing the combination of compounds. Alternatively, simultaneous administration can be achieved by administering two

separate compositions, one comprising the HDAC6 inhibitor (ACY-1215) and the other comprising BTK inhibitor (ibrutinib).

In other embodiments, the HDAC6 inhibitor and the BTK inhibitor are not administered simultaneously. In some embodiments, the HDAC inhibitor (ACY-1215) is administered before the BTK inhibitor (ibrutinib). In other embodiments, the BTK inhibitor (ibrutinib) is administered before the HDAC6 inhibitor (ACY-1215). The time difference in non-simultaneous administrations can be greater than 1 minute, five minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, two hours, three hours, six hours, nine hours, 12 hours, 24 hours, 36 hours, 48, Or 72 hours. In other embodiments, the first administered compound is provided time to take effect on the patient before the second administered compound is administered. Generally, the difference in time does not extend beyond the time for the first administered compound to complete its effect in the patient, or beyond the time the first administered compound is completely or substantially eliminated or deactivated in the patient.

In general, components of the combination therapy of the invention can be administered in therapeutically effective amounts via any of the usual and acceptable modes known in the art. A therapeutically effective amount may vary widely depending on the severity of the disease, the age and relative health of the subject, the potency of the compound used and other factors.

In certain embodiments, the amount of the HDAC6 inhibitor that is administered may range from about 0.1 mg/kg to about 500 mg/kg (about 0.18 mg/m² to about 900 mg/m²), alternatively from about 1 to about 50 mg/kg (about 1.8 to about 90 mg/m²). In general, treatment regimens according to the present invention comprise administration to a patient in need of such treatment from about 10 mg to about 1000 mg of the HDAC6 inhibitor per day in single or multiple doses. Therapeutic amounts or doses can vary depending on route of administration, as well as the possibility of co-usage with other agents.

ACY-1215 is an oral HDAC6 inhibitor. Multiple clinical trials on the use of ACY-1215 are currently conducted, and the information obtained from clinical trials can be used as a guide for dosage and frequency of administration. In certain instances, ACY-1215 160 mg can be administered orally on a once or twice daily basis as a single agent. In other instances, ACY-1215 can be administered in combination with several additional anti-cancer agents. When given in combination, ACY-1215 is typically administered once daily at doses ranging from 80 mg to 480 mg. Examples of suitable additional anti-cancer agents include: nab-

paclitaxel, paclitaxel, lenalidomide, pomalidomide, dexamethasone, and bortezomib. In some instances, patients receiving combination therapy are treated WITH acy-1215 continuously or on a 1-21 day cycle out of 28 day treatment schedule.

In some embodiments, the amount of a BTK inhibitor that is administered is from 10
5 mg/day up to, and including, 1000 mg/day. In some embodiments, the amount of a BTK inhibitor that is administered is from about 40 mg/day to 900 mg/day, about 40 mg/day to 840 mg/day, about 80 mg/day to 600 mg/day, about 100 mg/day to 500 mg/day, or about 140 mg/day to 420 mg/day. In some embodiments, the amount of a BTK inhibitor that is administered per day is about 10 mg, about 11 mg, about 12 mg, about 13 mg, about 14 mg,
10 about 15 mg, about 16 mg, about 17 mg, about 18 mg, about 19 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 110 mg, about 120 mg, about 125 mg, about 130 mg, about 135 mg, about 140 mg, about 180 mg, about 220 mg, about 260 mg, about 300 mg,
15 about 350 mg, about 400 mg, about 420 mg, or about 840 mg.

In some embodiments, the amount of ibrutinib that is administered is from 10 mg/day up to, and including, 1000 mg/day. In some embodiments, the amount of Ibrutinib that is administered is from about 40 mg/day to 900 mg/day, about 40 mg/day to 840 mg/day, about 80 mg/day to 600 mg/day, about 100 mg/day to 500 mg/day, or about 140 mg/day to 420
20 mg/day. In some embodiments, the amount of Ibrutinib that is administered per day is about 10 mg, about 11 mg, about 12 mg, about 13 mg, about 14 mg, about 15 mg, about 16 mg, about 17 mg, about 18 mg, about 19 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg,
25 about 110 mg, about 120 mg, about 125 mg, about 130 mg, about 135 mg, about 140 mg, about 180 mg, about 220 mg, about 260 mg, about 300 mg, about 350 mg, about 400 mg, about 420 mg, or about 840 mg. In some embodiments, the amount of ibrutinib that is administered is about 40 mg/day. In some embodiments, the amount of ibrutinib that is administered is about 50 mg/day. In some embodiments, the amount of ibrutinib that is
30 administered is about 60 mg/day. In some embodiments, the amount of ibrutinib that is administered is about 70 mg/day. In some embodiments, the amount of ibrutinib that is administered is about 420 mg/day. In some embodiments, the amount of ibrutinib that is administered is about 840 mg/day.

EXAMPLES

Materials and Methods

Drugs and Reagents

ACY-1215 was provided by Acetylon Pharmaceuticals, Inc. (Boston, MA). Bortezomib, ibrutinib, romidepsin, verapamil and vorinostat were obtained from Selleck Chemicals (Houston, TX). All drugs were diluted in DMSO. Deuterated internal standard ibrutinib-d5 was purchased from TLC Pharmaceutical Standards Ltd (Ontario, Canada). All solvents and other chemicals for sample extraction were LCMS grade.

Cell lines and culture

Hbl-1, OCI-Ly10, and Riva are activated B-cell (ABC) DLBCL cell lines; OCI-Ly1, OCI-Ly7, Su-DHL-6 are germinal center (GC) DLBCL cell lines; JVM2, MAVER, Hbl-2, Jeko-1, and Rec-1 are mantle cell lymphoma (MCL) cell lines; HH and H9 are T-cell lymphoma (TCL) cell lines. Su-DHL-6, HBL-1, Riva, HBL2, Jeko-1, Rec-1, MAVER, HH, and H9 were obtained from ATCC. OCI-Ly1, OCI-Ly7, and OCI-Ly10 were obtained from DSMZ. Su-DHL-6, Hbl-1, Hbl-2, HH, H9, Jeko-1, Jvm-2, MAVER, and Rec-1 were grown in RPMI (10% FBS). OCI-Ly7, OCI-Ly10 and Riva were grown in IMDM (10% FBS). All cell lines were authenticated and screened for mycoplasma using the ATCC/Promega STR Authentication Testing Kit and Lonza MycoAlert for mycoplasma testing. Primary patient lymphoma samples were collected on an approved IRB protocol. Peripheral blood was collected and peripheral blood mononuclear cells were extracted by Ficoll-Paque density gradient media and centrifugation.

BH3 Profiling

BH3 profiling was performed as previously described [19]. Briefly, whole cells were permeabilized using the detergent digitonin which selectively permeabilizes the cell membrane, but not the mitochondrial membrane. BH3 peptides (synthesized by the Tufts University Peptide Synthesis Core and Genscript) were administered to the cells in prescribed concentrations. Depolarization of the mitochondrial membrane potential was assessed by staining the cells with JC-1 dye in 384 well, black, flat bottom, non-treated plates. Following JC-1 exposure, the JC-1 emission was read on a GloMax (Promega) plate reader once every 5 minutes for 180 minutes to generate a kinetic trace of mitochondrial membrane potential over time.

Flow Cytometry/JC-1

FACS Calibur System was used to acquire the fluorescence signals ($\geq 1 \times 10^5$ events/sample); data was analyzed using Flowjo 8.8.6. Cells ($3 \times 10^5/\text{mL}$) were quantitated for apoptosis using Alex Fluor 488/Annexin V (Dead cell apoptosis kit Invitrogen #V13240) or for the determination of the transmembrane mitochondrial membrane potential ($\Delta\psi_m$) via staining with JC-1 1.3 $\mu\text{g}/\text{mL}$ JC-1 dye (Invitrogen, Carlsbad, CA) as previously described[17].

Western blotting

Western blotting (WB) was performed as previously described[17]. Antibodies used were as follows: anti-acetylated lysine, anti-GRP78/BiP, anti-HDAC6, anti-HDAC9, anti-PERK and p-PERK, anti-p-eif2 α , anti-IRE1 α , , anti-FYN, anti-eif2 α , anti-p-eif2 α , anti-BTK, anti-p-BTK, anti-PCL- γ 2, anti-p-PCL- γ 2, anti-CARD11, anti-ATF4, anti-AKT, anti-p-AKT, anti-CHOP, anti-Helios, anti- β -actin, anti-BIM, anti-Bcl2 (Cell Signaling Technology); anti-MAPK10, anti-p-IRE1 α and anti-SH3BP5 (Abcam); anti-XBP-1 (Santa Cruz). Densitometry analysis was performed on scanned images using ImageJ software (NIH).

Gene Expression Profiling

RNA was extracted from cells using the RNeasy minikit (Qiagen). RNA was quantified and assessed for integrity via Bioanalyzer 2100 (Agilent) for an RNA Integrity Number greater than 8 for RNA sequencing by the Columbia Genome Center. mRNA was enriched from total RNA samples using poly-A pull down and libraries were prepared using the TruSeq RNA prep kit(Illumina). Sample derived libraries were then sequenced using the HiSeq2500 platform (Illumina).

Base calling was performed using Real Time Analysis (Illumina) and bcl2fastq(v 1.8.4) for converting BCL to fastq format. Reads were then mapped to a reference genome (Human:NCBI/build 37.2) using TopHat (v2.0.4). Relative abundance was estimated using Cufflinks (version 2.0.2). Differentially expressed genes were tested for using the R package DESeq2.

Gene set enrichment analysis was performed using a pre-ranked tool from the Broad Institute's GSEA software[20]. The metric of $-\log_{10}$ of the p value*sign of the log fold change was used to rank the genes. Pathway analysis on this pre-ranked list of genes was then performed using Gene Ontology and Lymphoid biology gene sets from the Staudt lab[21].

Semi-Quantitative PCR

Semi-quantitative Multiplex RT-PCR was performed as previously described [22]. Reverse transcription (RT) was performed in a 20 μL reaction system with a total of 2 mg of

RNase free DNase treated RNA (Omniscript RT kit, Qiagen) and oligo-d(T). Multiplex PCR reactions were run in 30 cycles. Primers (Fisher Scientific) were designed to span multiple exons of the target genes. Primer sequences can be found in Table 1. Images were analyzed using the ImageJ software (NIH) and band densities were normalized to GAPDH.

5

Table 1: Primers Used for semi-quantitative RT-PCR

Gene		Forward		Reverse
MAPK10	SEQ: 1	5'-CGGACTCCGAGCACAATAAA-3'	SEQ: 2	5'-GGAGCAGGTAGATGCAAGATAG-3'
SH3BP5	SEQ: 3	5'-ATGAGGGTAGGAGGGACTTATAG-3'	SEQ: 4	5'-CGTCACCACCTCGTCTTTATC-3'
FYN	SEQ: 5	5'-GGATTGGCCCGATTGATAGA-3'	SEQ: 6	5'-GGTTTCACTCTCGCGGATAAG-3'
HDAC9	SEQ: 7	5'-GAGGCAAGAACAGGAAGTAGAG-3'	SEQ: 8	5'-GGATGGAGATGTTCCACTAAGG-3'
IKZF2	SEQ: 9	5'-CACCCAACCACTCCCAAATA-3'	SEQ: 10	5'-CTGAGCCATATCCACCTCTTC-3'
HDAC6	SEQ: 11	5'-AGTGCCCGCATTATCCTTATC-3'	SEQ: 12	5'-GAGGGTCCTTCTCTGTCTTCTA-3'
LCK	SEQ: 13	5'-CTACGGGACATTCACCATCAA-3'	SEQ: 14	5'-CTCTCTGTGGTCTCAGGAAATG-3'

In Vivo Studies

Animals were housed and maintained in accordance with an IUCAC-approved protocol. OCI-Ly10 1×10^7 (either resistant or parental) in 50% Matrigel (BD Biosciences) were subcutaneously injected into the flanks of 5-7-week-old beige/SCID mice (Taconic Farms, INC, NY). Treatment was initiated when tumor volume measured 80 mm³. Tumors volume was assessed using the 2 largest perpendicular axes (l=length; w=width) and calculated using the formula $v=0.5(l^2 \times w)$. Mice were divided into 4 cohorts of 8-10 mice per cohort as follows: (1) untreated control; (2) ACY-1215: 50 mg/kg days 1-5, 8-12, 15-19; (3) ibrutinib: 3 mg/kg days 1-20; (4) ACY-1215 plus ibrutinib. Drugs were diluted in sterile dextrose 5% in water and were administered via the intraperitoneal (IP) route[23]. Mice were assessed for weight loss and tumor volume 3x/week. Animals were sacrificed when the tumor volume exceeded 2000 mm³ or after sustained loss of >10% body weight in accordance with institutional guidelines.

Pharmacokinetic/Pharmacodynamic *In Vivo* Studies

Mice were studied for pharmacokinetic and pharmacodynamic effects of ACY-1215 and ibrutinib. Approximately 250 μ L of blood was collected by sub-mandibular vein bleed at 0.5, 1, and 2 hours after treatment. Mice were sacrificed at 4, 6, and 8 hours after treatment; blood and tumor tissue were collected for measurement of drug concentration and WB analysis.

-MS/MS Analysis for Quantitation of ACY-1215 and Ibrutinib

ACY1215 and ibrutinib were measured in mouse serum and tumor tissues using ultra performance Liquid Chromatography-tandem Mass Spectrometry (LC-MSMS) after liquid-liquid extraction using deuterated ibrutinib as internal standard. Serum samples (100 μ L) or aqueous tissue homogenate containing 10 mg wet tissue disrupted by a Tissue Tearer homogenizer were spiked with deuterated internal standard at a level of 50 ng/mL. This was mixed with 100 μ L of acetonitrile, vortexed and incubated for 10 minutes followed by addition of 750 μ L of methyl tert-butyl ether. The mixture was vortexed for 10 minutes, centrifuged and the organic layer was transferred to a LCMS vial, evaporated under nitrogen stream and re-suspended in 50% methanol. Calibration standards and QC samples for both the compounds were prepared spanning a range of 0.5 ng/mL to 1000 ng/mL and extracted same as the samples.

LC-MSMS analysis was performed on a platform comprising Agilent 1290 Infinity UHPLC integrated to Agilent 6410 triple quad mass spectrometer controlled by MassHunter v 3.1 (Agilent Technologies, Santa Clara, CA). Chromatographic separation was performed on an Agilent Poroshell C18 column (50x2.1mm, 2.7 μ , 100A) maintained at 40°C. The flow rate was maintained at 500 μ L/min. The initial flow conditions were 50% solvent A (water containing 0.1% formic acid) and 50% solvent B (Methanol with 0.1% formic acid). Solvent B was raised to 70% over 1.75 min and to 95% by 1.95 min, held until 3.5 min and back to initial conditions by 4 min with a total run time of 6 min. The retention time for ACY-1215 and ibrutinib was 1.34 and 2.08 min respectively. The mass spectrometer was operated under multiple reaction monitoring (MRM) mode with positive electrospray ionization. For MRM, following transitions were utilized for quantitation: ACY1215 434.2>274.0; ibrutinib 441.2>304.1 and ibrutinib-d5 446.2>309.1. Mass spectrometer was operated using the following parameters: gas temperature, 300°C; gas flow, 13 L/minute; nebulizer, 30 psi; capillary 3 Kv; desolvation gas flow, 500 L/h; cone gas flow, 50 L/h and collision energy, 30 v.

Lower limit of quantification (LLOQ), defined as the lowest concentration with an accuracy and precision of <20% was determined to be 0.5 ng/mL for ACY1215 and 2.5 ng/mL for ibrutinib in serum. In tumor samples the LLOQ was determined to be 1ng/mL for both ACY1215 and 2.5ng/mL for ibrutinib. The intra-assay accuracy and precision for ACY1215 was 96.1% and 1.32% respectively while for ibrutinib, the intra-assay accuracy was 99.6% with a precision of 1.72%. The assay showed an inter-assay precision for ACY1215 0.90% and for ibrutinib 1.52%.

Statistical Analysis

For determination of the inhibitory concentration of 50% of cells (IC₅₀) and synergy, all experiments were run in triplicate and repeated at least twice. IC₅₀ was calculated with Calcsyn software (Biosoft, Cambridge, United Kingdom). Relative risk ratio (RRR) was used as a model for establishing synergy between 2 drugs[24]. RRR is based on calculating the ratio between the actual value and expected value (EV). In the case of 2 cytotoxic compounds, EV is calculated by the formula: $EV = (N_A \times N_B)/100$, where N_A represents the percentage of viable cells treated with drug A and N_B represents the percentage of viable cells treated with drug B. RRR<1 represents a synergistic effect; values equal to 1 indicate the mean additive effect; and values>1 represent an antagonistic effect. Flow cytometry assays were performed in duplicate, repeated at least twice, and reported as the mean with associated standard deviations.

In vivo statistical analysis was performed using Prism GraphPad's Two-Way ANOVA Analysis. Overall survival (OS) was measured using the Kaplan-Meier method, and presented as the mean OS with 95% confidence intervals. Area under the serum concentration time curve (AUC) and half-life were determined non-compartmentally using Phoenix Winnonlin software version 6.3 (Certara, St. Louis, MO). All drug concentrations are represented as the mean with the standard deviation where applicable.

EXAMPLE 1

The development of an ACY-1215 resistant cell line is stable and not dependent on efflux pumps.

The diffuse large B-cell lymphoma cell line OCI-Ly10 was exposed to increasing concentrations of ACY-1215 over the course of 1 year. Systematic incremental increases in drug exposure led to the development of a distinct cell line with an IC₅₀ value 10-20 fold greater than that of the parental line. The resistant R10-OCI-LY10 (R10) exhibited an IC₅₀

of 10 μM as compared to parental IC₅₀ of 0.9 μM after 48 hours of exposure (Fig.1A). Resistance was maintained after repeated passages for ≥ 1 month in the absence of drug and was not overcome by inhibition of efflux pumps as determined via verapamil co-exposure (Fig. 1A) [25]. Interestingly, the resistant line was cross resistant to vorinostat which strongly inhibits HDAC6 (R10 IC₅₀=not reached vs P IC₅₀=0.6 μM). Treatment of the resistant line with bortezomib demonstrated intermediate activity underscoring the notion that although their molecular targets are vastly different (*i.e.*, proteasome vs HDAC6), both drug mechanisms converge on the processing of misfolded proteins (R10 IC₅₀=8 nM vs P IC₅₀=3.3 nM). In contrast, the resistant line was sensitive to romidepsin which is known to predominantly inhibit HDAC1, 2 and 3 with minimal activity against HDAC6 (R10 IC₅₀=3 μM vs P IC₅₀=2.25 μM) (Fig. 1B) [26].

Both the resistant and parental LY10 cell lines were xenografted into the flanks of SCID beige mice at 10^7 cells. Mice were divided into 4 cohorts, resistant (R) control, parental (P) control, (R) ACY-1215, (P) ACY-1215. Mice were treated with ACY-1215 50 mg/kg days 1-5, 8-12, 15-19 by intraperitoneal route. The tumor volume doubling time was calculated for each cohort using GraphPad Prism software. The resistant cohorts exhibited accelerated tumor growth and decreased survival demonstrating that the resistant line maintains a highly aggressive phenotype over time *in vivo*. Treating the resistant mouse with ACY-1215 had no impact on the tumor doubling time as compared to the untreated resistant control mice (6.857 versus 7.004 days). In addition, the rate of growth was faster in the resistant control mouse cohort as compared to the parental control cohort (7.004 versus 8.039 days to doubling). The volumetric doubling time of (P) ACY-1215 treated tumors was more than twice as long compared to those of the (R) line treated with ACY-1215 (15.62 versus 6.857 days) (Fig. 1C). This translated into a shorter survival for both the resistant cohorts as compared to parental cohorts. Median survival in days was as follows for each cohort: resistant control (26.5) < resistant ACY-1215 (38) < parental control (43.5) < parental ACY-1215 (54) (Fig. 1D).

EXAMPLE 2

The IRE-1/XBP-1 pathway is upregulated in cells resistant to ACY-1215

Basal levels, Bcl2 and Bim were correlated to IC₅₀ of a panel of lymphoma cell lines. The Bcl2:Bim ratio correlated to sensitivity to ACY-1215 and cell lines with relatively high levels of Bcl2 and low levels of Bim were relatively resistant to ACY-1215 (Fig. 2A). Based on these findings, mitochondrial membrane depolarization was evaluated following treatment

with ACY-1215 in parental and resistant cells. Compared to the parental line the resistant line did not depolarize the mitochondrial membrane following treatment with ACY-1215 2.5 μ M, confirming resistance to induction of apoptosis (Fig. 2B). Next, whether resistance to apoptosis in the resistant cell line was due to alterations in the BCL2 family proteins, which are known to play critical roles in regulating apoptosis, was investigated. BH3 profiling is a functional assay which informs the cellular dependence on anti-apoptotic proteins for evasion of cell death [27]. There was no difference in the BH3 profiles of the resistant and parental cell lines (Fig. 2C).

Given the known influence of ACY-1215 on the unfolded protein response (UPR) [7, 8], evaluation of PERK and IRE-1 pathways were evaluated by immunoblot (Fig. 2D). The resistant line displayed relatively stable GRP78, the master regulator of the UPR. The GRP78- PERK pathway also remained relatively stable. The IRE-1 pathway however was upregulated with concomitant activation of XBP-1 and AKT as compared to the parental line perhaps driving accelerated growth in these ACY-1215 resistant cells.

EXAMPLE 3

Gene expression profiling reveals distinct modulation of the B-cell receptor pathway in the resistant cell line as compared to the parental cell line

Gene expression profiling was performed on both the resistant and parental cell lines. The parental and resistant cell lines had distinct gene expression signatures as represented by principal component analysis and as can be visualized on the heat map (Fig. 2A). The gene expression data were analyzed by GSEA and gene cluster analysis. There were 1363 genes from resistant line 2-log fold up-regulated as compared to the parental and 1825 genes 2-log fold down-regulated as compared to the parental ($p < 0.05$). GSEA revealed increased expression of pathways known to drive lymphomagenesis of activated B-cell (ABC) lymphoma such as the BTK pathway (Fig. 2B). In addition to changes in the UPR, differentially expressed genes in the resistant line include up-regulation of MAPK10, HELIOS, HDAC9 and FYN, as well as down-regulation of SH3BP5 (a negative regulator of BTK) and LCK. The change in expression was confirmed by PCR (Fig. 2C) and western blot analysis (Fig. 2D). Given the up-regulation of FYN, a tyrosine kinase in the B-cell receptor pathway, and down-regulation of SH3BP5 a negative regulator of the Bruton's Tyrosine Kinase, as well as findings generated by GSEA, the resistant line was treated with ibrutinib. Cell viability of the resistant and parental lines were evaluated over time. Ibrutinib 2 μ M was able to overcome resistance with a 70% viability in the resistant line versus 64% viability in

the parental line at 72 hours (Fig. 2E). This data reveals that cells exposed to ACY-1215, a selective HDAC6 inhibitor, rely heavily on the B-cell receptor pathway for expansion. It also suggests that treatment with the combination of ACY-1215 and ibrutinib lead to a synergistic interaction.

5 EXAMPLE 4

ACY-1215 plus Ibrutinib is highly synergistic in lymphoma cell lines and primary human lymphoma samples

Based on the findings generated from the resistant cell line, a panel of lymphoma cell lines were treated with ACY-1215, ibrutinib, or the combination and viability and synergy
10 were measured over 24, 48, and 72 hours (Fig. 3A). Cell lines included the GCB-DLBCL OCI-Ly7, the ABC-DLBCL cell lines HBL1, OCI-Ly10, RIVA, mantle cell lymphoma (MCL) lines HBL2, JEKO1, REC1, and the T-cell lymphoma line H9. As expected, synergy was most pronounced in the ABC-DLBCL and MCL cell lines with synergy coefficients as low as 0.10 (Ly10) and 0.06(JEKO1) respectively (where $RRR < 1$ connotes synergy). Both of
15 these disease entities are known to be driven by tonic signaling of the B-cell receptor (BCR) pathway and are highly sensitive to BTK inhibitors. There was little-to-no synergy in GCB-DLBCL and T-cell lymphoma respectively as these cell lines do not rely on the BCR pathway for growth.

To confirm the clinical relevance of this observation, three primary patient samples of
20 leukemic phase lymphoma were collected under an IRB approved protocol. The patient samples spanned 3 subtypes of lymphoma including chronic lymphocytic lymphoma (CLL), lymphoplasmacytic B-cell lymphoma (LPL), and nodal marginal zone lymphoma (MZL) harboring a 17p deletion. These three diseases are known for their sensitivity to BTK inhibitors. Cells were treated with increasing concentrations of ibrutinib in combination with
25 ACY-1215 and viability and synergy were measured after 24, 48, 72, and 96 hours. All three of the lymphoma subtypes achieved marked synergy with RRR of 0.54, 0.43, and 0.22 respectively (Fig. 3B).

The molecular effects of the combination treatment was evaluated in three lymphoma cell lines, 2 ABC-DLBCL (LY-10 and HBL-1) and one MCL line (JEKO1) as well as the
30 primary human MZL 17p- patient sample by immunoblot (Fig. 3C). The combination led to a decrease in p-IRE1- α which has been shown to interplay with the BTK pathway [15, 16]. This corresponded to a decrease p-BTK, total BTK and its down-stream targets such as p-PLC2- γ and CARD11. There was decreased expression of SH3BP5, the negative regulator of

the BTK in all cell lines. This is expected as its function is to inhibit auto-phosphorylation of BTK and the cells treated with the combination exhibited complete inhibition of p-BTK [28]. These findings show that targeting the IRE1 pathway of the UPR and the BTK pathway with a selective HDAC6 inhibitor and ibrutinib are synergistic at both the cytotoxic and biologic level.

EXAMPLE 5

The combination of ACY-1215 and ibrutinib led to marked tumor growth delay and prolonged overall survival in a xenograft model of lymphoma

The parental LY10 cell line was xenografted into the flanks of SCID beige mice at 107 cells. Mice were divided into 4 cohorts, control, ACY-1215, ibrutinib, and the combination. Mice were treated with ACY-1215 50 mg/kg days 1-5, 8-12, 15-19, and ibrutinib 3 mg/kg once daily on days 1-20. Both drugs were given by intraperitoneal route (Fig. 4A). The combination of ACY-1215 and ibrutinib was well tolerated with weight loss observed in both the ACY-1215 and combination cohorts that returned to baseline by day 20 (Fig. 4B). The tolerance and weight loss data are similar to those from prior mouse studies of single agent and other combinations with ACY-1215 [7, 8]. Mice demonstrated significant tumor growth delay following treatment with the combination as compared to either ACY-1215, ibrutinib, or untreated mice ($p=0.0003$, $p=0.0006$, and $p<0.0001$ respectively) (Figure 4c). Mice treated with the combination had a prolonged overall survival compared to the control (Fig. 4D). Of the mice that completed one cycle of therapy, the mean survival in days was as follows: combination (66.5) > ibrutinib (57.6) > ACY-1215 (56.6) > control (40.1).

EXAMPLE 6

Pharmacokinetic and pharmacodynamic effects of ACY-1215 in combination with ibrutinib

The concentration of ACY-1215 and ibrutinib was measured at sequential time points in serum and tumor tissue after a single intraperitoneal injection of ACY-1215 (50mg/kg) or ibrutinib (3mg/kg) or a combination of both. The concentrations achieved for both drugs are similar or greater than the concentrations found to induce cytotoxicity in the *in vitro* experiments. In addition, these concentrations also recapitulate what has been described in human pharmacokinetic studies. There was no difference in the concentrations of either drug in resistant or parental mice therefore these data were pooled for analysis (Figs. 6A-B).

The average ACY-1215 serum concentration reached at 0.5 and 1 hour after administration was 438 (+/-85) and 327 (+/-133) ng/mL, respectively. The average serum

concentration of ACY-1215 at 6 and 8 hours was 182 (+/-80) and 200 (+/-89) ng/mL, resulting in an average half-life of 5.3 hours and an average AUC_{0-6h}, AUC_{0-8h} and AUC_{0-inf} of 1425, 2013 and 3262 h*ng/mL, respectively for both single administration of ACY-1215 and in combination with ibrutinib in both parental and resistant groups. There was no substantial difference in serum pharmacokinetics of ACY-1215 between groups and treatments, which was also observed for tumor concentrations of the drug. Mean (+SD) tumor concentrations for single administration of ACY1215 and the combination with ibrutinib in the parental and resistant group at 4, 6 and 8 hours after injection were 94 (+/-47), 80 (+/-38) and 45 (+/-18) ng/mL, respectively (Fig. 6C).

The serum pharmacokinetics of ibrutinib exhibited no substantial differences when the drug was administered alone or in combination with ACY-1215. The average ibrutinib serum concentration reached at 0.5 and 1 hour after administration was 778 (+/- 85) and 224 (+/- 183) ng/mL, respectively. The average serum concentration of ibrutinib at 6 and 8 hours was 13 (+/-22) and 6 (+/-6) ng/mL, resulting in an average half-life of 1.1 hours and an average AUC_{0-8h} and AUC_{0-inf} of 752, and 757 h*ng/mL, respectively for both single administration of ibrutinib and in combination with ACY-1215 in both parental and resistant groups. Ibrutinib tumor concentrations were below the lowest limit of quantification in most samples (Fig. 6B).

Immunoblot analysis of mouse tumor tissue was evaluated for modulation of the IRE1 and BTK pathways following 6 hours of exposure to ACY-1215, ibrutinib, or the combination. Following the short, single exposure to the drugs there was reduction of both IRE1 and p-BTK and corresponding reduction of the downstream targets p-PLC2- γ and CARD11 (Fig. 6D). These results confirm those demonstrated in the cell lines treated with the combination.

References

1. Duvic, M., et al., *Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL)*. Blood, 2007. **109**(1): p. 31-9.
2. Piekarz, R.L., et al., *Phase 2 trial of romidepsin in patients with peripheral T-cell lymphoma*. Blood, 2011. **117**(22): p. 5827-34.

3. O'Connor, O.A., et al., *Belinostat in Patients With Relapsed or Refractory Peripheral T-Cell Lymphoma: Results of the Pivotal Phase II BELIEF (CLN-19) Study*. J Clin Oncol, 2015. **33**(23): p. 2492-9.
4. Ning, Z.-Q., et al., *Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity*. Cancer Chemotherapy and Pharmacology, 2012. **69**(4): p. 901-909.
5. San-Miguel, J.F., et al., *Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial*. The Lancet Oncology, 2014. **15**(11): p. 1195-1206.
10. Amengual, J.E., et al., *Sirtuin and pan-class I/II deacetylase (DAC) inhibition is synergistic in preclinical models and clinical studies of lymphoma*. Blood, 2013. **122**(12): p. 2104-13.
15. 7. Amengual, J.E., et al., *Dual targeting of protein degradation pathways with the selective HDAC6 inhibitor ACY-1215 and bortezomib is synergistic in lymphoma*. Clinical Cancer Research, 2015. **21**(20): p. 4663-4675.
8. Santo, L., et al., *Preclinical activity, pharmacodynamic, and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma*. Blood, 2012. **119**(11): p. 2579-2589.
20. 9. Yee, A.J., et al., *Ricolinostat (ACY-1215), a selective HDAC6 inhibitor, in combination with lenalidomide and dexamethasone: results of a phase 1b trial in relapsed and relapsed refractory multiple myeloma*. Blood, 2014. **124**(21): p. 4772-4772.
25. 10. Niesvizky, R., et al., *ACY-241, a Novel, HDAC6 Selective Inhibitor: Synergy with Immunomodulatory (IMiD®) Drugs in Multiple Myeloma (MM) Cells and Early Clinical Results (ACE-MM-200 Study)*. Blood, 2015. **126**(23): p. 3040-3040.
11. Vogl, D.T., et al., *Phase 1B results of ricolinostat (ACY-1215) combination therapy with bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma (MM)*. Blood, 2014. **124**(21): p. 4764-4764.
30. 12. Raje, N.S., et al., *Ricolinostat (ACY-1215), the First Selective HDAC6 Inhibitor, Combines Safely with Pomalidomide and Dexamethasone and Shows Promising*

Early Results in Relapsed-and-Refractory Myeloma (ACE-MM-102 Study). Blood, 2015. **126**(23): p. 4228-4228.

13. Burchenal, J., et al., *The induction of resistance to 4-amino-N10-methylpteroyl-glutamic acid in a strain of transmitted mouse leukemia*. American Association for the Advancement of Science. Science, 1950. **111**: p. 116-117.

14. Alt, F.W., et al., *Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells*. Journal of Biological Chemistry, 1978. **253**(5): p. 1357-1370.

15. Kriss, C.L., et al., *Overexpression of TCL1 activates the endoplasmic reticulum stress response: a novel mechanism of leukemic progression in mice*. Blood, 2012. **120**(5): p. 1027-1038.

16. Tang, C.-H.A., et al., *Inhibition of ER stress-associated IRE-1/XBP-1 pathway reduces leukemic cell survival*. The Journal of clinical investigation, 2014. **124**(6): p. 2585-2598.

17. Paoluzzi, L., et al., *Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in in vitro and in vivo models of B-cell lymphoma*. Blood, 2008. **111**(11): p. 5350-5358.

18. Marchi, E., et al., *Pralatrexate is synergistic with the proteasome inhibitor bortezomib in in vitro and in vivo models of T-cell lymphoid malignancies*. Clin Cancer Res, 2010. **16**(14): p. 3648-58.

19. Ryan, J. and A. Letai, *BH3 profiling in whole cells by fluorimeter or FACS*. Methods, 2013. **61**(2): p. 156-164.

20. Subramanian, A., et al., *Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles*. Proceedings of the National Academy of Sciences, 2005. **102**(43): p. 15545-15550.

21. Shaffer, A.L., et al., *A library of gene expression signatures to illuminate normal and pathological lymphoid biology*. Immunological Reviews, 2006. **210**(1): p. 67-85.

22. Kalac, M., et al., *HDAC inhibitors and decitabine are highly synergistic and associated with unique gene-expression and epigenetic profiles in models of DLBCL*. Blood, 2011. **118**(20): p. 5506-5516.

23. Yang, Y., et al., *Exploiting Synthetic Lethality for the Therapy of ABC Diffuse Large B Cell Lymphoma*. Cancer Cell, 2012. **21**(6): p. 723-737.

24. Zhao, L., M.G. Wientjes, and J.L. Au, *Evaluation of combination chemotherapy integration of nonlinear regression, curve shift, isobologram, and combination index analyses*. Clinical Cancer Research, 2004. **10**(23): p. 7994-8004.
25. Gottesman, M.M., T. Fojo, and S.E. Bates, *Multidrug resistance in cancer: role of ATP-dependent transporters*. Nat Rev Cancer, 2002. **2**(1): p. 48-58.
26. Bradner, J.E., et al., *Chemical phylogenetics of histone deacetylases*. Nat Chem Biol, 2010. **6**(3): p. 238-243.
27. Moore, V.D.G. and A. Letai, *BH3 profiling—measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions*. Cancer letters, 2013. **332**(2): p. 202-205.
28. Yamadori, T., et al., *Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein*. Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(11): p. 6341-6346.
29. Woyach, J.A., et al., *Resistance Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib*. New England Journal of Medicine, 2014. **370**(24): p. 2286-2294.
30. Landau, D., et al., *Clonal evolution in patients with chronic lymphocytic leukemia (CLL) developing resistance to BTK inhibition*. Blood, 2013. **122**(21): p. 866-866.

INCORPORATION BY REFERENCE

All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those

described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

5 The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The entire disclosure of each of the patent documents, including certificates of correction, patent application documents, scientific articles, governmental reports, websites, and other references referred
10 to herein is incorporated by reference herein in its entirety for all purposes. In case of a conflict in terminology, the present specification controls.

CLAIMS

1. An isolated diffuse large B-cell lymphoma cell line resistant to an HDAC6 inhibitor.
2. The isolated diffuse large B-cell lymphoma cell line of claim 1, wherein the cell line is R10-OCI-LY10 (R10).
- 5 3. The isolated diffuse large B-cell lymphoma cell line of claim 1, wherein the HDAC6 inhibitor is ACY-1215.
4. The isolated diffuse large B-cell lymphoma cell line of claim 1, wherein the HDAC6 inhibitor is vorinostat.
- 10 5. The isolated diffuse large B-cell lymphoma cell line of any claims 1-4, wherein the cell line exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN.
6. The isolated diffuse large B-cell lymphoma cell line of any claims 1-4, wherein the cell line exhibits down-regulation of SH3BP5 and LCK.
7. The isolated diffuse large B-cell lymphoma cell line of any claims 1-4, wherein the cell line exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.
- 15 8. A kit comprising diffuse large B-cell lymphoma cell line according to any claims 1-4, and a culture medium.
9. The kit of claim 8, wherein the kit further comprises parental OCI-Ly10 cell line.
- 20 10. An isolated diffuse large B-cell lymphoma cell line resistant to a proteasome inhibitor.
11. The isolated diffuse large B-cell lymphoma cell line of claim 10, wherein the proteasome inhibitor is bortezomib.
12. A method for treating lymphoma in a patient comprising administering to a patient a therapeutically effective amount of (i) an HDAC6 inhibitor and (ii) a BTK inhibitor.
- 25 13. The method of claim 12, wherein the HDAC6 inhibitor is selected from the group consisting of ACY-1215, ACY-241, Tubacin, Tubastatin A, ST-3-06, ST-2-92, Nexturastat A, and Nexturastat B, and optionally combinations thereof.
14. The method of claim 13, wherein the HDAC6 inhibitor is ACY-1215.
- 30 15. The method of claim 12, wherein the BTK inhibitor is selected from the group consisting of ibrutinib, HM-71224, BGB-3111, CG-036806, CC-292, ACP- 196,

GDC-0834, ONO-4049, RN-486, SNS-062, TAS-5567, AVL-101, AVL-291, PCI-45261, HCI-1684, and PLS-123, and optionally combinations thereof.

16. The method of claim 15, wherein the BTK inhibitor is ibrutinib.

17. The method of claim 12, wherein the HDAC6 inhibitor is ACY-1215 and the BTK inhibitor is ibrutinib.

18. The method of any of claims 12-17, wherein the lymphoma is selected from the group comprising of small lymphocytic lymphoma, lymphoplasmacytic B cell lymphoma, Waldenström macroglobulinemia, splenic marginal zone lymphoma, plasmacytoma, extranodal marginal zone B cell lymphoma, MALT lymphoma, nodal marginal zone B cell lymphoma (NMZL), follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma (DLBCL), mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt lymphoma, chronic lymphocytic lymphoma, classical Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, adult T cell lymphoma, nasal type extranodal NK/T cell lymphoma, enteropathy-type T cell lymphoma, hepatosplenic T cell lymphoma, blastic NK cell lymphoma, mycosis fungoide, Sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, angioimmunoblastic T cell lymphoma, unspecified peripheral T cell lymphoma, and anaplastic large cell lymphoma.

19. The method of claim 18, wherein the lymphoma is selected from the group consisting of DLBCL, mantle cell lymphoma, chronic lymphocytic lymphoma, lymphoplasmacytic B-cell lymphoma, and nodal marginal zone lymphoma.

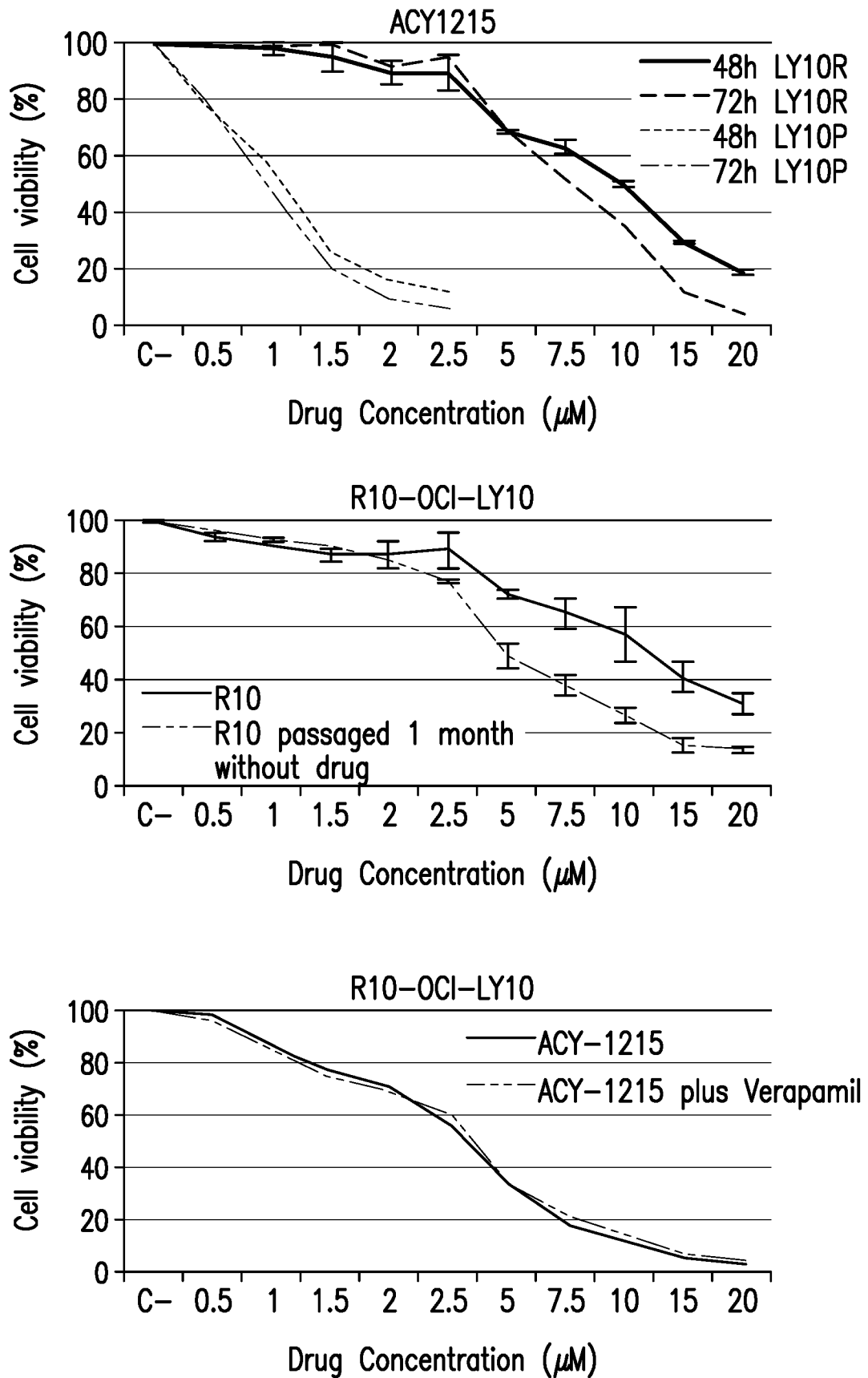
20. The method of claim 19, wherein the lymphoma is diffuse large B-cell lymphoma (DLBCL).

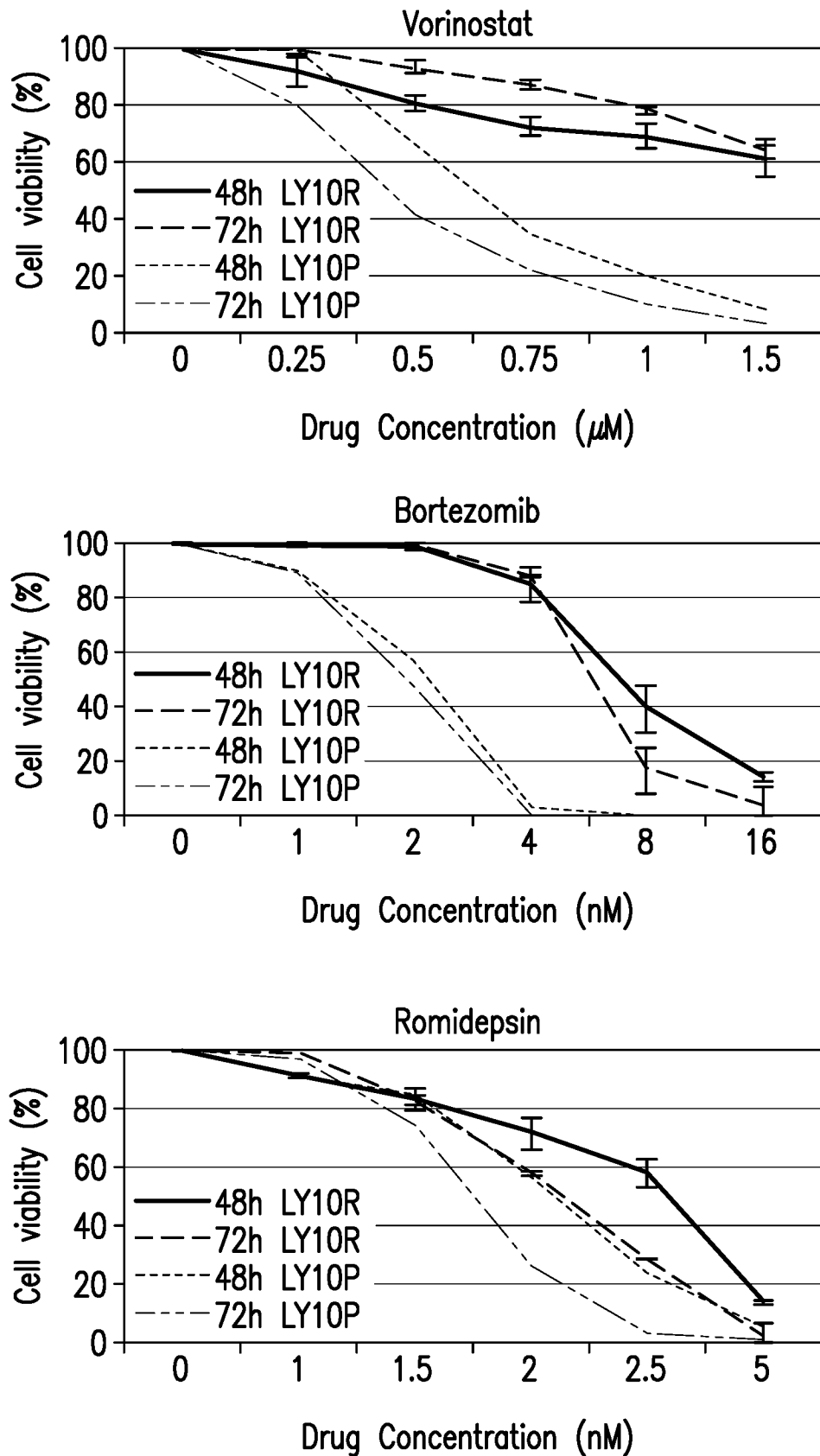
21. The method of any of claims 12-17, wherein the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN.

22. The method of any of claims 12-17, wherein the lymphoma exhibits down-regulation of SH3BP5 and LCK.

23. The method of any of claims 12-17, wherein the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.

24. A method of selecting a patient diagnosed with lymphoma for a method of treatment according to claim 12, wherein the lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN.
25. A method of selecting a patient diagnosed with lymphoma for a method of treatment according to claim 12, wherein the lymphoma exhibits down-regulation of SH3BP5 and LCK.
26. A method of selecting a patient diagnosed with lymphoma for a method of treatment according to claim 12, wherein the patient's lymphoma exhibits up-regulation of MAPK10, HELIOS, HDAC9, and FYN and down-regulation of SH3BP5 and LCK.

**FIG.1A**

**FIG.1B**

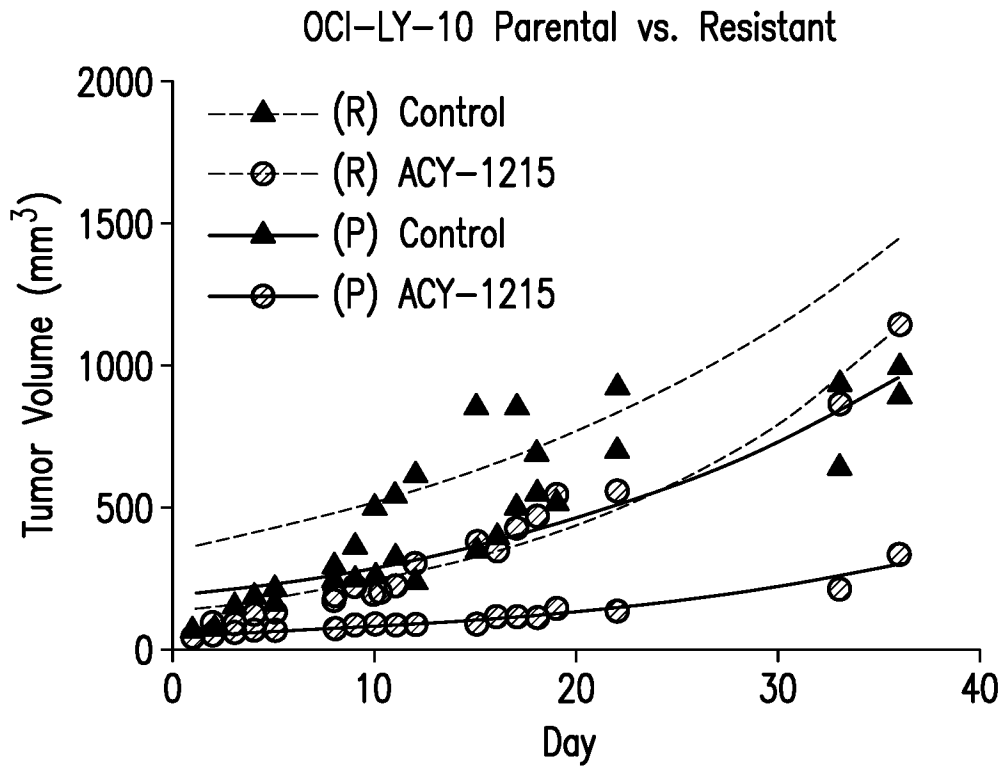


FIG.1C

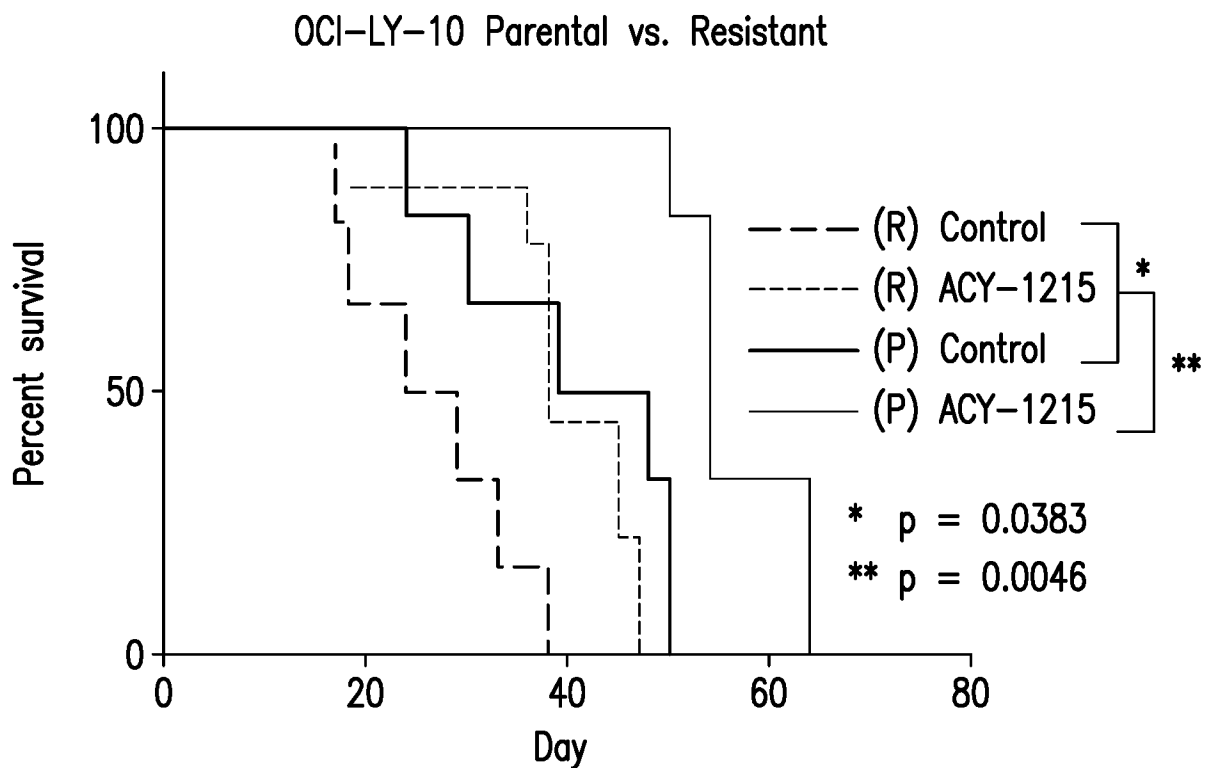


FIG.1D

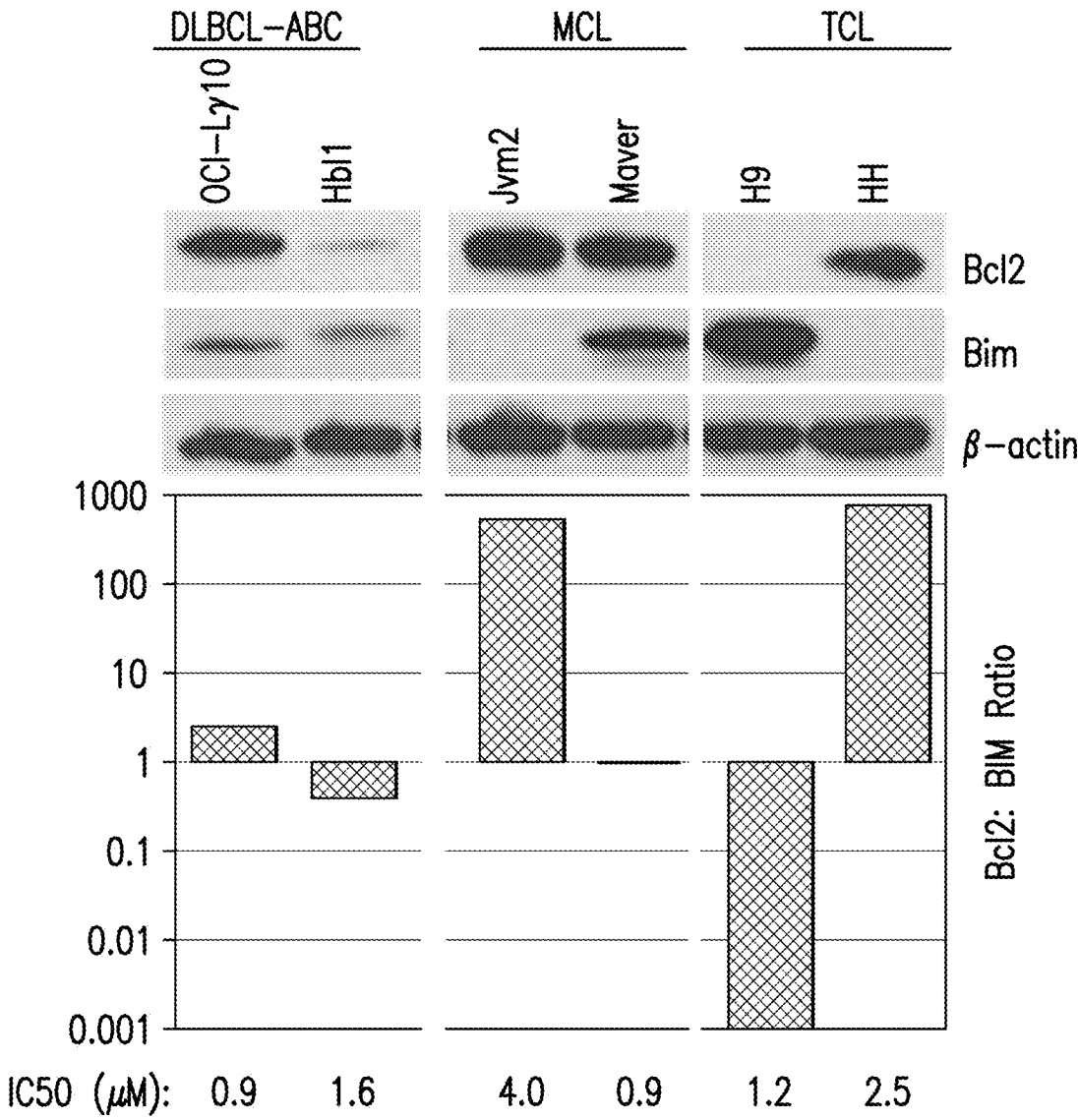
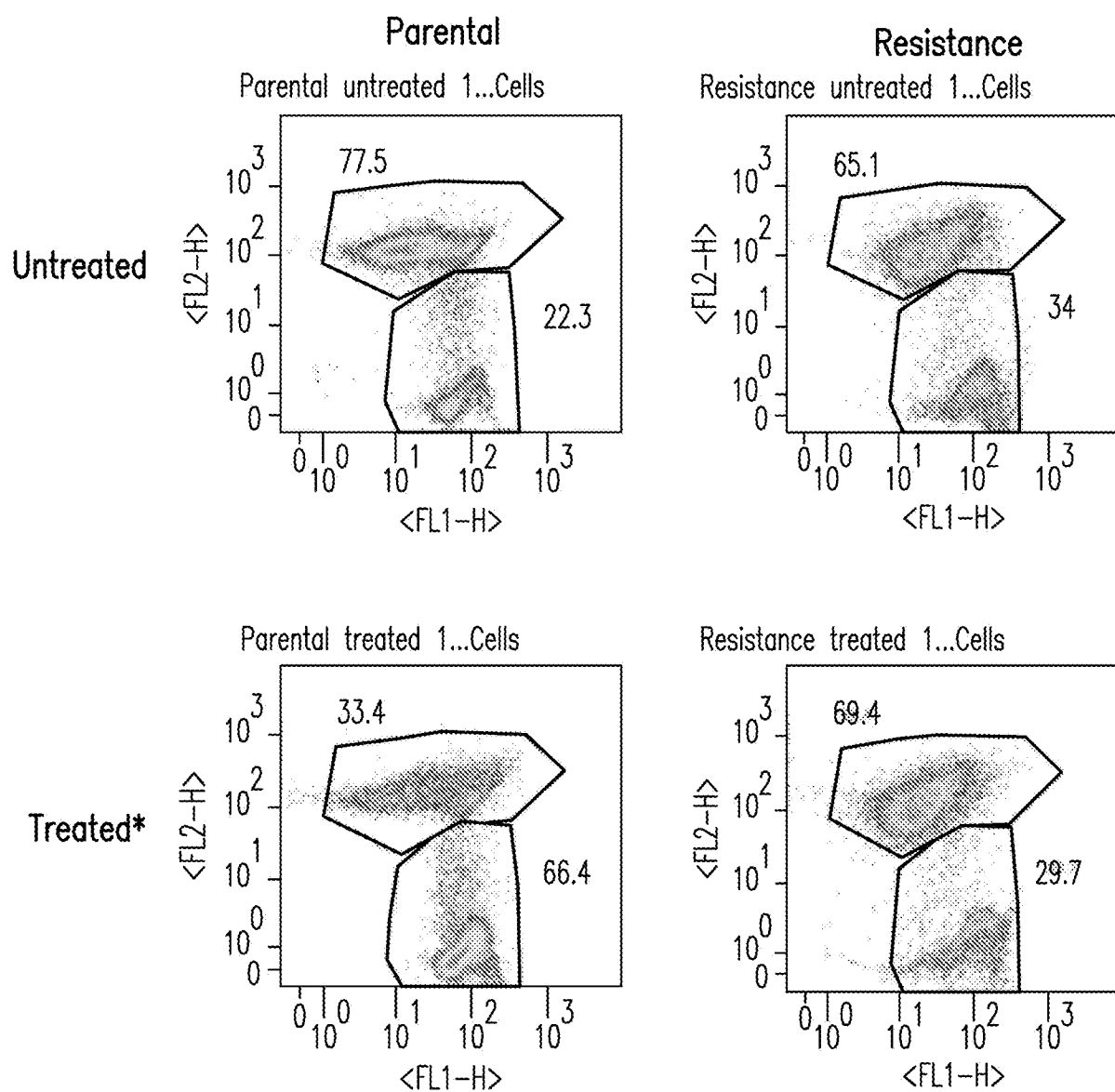


FIG.2A



(%)	p- Untreated	p- Treated	R- Untreated	R- Treated
Live	74	35	69	65
Apoptotic	26	65	28	26

FIG.2B

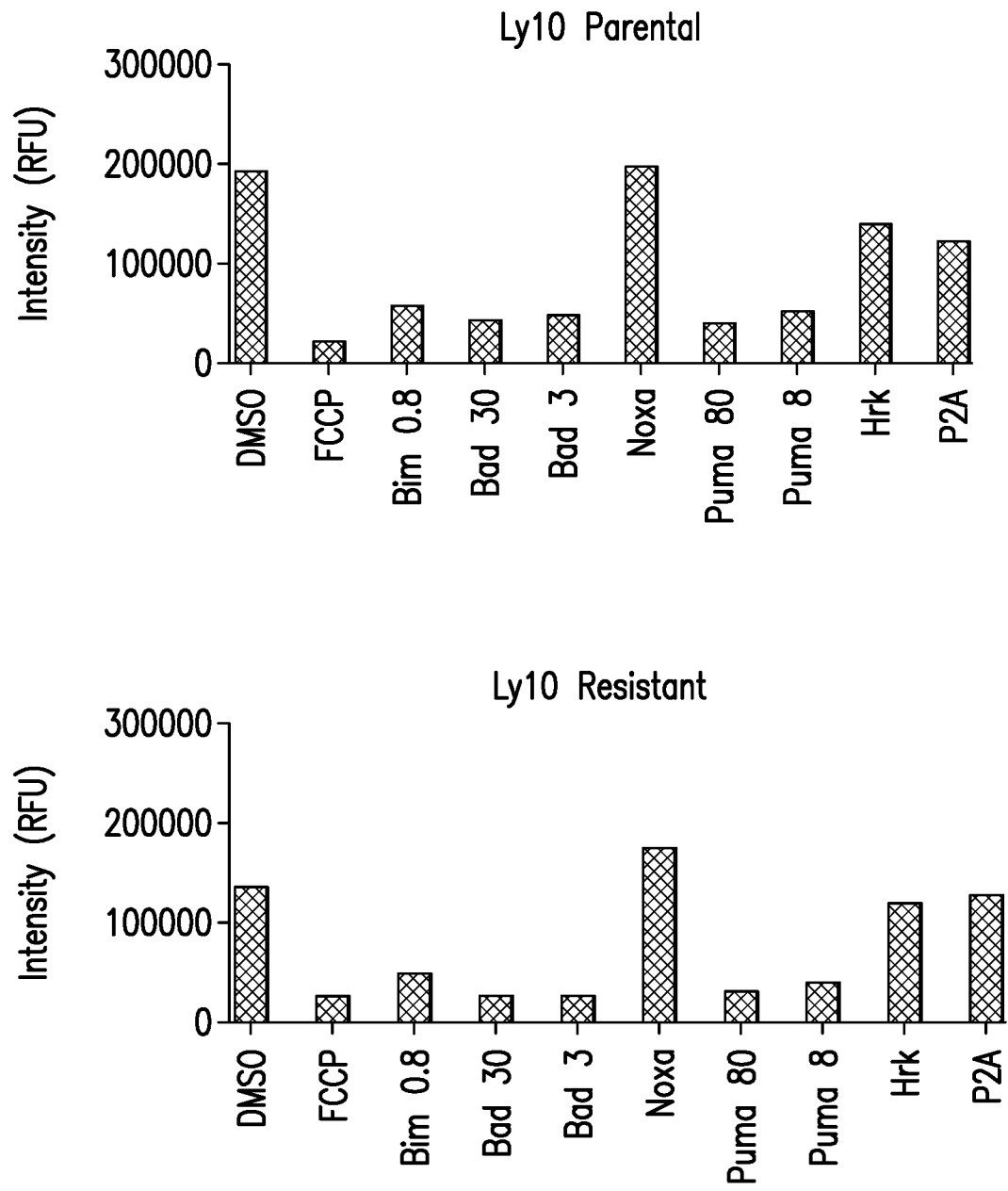


FIG.2C

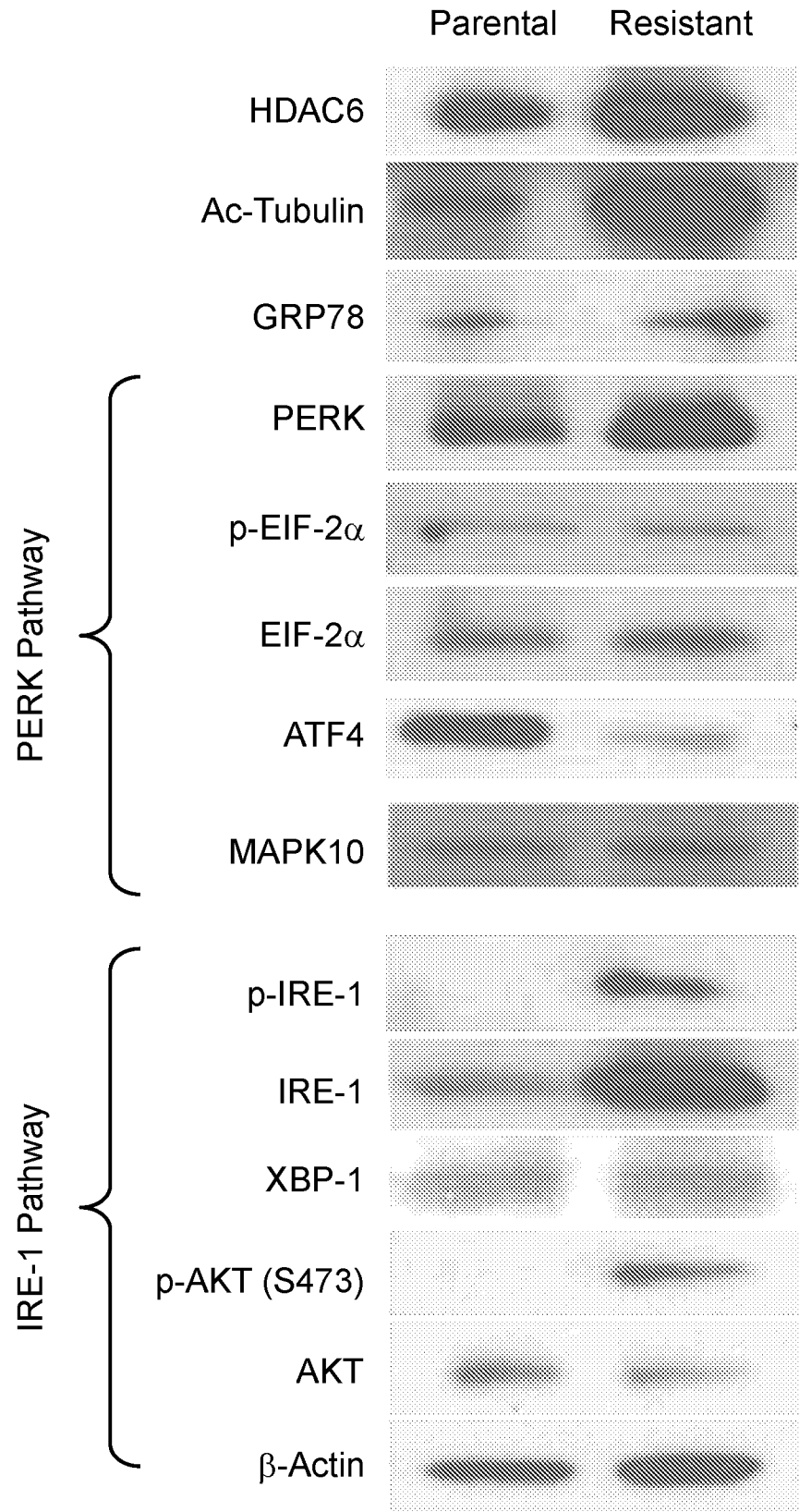


FIG.2D

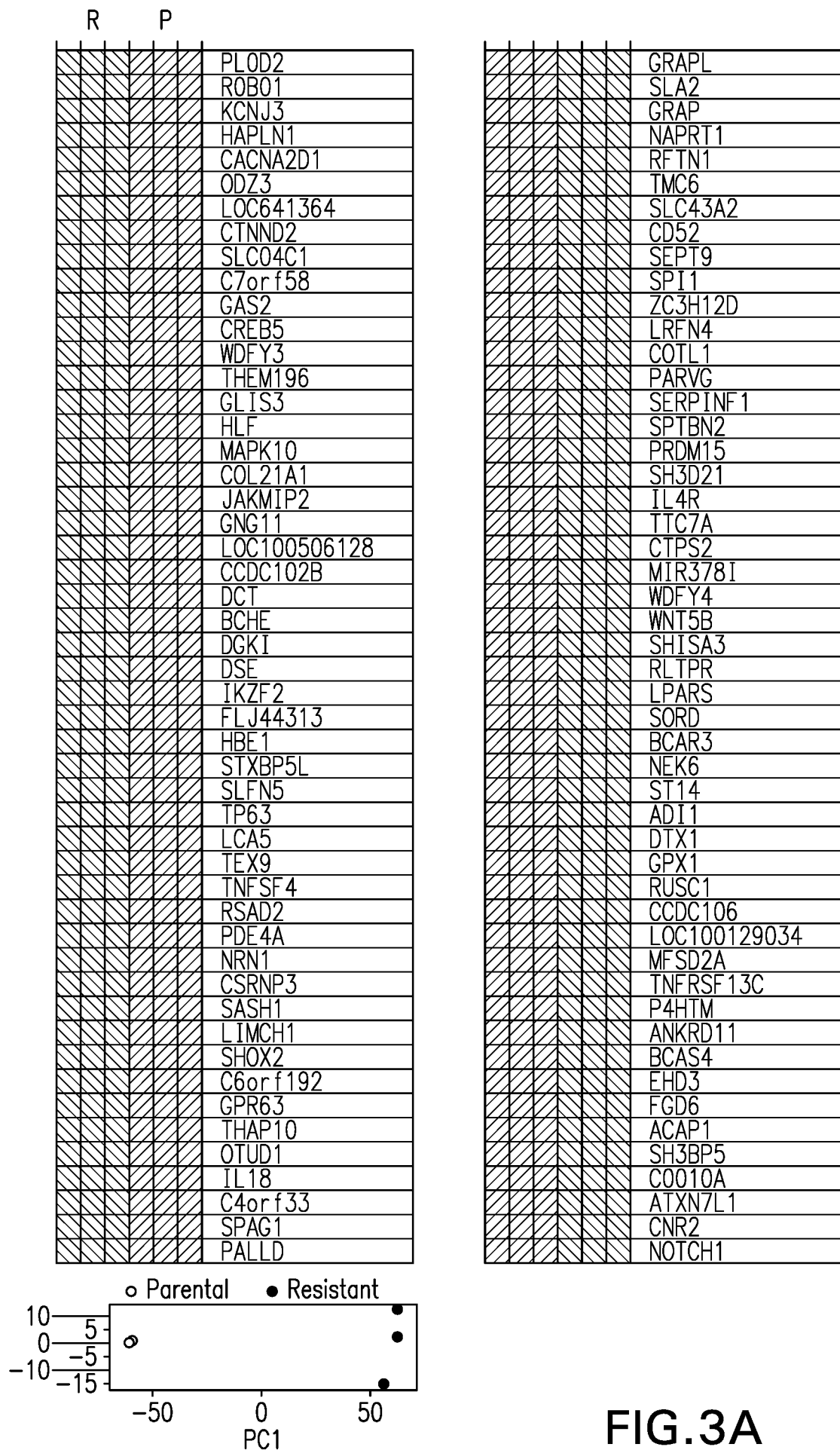


FIG.3A

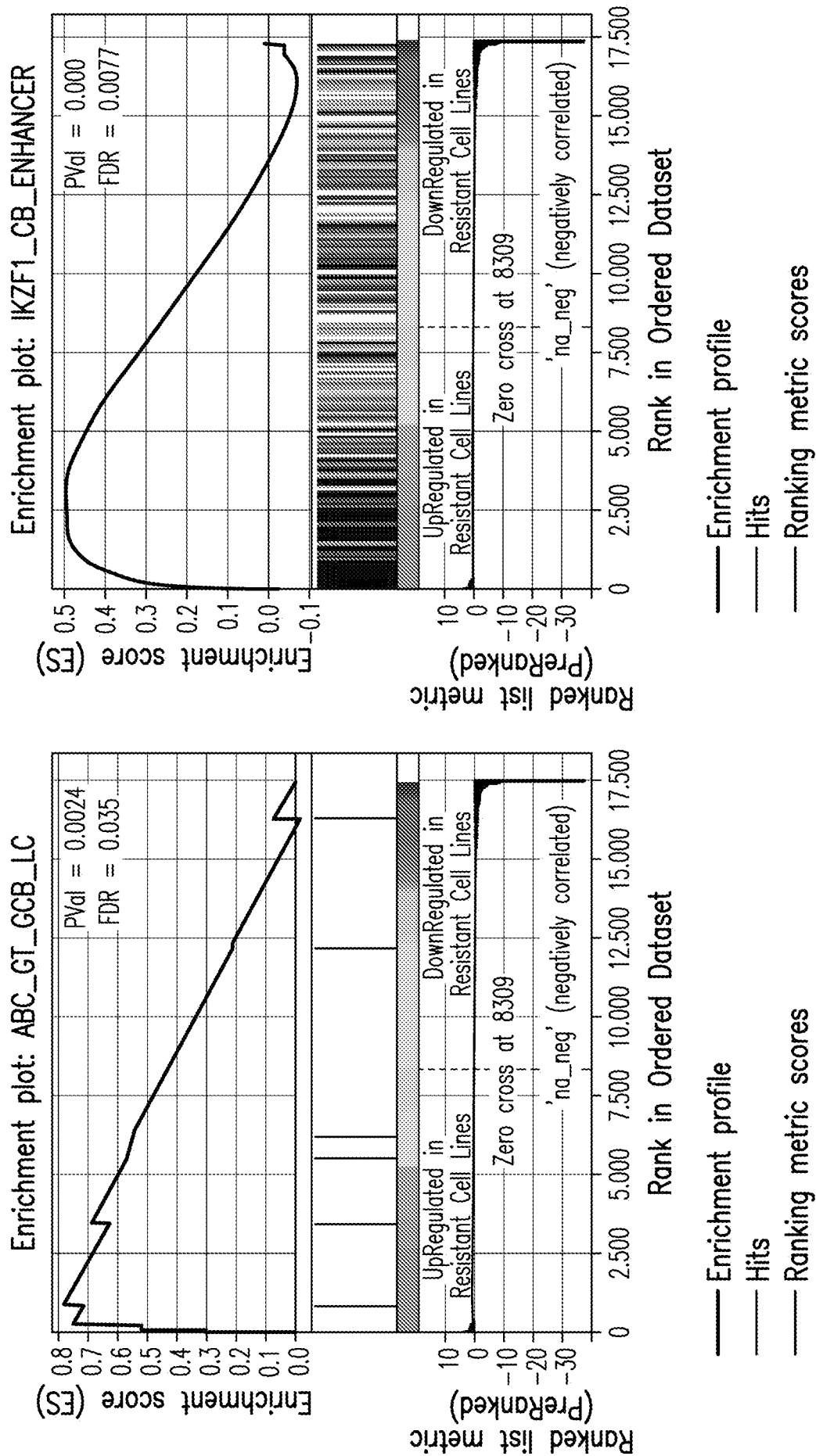


FIG.3B

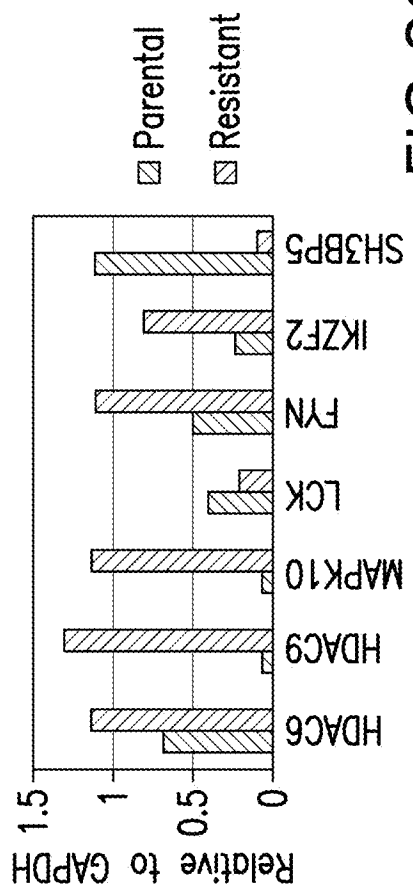
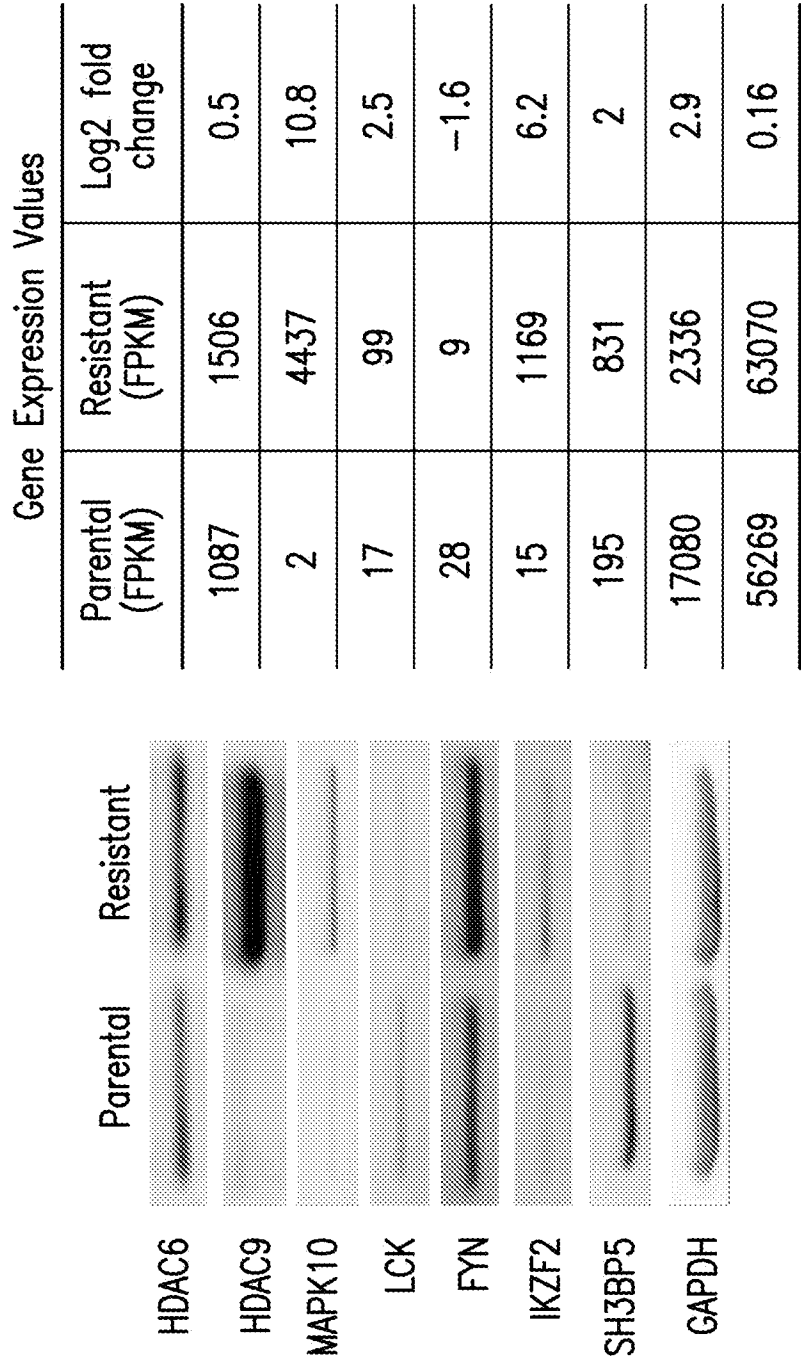


FIG.3C

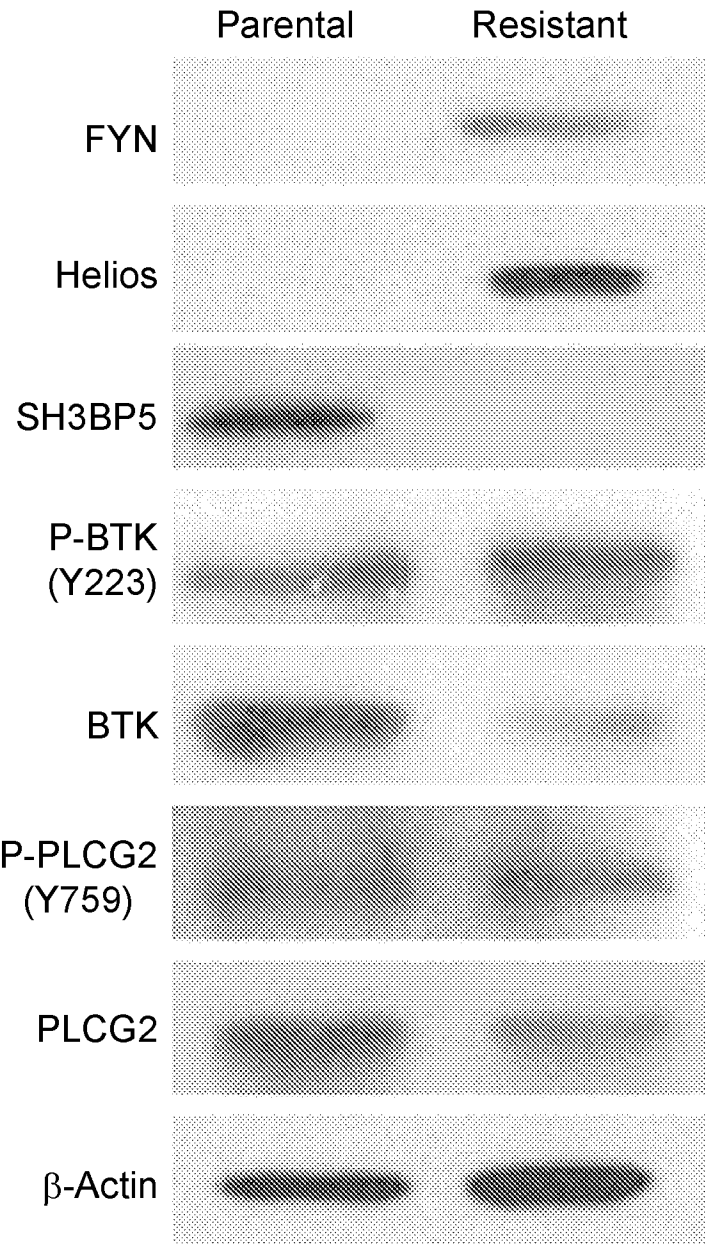


FIG.3D

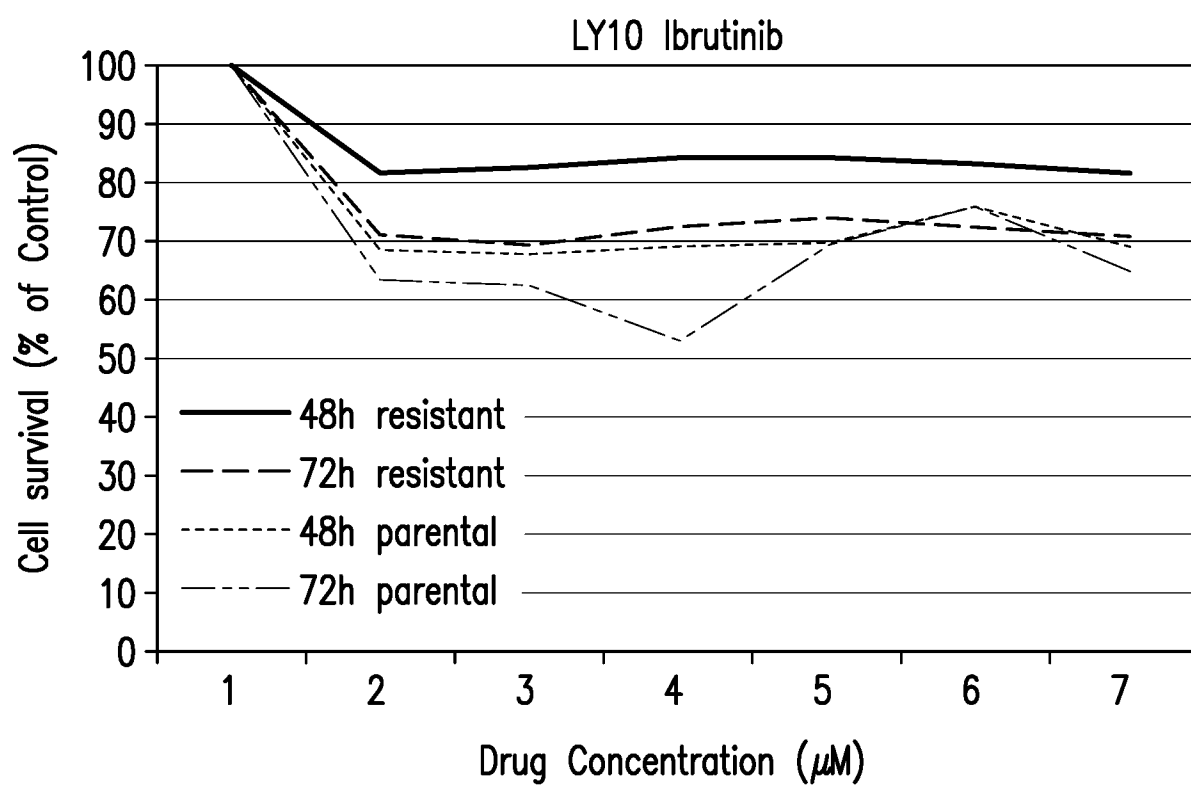


FIG.3E

ibrutinib (μM)																								
			1	5	10	1	5	10	1	5	10													
			24 Hours			48 Hours			72 Hours															
ACY-1215 (μM)																								
Cell Line	AC1-1215 (μM)	DLBCL (GC)	OCI-LY7	HBL1	DLBCL (ABC)	OCI-LY10	RIVA	HBL2	MANTLE CELL	JEK01	REC1	T-CELL H9	RRR											
													0	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1		
Viability (Percent of Control)	0	84.9	63.2	39.6	86.0	52.3	17.4	96.6	76.2	17.5														
	1	83.1	63.8	40.3	91.2	50.4	13.9	101.1	73.0	11.7														
	1.5	76.4	62.0	42.3	85.6	48.8	9.4	88.0	62.2	11.1														
	0	99.0	91.6	84.1	72.5	74.0	58.5	67.2	64.4	40.5														
	1	90.5	85.2	63.7	52.8	47.9	28.9	46.5	34.9	19.9														
	1.5	83.3	68.3	53.6	48.2	38.5	14.4	32.4	23.8	9.2														
	0	75.1	63.0	37.6	43.8	24.7	10.8	25.9	9.6	2.7														
	1	56.7	40.1	13.9	16.4	5.8	1.7	4.8	0.9	0.9														
	1.5	34.8	22.4	6.0	5.4	1.3	0.7	0.8	0.5	0.6														
	0	88.6	87.9	72.8	78.7	76.3	68.1	72.3	67.5	59.2														
	1	82.5	70.4	25.1	69.9	63.0	23.7	62.9	54.0	19.1														
	1.5	71.5	56.1	17.1	66.3	49.7	15.7	56.0	41.0	11.8														
0	107.0	103.3	56.5	108.1	93.9	49.4	94.0	79.8	43.8															
1	105.5	100.2	50.1	99.2	91.9	41.9	98.8	81.0	38.9															
1.5	108.0	100.3	72.9	101.3	96.4	62.4	91.8	88.5	62.2															
0	99.6	110.5	69.6	84.0	58.0	20.1	142.5	75.2	0.9															
1	85.4	65.4	24.1	57.8	38.1	4.2	98.0	26.3	0.2															
1.5	57.8	49.3	23.5	35.8	19.2	2.0	56.6	9.2	0.1															
0	108.8	102.5	63.1	85.8	68.0	38.8	110.0	91.6	31.3															
1	121.2	94.7	52.4	77.1	53.4	18.4	104.3	61.3	12.4															
1.5	108.3	72.5	46.6	67.4	35.5	9.5	89.3	31.4	6.7															
0	100.7	99.5	92.3	100.2	99.1	85.1	105.2	95.1	85.3															
1	100.5	101.0	72.2	101.8	96.8	85.3	101.7	101.6	80.7															
1.5	99.0	101.1	93.3	99.2	99.8	83.3	100.1	92.4	82.9															
													0.0	10.0	20.0	30.0	40.0	50.0	60.0	70.0	80.0	90.0	100.0	
% Viability																								
													0	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1		
													RRR											

FIG. 4A

% Viability	0.0	10.0	20.0	30.0	40.0	50.0	60.0	70.0	80.0	90.0	100.0	RRR	0	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1

		Ibrutinib (μM)																							
		24 Hours						48 Hours						72 Hours						96 Hours					
		0	2	4	8	0	2	4	8	0	2	4	8	0	2	4	8	0	2	4	8	0	2	4	8
Viability (Percent of Control)	CLL	ACY-1215 (μM)																							
		0	100.0	99.1	95.2	83.5	100.0	75.9	68.9	52.8	100.0	58.1	59.1	38.1	100.0	60.0	48.8	27.0							
		2	98.2	90.4	87.9	69.1	83.8	57.0	49.6	32.2	81.9	43.4	37.0	18.7	83.8	36.8	29.4	12.1							
		0	100.0	145.9	152.2	114.3	100.0	125.9	118.5	126.6	100.0	133.9	142.0	97.1	100.0	117.5	116.6	81.5							
		2	107.5	118.8	111.1	94.9	88.7	88.5	80.9	48.1	94.7	94.3	81.2	71.9	75.8	51.3	59.8	31.9							
MZL (17p-)		0	100.0	52.29	62.56	81.49	100.0	87.58	76.89	65.84	100.0	103.64	94.49	66.85	100.00	96.74	88.38	50.59							
		0.5	84.60	94.55	73.24	89.62	60.86	82.47	42.02	66.38	64.64	49.40	13.84	51.28	40.08	35.89	7.81	35.47							

		Ibrutinib (μM)															
		2	4	8	2	4	8	2	4	8	96 Hours						
ACY-1215 (μM)		24 Hours			48 Hours			72 Hours			96 Hours						
CLL	2	0.93	0.94	0.84	0.9	0.86	0.73	0.91	0.77	0.6	0.73	0.72	0.54				
LPL	2	0.76	0.68	0.77	0.79	0.77	0.43	0.74	0.6	0.78	0.58	0.68	0.52				
MZL (17p-)	0.5	2.14	1.38	1.3	1.55	0.9	1.66	0.76	0.23	0.53	0.93	0.22	1.75				

FIG.4B

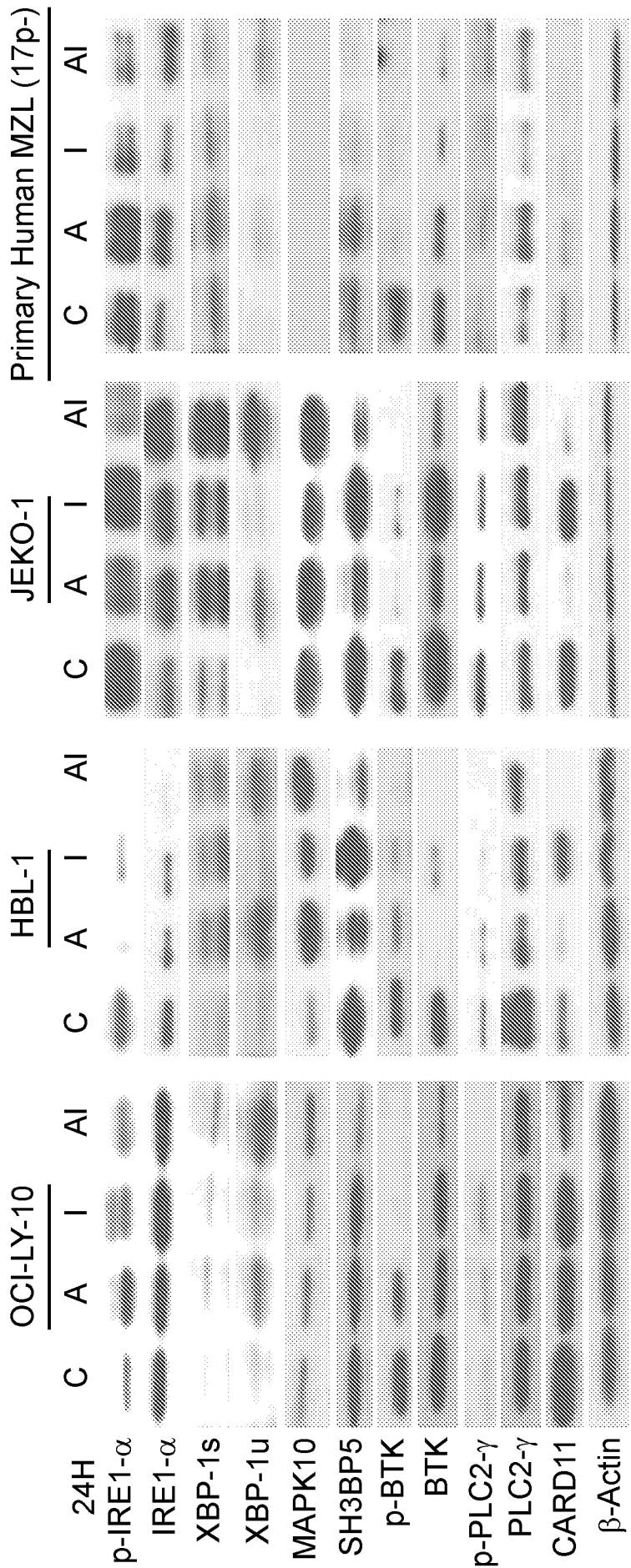


FIG.4C

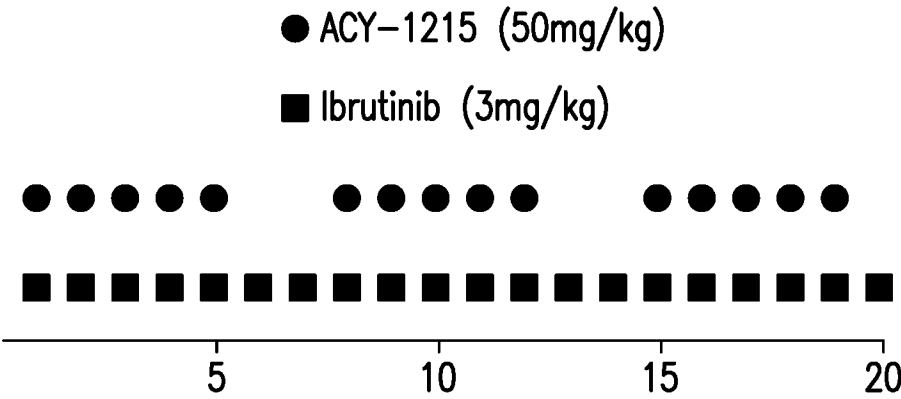


FIG.5A

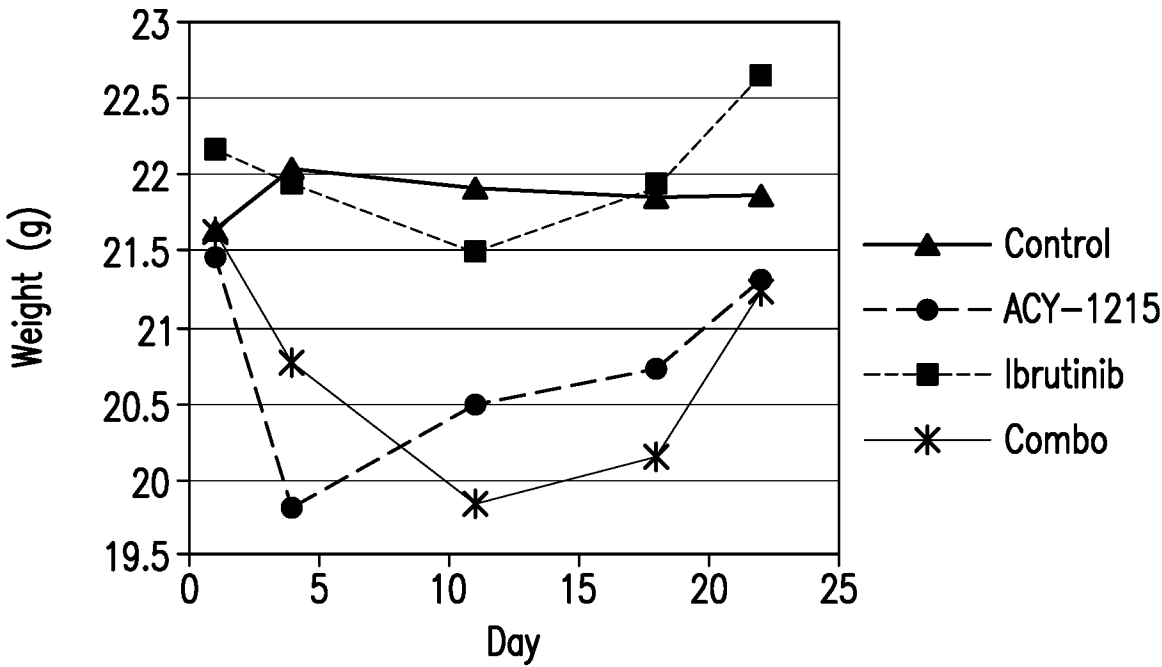


FIG.5B

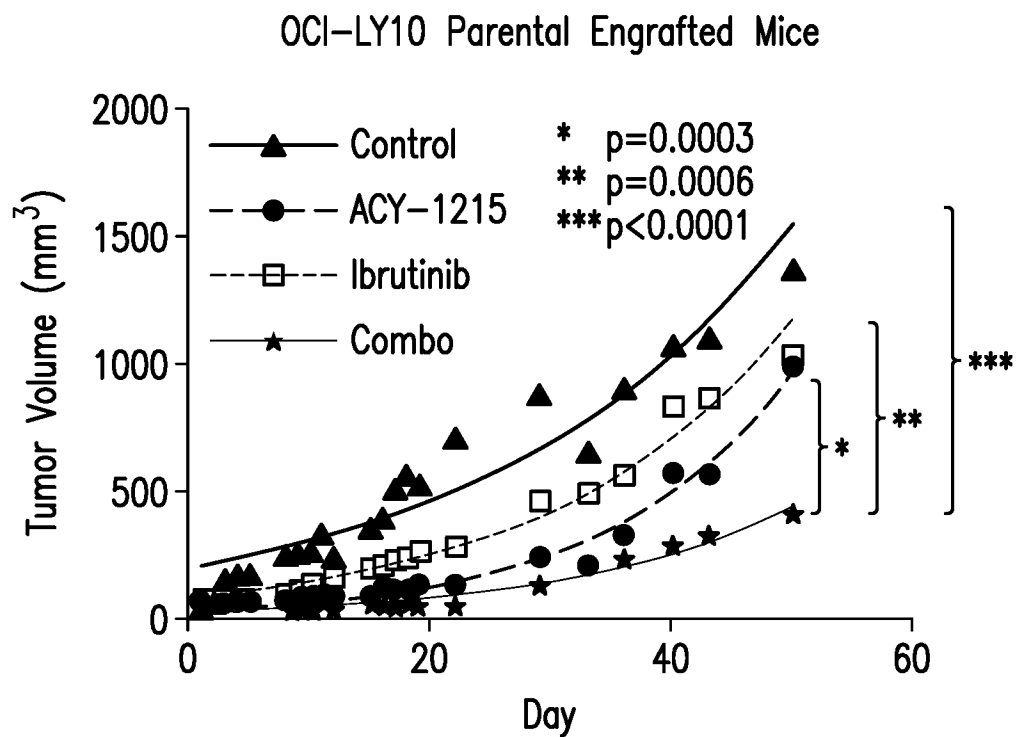


FIG.5C

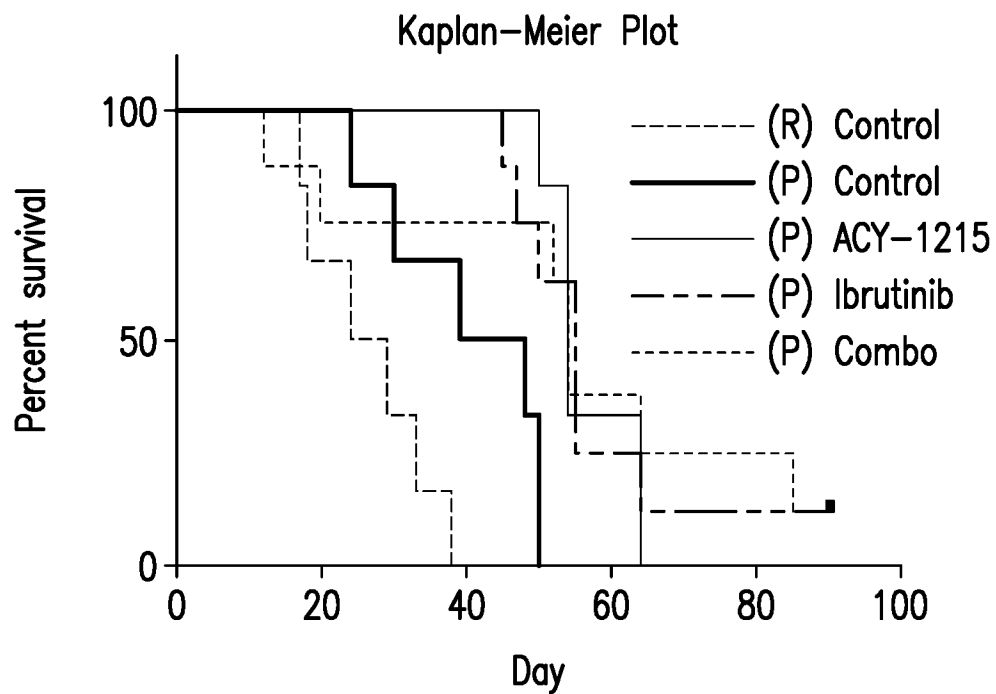


FIG.5D

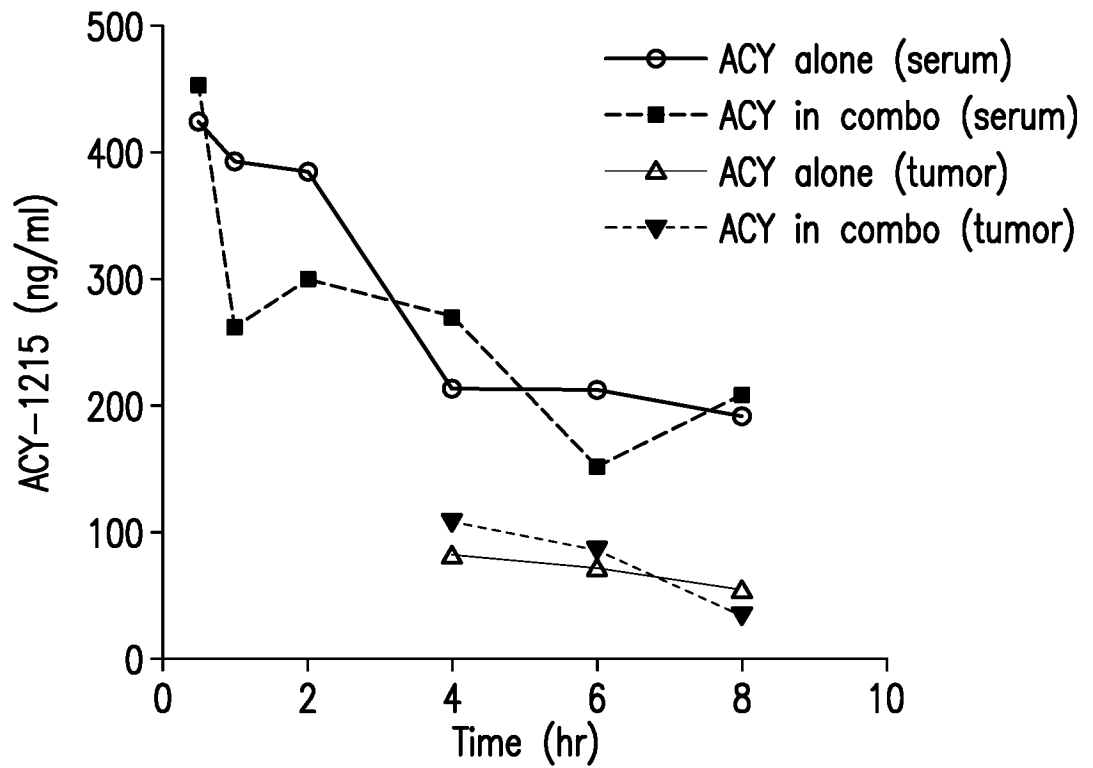


FIG.6A

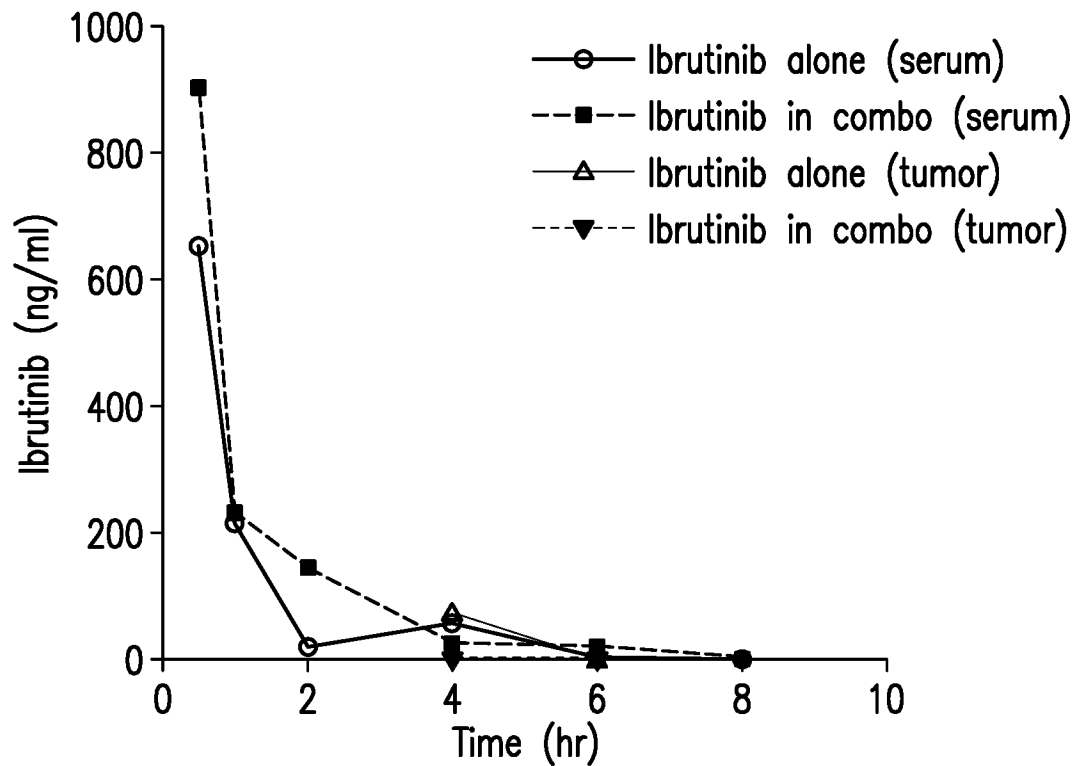


FIG.6B

	ACY-1215	Ibrutinib
SERUM		
(ng/mL)		
0.5h	438 (+/-83)	778 (+/-85)
1.0h	327 (+/-133)	224 (+/-183)
$T_{1/2}$ (h)	5.3	1.1
AUC (h*ng/mL)		
0-6 h	1424	---
0-8 h	2013	752
0-inf h	3262	757
TUMOR		
(ng/mL)		
4 h	94 (+/-47)	Below the
6 h	80 (+/-38)	limit
8 h	45 (+/-18)	of detection

FIG.6C

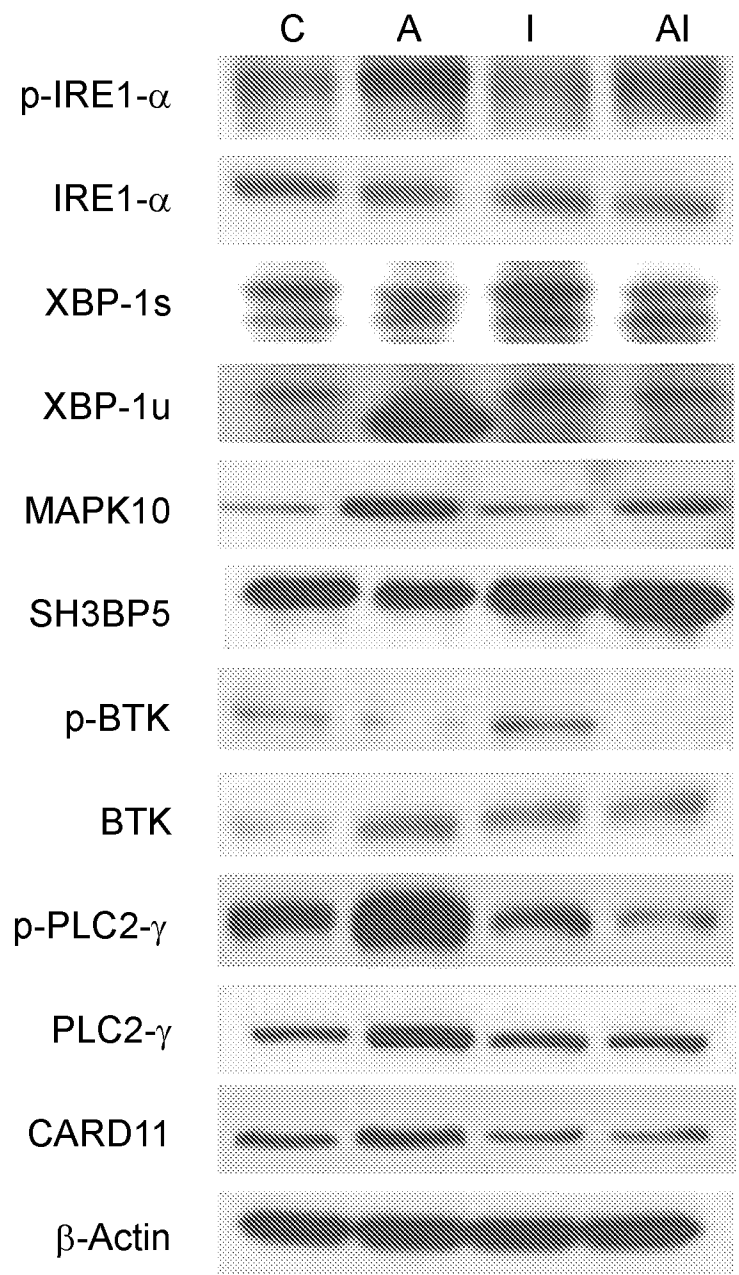


FIG.6D